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BIOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF LIVER OILS OF SELECTED DEEP SEA SHARKS AND CHIMAERAS OF THE INDIAN EEZ

Thesis submitted to the

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in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

By

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Declaration

I, Mathen Mathew (Reg.No.3289, Faculty of Marine Sciences, Cochin University of Science and Technology, Cochin-16) hereby declare that the thesis entitled "***Biochemical & Pharmacological evaluation of liver oils of selected deep sea sharks and chimaeras of the Indian EEZ***" is based on the original research work carried out by me under the guidance and supervision of Dr. Suseela Mathew, Senior Scientist, Biochemistry & Nutrition Division, Central Institute of Fisheries Technology, Cochin-29 and no part of this work has previously formed the basis for award of any degree, associate-ship, fellowship or any other similar title or recognition of this or in any other institution or university.

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CERTIFICATE

This is to certify that the thesis entitled "***Biochemical & Pharmacological evaluation of liver oils of selected deep sea sharks and chimaeras of the Indian EEZ***" embodies the original work carried out by Shri. Mathen Mathew, Full time Ph.D. student (Reg.No.3289, Faculty of Marine Sciences, Cochin University of Science and Technology, Cochin-16) under my guidance in the Biochemistry & Nutrition Division, Central Institute of Fisheries Technology, Cochin-29, in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry. I further certify that no part of this thesis has previously been formed the basis of award of any degree, diploma, associate-ship, fellowship or any other similar titles of this or in any other university or institution.

Dr.Suseela Mathew
Senior Scientist & Research Guide,
Biochemistry & Nutrition

To you, Mom & Dad
for all the love and support
you have given me...

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Mathen Mathew

Abstract

Chapter 1. The Marine Fishery Resources of the Indian Exclusive Economic Zone

With a seacoast of 8,118 km, an exclusive economic zone (EEZ) of 2 million square km, and with an area of about 30,000 square km under aquaculture, India produces close to six million tonnes of fish, over 4 per cent of the world fish production. While the marine waters upto 50m depth have been fully exploited, those beyond, remain unexplored. There is an ever increasing demand for fishery resources as food. The coastal fishery resources of the country are dwindling at a rapid pace and it becomes highly imperative that we search for alternate fishery resources for food. The option we have is to hunt for marine fishery resources. Studies pertaining to proximate composition, amino acid and fatty acid composition are essential to understand the nutraceutical values of these deep sea fishery resources.

For over two decades, accepted dietary guidance has stressed the importance of choosing a diet that is low in fat, saturated fat, and cholesterol. Dietary omega-3 fatty acids play a major role in the regulation of blood pressure, clotting and in reducing blood cholesterol. Fresh water fish generally have lower levels of omega-3 PUFAs than marine fish. It is essential to understand the biochemical compositions of deep sea creatures belonging to the Indian EEZ to identify potential resources that could serve as food supplements in the fisheries industry.

The main objectives of the study were to carry out proximate composition of deep sea fishery resources obtained during cruises onboard the *FORV* Sagar Sampada, to identify fishery resources which have appreciable lipid content and thereby analyse the bioactive potentials of marine lipids, to study the amino acid profile of these fishery resources, to understand the contents of SFA, MUFA and PUFA and to calculate the n3/n6 fatty acid contents.

Deep sea fishery resources were obtained as a result of trawling (IISDT, EXPO) operations on the FORV *Sagar Sampada* during cruises 241, 250, 252. The catch was sorted onboard and the samples were frozen immediately at -20^{0}C, in the fish-hold. These were then brought to the laboratory for further studies. Proximate composition of six different marine species were carried out following the Association of the Official Analytical Chemists (AOAC, 1984) methods. The amino acid composition was determined using

HPLC- LC 10 AS) equipped with cation exchange column packed with a strongly acidic cation exchange resin whereas the fatty acid composition, with a Gas Chromatograph clubbed with a Flame Ionisation detector. The extent of unsaturation in the lipids was determined by determining their corresponding Iodine values; the phospholipid contents were also analysed.

The main results of the study were as follows. Over 20 different marine fishery resources, obtained from the south west & south east coasts of the India as well as the Andaman Seas, were analysed for their biochemical compositions ranging from the elasmobranchs *Echinorhinus brucus, Neoharritotta raleighana* to other demersal creatures like *Alipocephalus blanfordii, Bathytroctes squamosus, Lamprogrammus niger, Neopinnula orientalis, Hoplostethus mediterraneus, Aristeus alcockii, Bathyuroconger braueri,Synopsis cyanea, Luciobrotula corethromycter, Chelidoperca investigatoris, Lophiomus setigerus, Bembrops caudimaculata, Pterygotrigla Hemistica and Xenomystax trucidens.* Out of these six species were studied for their amino acid and fatty acid contents based on their catch frequency and high lipid contents. Significantly ($p<0.05$) high amounts of histidine, tyrosine, leucine and proline were observed in the tissues of *N.raleighana.* The deep sea shrimp *A.alcockii* was found to possess significantly high amounts of arginine and glutamic acid. The snake mackeral *P.cyanea* contained significantly high amounts of threonine and leucine while significantly high amounts of the essential amino acid methionione were observed in the muscle tissues of the slick head *B.squamosus.* Liver of *E.brucus* and *N.raleighana* contained 70% lipids which possessed a high fraction of the non-saponifiable matter (78%) and unsaturated fatty acids. Palmitic and myristic acids were the dominant saturated fatty acids in most of the deep sea fishery resources analysed. Significantly high amounts of oleic acid was observed in the oils of *P.cyanea, B.squamosus and A.alcockii.* Among the PUFAs analysed, n-3 PUFA (EPA, DHA) formed the dominant class of fatty acids in *E.brucus* and *N.raleighana* liver oils. Moreover the n-3:n-6 fatty acid ratio was significantly high in *P.cyanea* and *B.squamosus.*

Though the presence of nutraceuticals was identified in the marine fishery resources analysed in the study, their use as potential food resources deserve further investigation.

Chapter 2. Biochemical Analyses of liver oils of selected sharks & chimaeras of the Indian EEZ

The annual average landings of sharks, skates and rays during 1996-2006 were 60,866 t of which sharks constituted 60.1% (36,592 t) (CMFRI statistics, 1996-2006). Though a harvest potential of 1,85,000 t of elasmobranchs has been indicated for the Indian EEZ, they are not fully exploited. Sharks are of great commercial importance the world over, apart from being a significant link in the marine ecology. Sixty-five species of shark have been sighted in Indian waters and over 20 of these, of the Carcharhinidae and Sphyrnidae families, contribute to the fishery. Tamil Nadu, Gujarat, Maharashtra, Kerala Karnataka and Andra Pradesh supply around 85% of the shark landings in India. Despite the commercial importance, no serious attempts have so far been made at any targeted exploration of this valuable resource.

The main objectives of the study were to calculate the hepatosamatic indices of sharks & chimaeras and conduct biochemical characterisation of liver oils of *Apristurus indicus, Centrophorus scalpratus, Centroselachus crepidater, Neoharriotta raleighana and Harriotta pinnata* obtained during cruises onboard the FORV *Sagar Sampada.*

The major results of the study were as follows. The liver weights were found to be approximately 1/5th of the total body weights of the selected elasmobranchs. The livers of all the species contained high lipid fractions ranging from 69% in the *Neoharritotta* sp. to 79% in the *Centrophorus* sp. The liver oils of *Centrophorus scalpratus, Centroselachus crepidater, Neoharriotta raleighana and Harriotta pinnata* contained high non-saponifiable matter compared that of *Apristurus* sp. Squalene was the predominant hydrocarbon present in the liver oils of the *Neoharriotta* and the *Centrophorus* species at approximately 60% of their liver lipids while they constituted only 18% of the total lipid contents in the *Apristurus sp.* Triacylglycerols composition ranged from 2-4% of total lipids in liver of the selected elasmobranchs. Significantly high amounts of monoacylglycerols (7% total lipid) were observed in the livers of the *Neoharriotta* sp. Vitamin E at 2% lipid levels of liver were observed in both the *Neoharriotta sp.* and the *Centrophorus sp.* Traces of Vitamin A were also recorded in the selected species. However the lipids of *Apristurus sp* recorded high levels of saponifiable matter (68% total lipids) compared to the others. The total free fatty acids and polar lipids were found to be at less than 1% levels in all the species evaluated.

High levels of mono-unsaturated fatty acids were observed in the liver oils of all the species studied ranging from 52% in the *Neoharritotta sp.* to 69% in the *Centrophorus sp.*

of the total saponifiable matter of the lipids. The results of the present study indicated that the biochemical evaluation of liver oils of deep sea sharks and chimaeras provides insight into the various lipid bioactives contained within them, that would serve as potential natural remedies for the treatment of various human disorders/diseases.

Chapter 3. Shark liver oils as analgesic, anti-inflammatory, antipyretic and anti-ulcer agents.

Therapeutic use of shark liver oil is evident from its use for centuries as a remedy to heal wounds and fight flu (Neil *et al.* 2006). Japanese seamen called it *samedawa*, or "cure all". Shark liver oil is being promoted worldwide as a dietary supplement to boost the immune system, fight infections, to treat cancer and to lessen the side effects of conventional cancer treatment. These days more emphasis is laid on the nutritive benefits of shark liver oils especially on the omega 3 polyunsaturated fatty acids (PUFAs) (Anandan *et al.* 2007) and alkylglycerols (AKGs) (Pugliese *et al.* 1998) contained in them due to the high rise of inflammatory disorders such as arthritis, asthma and neurodegenerative diseases like Alzheimer's, Parkinson's and Schizophrenia.

Higher concentrations of AKGs in shark liver oils are now considered to be responsible for their high immune boosting ability (Pugliese et al. 1998). These AKGS are essentially a class of lipids with an ether linkage and a glycerol backbone. In addition, shark liver oils also contain, antioxidant vitamins and squalamine (Brunel et al. 2005), a substance which has shown a promising behavior towards fighting cancers of the breast, lung, brain, and skin (melanoma specifically) by choking off the tumor's blood supply. The pharmaceutical values associated with shark liver oils are abundant; they form the active ingredients of many different formulations ranging from vitamin supplements to skin based ointments and creams (Neil et al. 2006).

The aim of the present study was to evaluate the pharmacological properties w.r.t analgesic, anti-inflammatory, anti pyretic and anti-ulcer effects of four different liver oils of sharks belonging to the Indian EEZ and to identify the components of oil responsible for these activities. The analgesic and anti–inflammatory activities of liver oils from *Neoharriotta raleighana* (NR), *Centrosymnus crepidater* (CC), *Apristurus indicus* (AI) *and Centrophorus sculpratus* (CS) sharks caught from the Arabian Sea and the Indian Ocean were compared. As the information available with regard to these properties is relatively scanty, an attempt has been made to explore their ability as therapeutic agents.

mono and polyunsaturated fatty acids would have played a major role in lowering the incidence of inflammatory diseases by blocking the activity of prostaglandins and leukotrienes. AI oil recorded the lowest fraction of NSM (25%) and hence exhibited lower effects.

Chapter 4. The hypocholesterolemic fish oil

The Indian subcontinent is home to 20% of the world's population and may be one of the regions with the highest burden of cardiovascular diseases (CVD) in the world. Most CVD cases arise due to lifestyle, unhealthy eating habits, smoking etc. and are more common in men. In 1990, there were an estimated 1.17 million deaths from CVD in India, and the number is expected to almost double by the end of 2010. Studies have indicated that South Asians have elevated levels of LDL cholesterol and triglycerides, while also suffering from a deficiency in HDL cholesterol (good cholesterol, which helps clear fatty buildups from blood vessels). In addition, South Asians are more vulnerable and are at greater risk of developing heart disease.

The main objectives of the study were to determine the cholesterol lowering effects of liver oils of *Neoharriotta raleighana* (NR) and *Centrophorus scalpratus* (CS) on the high fat diet induced dyslipidemia and to compare the impact of four isolipidemic diets, on levels of serum diagnostic marker enzymes, on lipid profile of blood and liver and antioxidant status of heart, in male Albino rats.

All animals were fed a basal diet enriched with either: coconut oil (group CO), NR oil (group NR), CS oil (group CS) or poly-unsaturated fatty acids concentrate (group PUFA) for a period of 8 weeks. The composition of the basal diet was as follows: corn starch (60.6%), casein (18.3%), salt mixture (4%), vitamin mixture (1%), cellulose (5%), cholesterol (1%) and methionine (0.1%). Five days after acclimatization, the rats were divided into four groups of 6 animals each and were allowed free access to the experimental diets and water. The first group of animals (Group CO) was fed, in addition to the basal diet, coconut oil at 10% feed levels. The remaining groups were also fed with the basal diets and fish oil (Group NR and CS) and polyunsaturated fatty acids (Group PUFA) at 5%, 5% and 1% feed levels respectively and made isolipidemic with coconut oil. At the end of the experimental period the animals were fasted overnight, thereafter, ether anesthetized and blood samples collected. They were then sacrificed: liver and heart tissues were excised, homogenates prepared and subjected to further analyses.

The main results of the study were as follows. Rats on CS and PUFA diets had better feed conversion ratios than those on NR and CO diets from the 6th week of feeding. Significant ($p<0.05$) decrease in protein content observed between rats on CO and NR and than those on CS and PUFA diets in liver and heart tissues. A significant increase ($P<0.05$) in the levels of cholesterol,

triglyceride & free fatty acids in CO and NR rats compared to those on CS and PUFA diets, was observed in the blood sera and heart tissues of the corresponding animals. Phospholipids were significantly higher in the ($P<0.05$) PUFA and CS rats than in the CO rats. Significant ($p<0.05$) increase in low density lipoprotein levels and a corresponding decrease in high density lipoprotein levels in the blood sera of CO and NR rats compared to those on CS and PUFA diets was recorded. The elevated cholesterol level observed in the liver tissues of CO and NR rats might be due to the increased uptake of LDL-cholesterol from the blood by the hepatic membranes. Significant differences in the levels of diagnostic marker enzymes (DME) of AST, ALT and LDH were observed in CO fed rats compared to those on NR, CS or PUFA diets. Rats fed on high energy CO diets would have increased oxidative stress in vivo, thereby increased the DME levels. Significantly higher amount of lipid peroxides were observed in Group PUFA which may be attributed to the high extent of unsaturation in n-3 PUFA. Natural antioxidants (vitamin E and squlalene) in the CC and NR diets would have been responsible for the low level of peroxides in the rat liver. Administration of CS and PUFA diets had a significant increase ($P<0.05$) in the levels of glutathione dependent antioxidant enzymes, glutathione peroxidise (GPx) and glutathione-S-transferase (GST) and antiperoxidative enzymes, catalase (CAT) and superoxide dismutase (SOD), in the heart tissues of the animals. But no significant differences were observed in the vitamin E and reduced glutathione (GSH) contents between CO and PUFA fed rats. CS supplementation significantly elevated ($P<0.05$) the vitamin E and GSH contents. In the present study n6:n3 ratio in CO animals was 6.9:1 as compared to 0.7:1,0.6:1 and 0.4:1 in NR, CS and PUFA animals respectively. This implied that NR, CS and PUFA diets provide balanced amounts of n6 and n3 fatty acids compared to CO diets. Moreover, significant ($P<0.05$) and positive correlations were observed between saturated fatty acid and triglyceride/cholesterol and between lipid peroxides and n3 PUFA whereas the correlations were found to be significant ($P<0.05$) and negative between n3 PUFA and triglyceride/cholesterol, lipid peroxides and vitamin E and between phospholipids and cholesterol, when subjected to Pearson's correlation tests.

The results of the present study indicated that consuming a coconut oil diet with a partial replacement by liver oil of *Centrophorus* sp. lowered the LDL or bad cholesterol and lipid peroxide contents and improved the antioxidant defense status in the blood, liver and heart tissues of Albino rats. However the diets with liver oils of *Neoharriotta* sp. significantly raised the cholesterol levels in blood; screening of fish oils for hypocholesterolemic effects is essential. The reduced levels of diagnostic marker enzymes, triglycerides, LDL cholesterol, lipid peroxides and enhanced levels of phospholipids, HDL cholesterol, antioxidants such as vitamin E and GSH, in the blood serum and heart tissues of albino rats upon consumption of CS diets could be attributed to the presence of anti-inflammatory LC-PUFAs and vitamin E in the fish oils. Further, the protective effects of fish oil on the risk of CVD may be due to the synergistic effects of the nutrients in fish and not solely to the

presence of LC-PUFAs. To summarize, fish oils may be effective dietary supplements in the management of various diseases in which oxidant/antioxidant defense mechanisms are decelerated.

Chapter 5. Effect of Centrophorus scalpratus liver oil against CFA-induced arthritis in Albino rats

Rheumatoid arthritis is a systemic autoimmune disease that causes chronic inflammation of the joints, a potential debilitating inflammatory disorder that affects more than 7 million people in India every year. The most suitable remedy for the treatment of arthritis is to rely on the use of non-steroidal anti inflammatory drugs (NSAIDs). But most NSAIDs available today have their negative side effects as they block the activity of the enzyme cyclooxygenase I (COX I) which otherwise produces metabolites required for the normal homeostasis. Natural drugs/ nutraceuticals which can selectively inhibit the activity of COX II during inflammation are the need of the hour. Clinical trials on rheumatoid arthritis patients who relied on fish oil as a supplement of their diet have shown decreased joint tenderness, joint pain, swelling and morning stiffness (Kremer *et al.*, 1990).

The objectives of the present study were to study the efficacy of *Centrophorus scalpratus* (CS) liver oil against Complete Freund's Adjuvant-induced arthritis and to compare the anti-inflammatory activity of this oil with a traditionally used anti-inflammatory substance gingerol (oleoresin extracted from ginger.)

Thirty wistar strain male Albino rats were divided into 5 groups of 6 animals each and were fed with standard rat feed throughout the experiment (36 days). Group I served as normal control, groups II, III & IV were induced with arthritis using CFA (0.01 ml suspension in paraffin oil--10mg/ml heat killed *Mycobacterium tuberculosae* injected into right hind paw), the animals were allowed to develop maximum inflammation (as recorded by monitoring the paw size) till the 14th day and from the 15th day onwards, groups III and IV animals were treated with the CS liver oil and gingerol respectively. Group V served as the control group for the fish oil administered – no inflammation induced but treated with the *Centrophorus* oil from the 15th day.

The major results of the study were as follows. Significant lowering ($p<0.05$) of paw size/edema and a significant increase in bodyweights for III and IV animals were noted from the 15th day of the experiment. Significant ($p<0.05$) increase in liver weight of Group II animals was also observed. The faster multiplication of acute phase proteins during inflammation might be the probable reason for increase in liver mass noticed in group II animals. The results of bone histopathology indicated periarticular inflammation with edema and infiltration of polymorphonuclear neutrophils and lymphocytic cells, synovitis and synovial hyperplasia as well as a severe loss of cartilage and bone in II animals whereas administration of CS oil reduced leukocyte infiltration, inflammation, hyperplastic synovitis, erosion of articular cartilage and osteolysis and stabilized lesions in III and IV animals.

The presence of inflammation was confirmed with the significantly ($p<0.05$) elevated levels of marker enzymes of cyclooxygenase (COX), myeloperoxidase (MPO) and nitric oxide (NO) levels. COX levels were significantly high in paw tissue homogenates of II, III and IV animals; however there was a significant inhibition of COX II (70%) activity in III and IV treated animals, thus highlighting the anti-inflammatory properties of the CS oil and gingerol extracts. Corresponding and significant decline ($p<0.05$) in MPO and NO levels were observed in III and IV animals compared to II animals.

A significant rise in blood and liver proteins was observed in animals during CFA induced inflammation. The decline in protein levels in III and IV to near normal levels upon treatment with CS oil and gingerol showed the membrane stabilizing and tissue regenerative capacity of CS and gingerol. The significant increase ($p<0.05$) in positive acute phase proteins observed during inflammation was evident from the increase in protein bands as seen in their SDS-PAGE results. This was further confirmed with the increase in Asp, Thr, Pro, Ala, Val, Ile, Leu, Phe, Lys and Arg observed in inflammation induced untreated Group II. The increase in sulphur-containing amino acids Cys and Met in II might be interpreted as a sign of an enhanced glutathione (GSH) catabolism during inflammation. There was a significant decline ($p<0.05$) in diagnostic marker enzymes AST, ALT and LDH and a corresponding decrease in end products of protein metabolism creatinine and urea was observed in inflammation treated Groups III & IV compared to II. There was a significant rise in the levels of hexose, hexosamine and sialic acid in both blood and liver of arthritic rats. The significant ($p<0.05$) elevations observed in II animals might be due to the enhanced synthesis and release of these glycoprotein components from liver to systemic circulation.

The impact of CFA-induced arthritis on carbohydrate metabolism in blood was studied. Significant ($p<0.05$) drop was observed in the blood glucose level in II animals. Inhibited activities of glucose-6-phosphatase and fructose-1,6-biphosphatase during gluconeogenesis would have resulted in the reduced blood glucose levels. The activities of glycolytic enzymes hexokinase and aldolase were high in inflammatory conditions as evident from the observed low levels of glucose in the blood. Since inflammatory cells exhibit higher catabolism of glucose for energy a significant ($p<0.05$) drop in the activity of the glycolytic enzymes in III and IV Indicated the ability of the samples to limit the supply of ATP to inflamed cells through glycolysis. The elevated activites of glycolytic enzymes and the decreased activites of gluconeogenic enzymes in inflammation indicated faster utilisation of glucose from the hepatic glycogen storage. This was evident from the significant drop ($p<0.05$) in glycogen levels in inflamed liver tissues.

CFA-induced arthritis increased oxidative stress within the body as recorded by the high levels of lysosomal enzymes B-glucosidase, B-glucoronidase, B-galactosidase, acid proteinase and alkaline phosphatase in liver tissues of the respective animals. The membrane stabilizing properties of n3 PUFA,squalene & alkyl-oxy-glycerols contained in CS oil and flavanoids in gingerol would have

rendered beneficial effects in controlling the spread of inflammation in III and IV animals respectively. There was a significant reduction ($p<0.05$) in activity of ATPases in II animals compared to the other groups, which might have been due to the disruption of membrane structure during inflammation. A significant ($p<0.05$) rise was observed in the level of calcium in the plasma and liver tissues of CFA-administered Group II rats compared with Group I control animals. CFA administration would have induced lipolysis and production of reactive oxygen species that destabilized the hapatic membrane, resulting in the inactivation of Na^+, K^+-ATPase and the depletion of plasma potassium and rise in sodium concentration.

Inflammation impairs the normal homeostasis process leading to alterations in plasma lipid and lipoprotein patterns. Increased mobilization of LDL cholesterol from the blood into the hepatic membranes might have resulted in abnormal deposition of cholesterol in the liver of II animals. The impact of oral supplementation of CS oil and gingerol extracts to animals under inflammatory conditions markedly reversed the plasma ($p<0.05$) and hepatic levels ($p<0.05$) of cholesterol & triglycerides to normal condition in group III & IV rats. The ability of n-3 PUFA in CS oil to enhance the activity of lipoprotein lipase may facilitate the utilization of triglycerides and thus lower the triglyceride level in III animals.

The impact of CFA-induced arthritis on enzymatic and non-enzymatic antioxidant defenses in the liver tissues were studied. High concentration of lipid peroxides and reduced rate of restoration of GSH by lower Glutathione reductase activity might be responsible for the lower level of GSH observed in hepatic tissue of group II rats ($p<0.05$) compared to group I rats. Added to this, excessive utilization of antioxidant vitamins for quenching elevated levels of free radicals produced by CFA and lack of efficient antioxidant system in the inflammed tissue to restore vitamin levels back into normalcy, resulted in decrease in antioxidant vitamin C and vitamin E levels in II. The significant decrease in the activities of glutathione S transferase ($p<0.05$), glutathione peroxidase ($p<0.05$) and glutathione reductase ($p<0.05$) in group II compared to group I rats might be due to the high demand for the antioxidants to detoxify the electrophiles produced by CFA. Diet supplemented with fish oil and gingerol concentrate significantly ($p<0.05$) increased the level of superoxide dismutase ($p<0.05$) and catalase ($p<0.05$) in groups III & IV rats compared to group II rats. Higher activity of both the enzymes is thought to be responsiveness of body's defense mechanism to deleterious radicals produced excessively upon exposure to inflammatory agents.

The results of the present study indicated that both *Centrophorus scalpratus* liver oils as well as gingerol extracts proved to be effective natural remedies against CFA-induced arthritis in Albino rats.

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AA	-	Arachidonic acid
ACP	-	acid phosphatase
ADP	-	Adenosine -5-diphosphate
AI	-	*Apristurus indicus*
AICR	-	American Institute for Cancer Research
AIDs	-	Acquired Immuno deficiency syndrome
ALP	-	Alkaline Phosphatase
ALT	-	Alanine aminotransferase
ANOVA	-	Analysis of variance
ANSA	-	Aminonaphthosulfonic acid
AOEs	-	Antioxidant Enzymes
AOM	-	Azoxymethane
APS	-	Ammonium per sulphate
ARA	-	Arachidonic Acid
AST	-	Aspartate aminotransferase
ATP	-	Adenosine triphosphate
a-TE	-	alpha tocopherol equivalents
BSA	-	Bovine serum albumin
Ca^{2+}	-	Calcium ion
Cal	-	Calories
cAMP	-	Cyclic adenosine monophosphate
CAT	-	Catalase
°C	-	Degree celsius
CC	-	*Centroselachus crepidater*
CDNB	-	1-Chloro-2, 4-dinitrobenzene
CFA	-	Complete Freund's Adjuvant
CHM	-	Chloroform, Heptane, Methanol
CHD	-	Coronary Heart Disease
CIFT	-	Central Institute of Fisheries Technology
CLA	-	Conjugated linoleic acid
CoA	-	Coenzyme A
COX	-	Cyclooxygenase
CPCSEA	-	Committee for the Purpose of Control and supervision of experimental animals
CPUE	-	Catch per unit effort
CS	-	*Centrophorus scalpratus*
Cu	-	Cubic
$CuSO_4$		Copper sulphate
CVD	-	Cardiovascular disease
DETC	-	Diethyldithiocarbomate
DHA	-	Docosahexaenoic acid
DHGLA	-	Dihomo gamma linolenic acid
dl	-	Decilitre
DMBA	-	Dimethyl benzanthracene
DMARD	-	Disease modifying anti-rheumatic drugs
DNA	-	Deoxyribonucleic acid
DNPH	-	2,4 Dinitrophenyl hydrazine
DOX	-	Doxorobucin

DPA	-	Docosapentaenoic acid
DTNB	-	5,5'-Dithiobis(2-nitrobenzoic acid)
EBV	-	Epstein bar virus
ECM	-	Extracellular Matrix
EDTA.	-	Ethelene diamine tetraacetic acid
EFAs	-	Essential fatty acids
EPA	-	Eicosapentaenoic acid
ESR	-	Erythrocyte Sedimentation rate
FA	-	Fatty acids
FAO	-	Food and Agriculture Organization
FDA	-	Food and Drugs Administration
$FeCl_3$	-	Ferric chloride
FFA	-	Free fatty acid
Fig	-	Figure
FORV	-	Fisheries Oceanic Research Vessel
FPTase	-	Farnesyl protein transferase
FSI	-	Fisheries Survey of India
g	-	grams
GLA	-	Gamma linolenic acid
GPx	-	Glutathione peroxidase
GR	-	Glutathione reductase
GSH	-	Reduced glutathione
GSSG	-	Oxidised glutathione
GST	-	Glutathione-S-tranferase
GT	-	Glutamyl transferase
h	-	Hours
H_2O_2	-	Hydrogen peroxide
H_2SO_4	-	Sulphuric acid
HCl	-	Hydrochloric acid
HDL	-	High density lipoprotein
HFA	-	Human-flora-associated
HGF/SF	-	Hepatocyte growth factors/scatter factor
HK	-	Hexokinase
HMG CoA	-	3-hydroxy-3-methylglutaryl coenzyme A
HODE	-	Hydroxy Octadecadienoic acid
HPLC	-	High performance liquid chromatography
HUFA	-	Highly Unsaturated Fatty Acids
ICMR	-	Indian Council of Medical Research
i.p	-	Intra peritoneal
IAEC	-	Institutional Animal Ethics Committee
IARC	-	International Agency for Research on Cancer
ICAM	-	Intercellular adhesion molecule
IFN	-	Interferon
IGF	-	Insulin-like growth factor
IL	-	Interleukins
iNOS	-	Isoforms of nitric oxide synthase
IU	-	International unit
K^+	-	Potassium ion
K_2HPO_4	-	Dipotassium hydrogen phosphate
KCl	-	Potassium chloride

Kg	-	Kilogram
KH_2PO_4	-	Potassium dihydrogen phosphate
KOH	-	Potassium hydroxide
l	-	litre
LA	-	Linoleic acid
LDH	-	Lactate dehydrogenase
LDL	-	Low density lipoprotein
LIF	-	Leukemia inhibitory factor
LPL	-	Lipoprotein lipase
LPS	–	Lipopolysaccharide
LPO	-	Lipid peroxides
LTB	-	Leukotrienes
MDA	-	Malondialdehyde
mg	-	Milligram
Mg^{2+}	-	Magnesium ion
µg	-	Microgram
min	-	Minutes
ml	-	Millilitre
mm	-	millimeter
µmol	-	Micromol
mM	-	Millimolar
MSY	-	Maximum Sustainable Yield
mRNA	-	Messenger Ribonucleic acid
MTD	-	maximum tolerated dose
MUFA	-	Mono unsaturated fatty acid
N	-	Normal
n-3	-	Omega 3
n-6	-	Omega 6
NR	-	*Neoharriotta raleighana*
HP	-	*Harriotta pinnata*
Na^+	-	Sodium ion
Na_2CO_3	-	Sodium carbonate
Na_2SO_4	-	Sodium sulphate
NaCl	-	Sodium chloride
NAD+	-	Nicotinamide adenine dinucleotide
NADH	-	Reduced nicotinamide adenine dinucleotide
NADP	-	Nicotinamide adenine dinucleotide phosphate
NADPH	-	Nicotinamide adenine dinucleotide phosphate (reduced)
$NaHCO_3$	-	Sodium bi carbonate
NaN_3	-	Sodium azide
NaOH	-	Sodium hydroxide
NCRP	-	National cancer registry programme
NF-kB	-	Nuclear transcription factor kB
Nitrosamine	-	N-nitroso-diethylamine
NSAID	-	Non-steroidal anti-inflammatory drug
nm	-	Nanometer
nmol	-	nano mole
NO	-	Nitric oxide
OD	-	Optical density
ODC	-	Ornithine decarboxylase

OPA	-	O- Phthaladehyde
O_2	-	oxygen
p	-	Probalility
PAF	-	Platelet-activating factor
PB	-	Phenobarbital
PBS	-	Phosphate buffer saline
PDGF	-	Plate derived growth factors
PGE	-	Prostaglandin E
Pi	-	Inorganic phosphorus
PIF	-	Proteolysis inducing factor
PKC	-	Protein kinase C
PMCA	-	Plasma membrane calcium ATPase
PMF	-	Protein mobilizing factor
PPARs	-	Peroxisome proliferator activated receptors
PUFA	-	Poly unsaturated fatty acids
RA	-	Rheumatoid arthritis
RBC	-	Red Blood Corpuscles
RDA	-	Recommended daily allowances
RNA	-	Ribonucleic acid
ROS	-	Reactive oxygen species
rpm	-	Revolution per minute
SD	-	Standard deviation
SFA	-	Saturated fatty acid
SDS-PAGE	-	Sodium dodecil sulphate – polyacrylamide gel electrophoresis
SOD	-	Superoxide dismutase
TBA	-	Thiobarbituric acid
TCA	-	Trichloroacetic acid
TEMED	-	N,N,N^1,N^1 -Tetra ethyl methylene diamine
TEP	-	Tetraethoxy propane
TNF	-	Tumor necrosis factor
TM	–	Trade mark
U	-	Unit
UICC	-	International Union against cancer
US	-	United States
UV	-	Ultra violet
v/v	-	Volume / Volume
VCAM	-	Vascular cell adhesion molecule
VEGF	-	Vascular endothelial growth factor
VLDL	-	Very low-density lipoprotein
w/v	-	Weight / Volume
WBC	-	White blood count
WHO	-	World health organization

Chapter 1.

The Marine Fishery Resources of the Indian Exclusive Economic Zone

Chapter 1. The Marine Fishery Resources of the Indian Exclusive Economic Zone

1.1 INTRODUCTION

With a seacoast of 8,118 km, an exclusive economic zone (EEZ) of 2 million square km, and with an area of about 30,000 square km under aquaculture, India produces close to six million tonnes of fish, over 4 per cent of the world fish production (Internet reference[a]). While the marine waters upto 50m depth have been fully exploited, those beyond, remain unexplored. There is an ever increasing demand for fishery resources as food. The coastal fishery resources of the country are dwindling at a rapid pace and it becomes highly imperative that we search for alternate fishery resources for food. An alternative option with us is to hunt for marine fishery resources. Studies pertaining to proximate composition, amino acid and fatty acid composition are essential to understand the nutraceutical values of the deep sea fishery resources that harbor the seas of the Indian EEZ.

For over two decades, accepted dietary guidance has stressed the importance of choosing a diet that is low in fat, saturated fat, and cholesterol. Dietary omega-3 fatty acids play a major role in the regulation of blood pressure, clotting and in reducing blood cholesterol. Fresh water fish generally have lower levels of omega-3 PUFAs than marine fish. It is essential to understand the biochemical compositions of deep sea creatures belonging to the Indian EEZ to identify potential resources that could serve as food supplements in the fisheries industry.

The main objectives of the study were formulated

1. With a view of searching new potential fishing grounds because of the depletion of the inshore/coastal fisheries resources and due to the need for survey of deep-sea fishery resources in the EEZs of the countries in the Southeast Asian region.
2. to carry out proximate composition of deep sea fishery resources obtained during cruises onboard the FORV Sagar Sampada,
3. to study the amino acid profile of these fishery resources, since deep sea fishery resources possess large quantities of essential amino acids and an understanding of their profile would be beneficial to the industry.
4. to identify fishery resources which have appreciable lipid content and thereby analyse the bioactive potentials of marine lipids, to understand the contents of SFA, MUFA and PUFA and to calculate the n3/n6 fatty acid contents.

1.2 REVIEW OF LITERATURE

1.2.1 The deep sea

For almost all of its vast extent, the deep sea is a very dark and cold environment. Although about half of the Earth's surface lies 3,000 or more meters below water, the deep ocean is a fairly new area of study for scientists. In 1977, the ALVIN, a specially designed submersible, first entered this area of permanent darkness (Ericson, 2001). ALVIN can dive to 4,000 meters (about 13,000 feet). For a point of reference, large submarines dive to about 1,000 meters (about 3,280 feet), while scuba divers have gone to a record depth of 133 meters (about 436 feet). The pressure of the water is so great at depths below 1,000 meters that it would crush a regular submarine. All of the animals that live in the deep sea must contend with these incredible pressures.

Besides being a "high pressure" environment, the deep sea is a very dark and very cold environment. Darkness poses real problems for life as we generally think about it. The depth at which light penetrates the ocean varies greatly. However, very little light reaches below 260 meters (about 853 feet) anywhere in the ocean.

Sunlight is the original source of energy for photosynthesis. Most of the life in the ocean, then, is ultimately dependent on sunlight. Phytoplankton and algae capture solar energy and then are food for zooplankton, fish and so many other animals of the sea. It would be because of the lack of light, there would be no organisms to begin food chains, no source of energy and no flow of nutrients. This is almost true, no plants live in the deep sea. Without sunlight for phytoplankton or seaweeds, it seemed there could be no life at all in the deep sea. Scientists long thought of these regions as dark and lifeless. This made sense to everybody except that every once in a while, fishermen trawling for their catch or oceanographers on research vessels would dredge up bizarre fish from deep waters. What could these animals be eating?

The answer was found nearer the surface. When surface organisms die, their bodies decompose. The resulting decayed organic material, called detritus sinks. It looks so much like falling snow that biologists call it "marine snow". Detritus is like "slightly used food". There's still some energy and some nutrients stored in that marine snow. The animals that live in the dark waters of the deep ocean make a living feeding on the marine snow. Adapted to the dark, pressure, cold, and scarcity of food of the deep sea environment, many of these

animals look as if they could be from a science-fiction movie. At first, people thought the strange appearances of the fish might be a result of bringing them to the surface; the fish never survived the drastic temperature and pressure changes of the trip up from the depths. Now, with the development of submersibles, biologists have visited these creatures in their own habitat and brought back footage of bizarre beings that inhabit the deep, dark, cold waters.

Deep sea fish, in particular, have caught the imagination of scientists all over. Though these creatures tend to look fierce, most, but not all, are quite small. Their fierce appearance comes from adaptations that allow them to eat anything that comes their way in the sparsely populated waters (Ericson, 2001). Many have sharp fang-like teeth to hold prey. Many have greatly expandable stomachs and jaws that unhinge to allow them to swallow prey larger than themselves. Some have photophores, pockets of bioluminescent bacteria, that they may use as fishing lures to attract prey close to their hungry mouths. For example, the lanternfish, which has rows of phosphorescent spots along its sides, looks like a miniature ocean liner with all its portholes illuminated.

Photophores may also function as identification tags so fish can find others of their species in order to reproduce. Special adaptations for reproduction must be important in an unlighted sea where fellow fish may be few and far between. The male anglerfish, for example, is much smaller than the female and attaches to the female, literally feeding off her blood. In return, he provides sperm to fertilize her eggs. The anglerfish also has a unique way of catching its prey. Attached to its head just above the mouth, an anglerfish has a thread-like line with a light at the end of it. The light dangles like a bit of shining bait. The anglerfish swims with its mouth open, ready to snap up any small fish that is attracted to the shining "bait".

The scarcity of food makes energy conservation a high priority for deep sea fish. Since large fish require correspondingly large amounts of energy to survive, most of the fish are small. Also, most of the deep sea fish are passive hunters, waiting for a meal to swim by; active hunting consumes more energy. Our knowledge of deep sea life has been greatly expanded by the use of a variety of mechanical devices from bathyspheres and underwater cameras to deep submersible vehicles like ALVIN.

1.2.2 Ecological and life-history patterns

Of the primary families of fishes commercially exploited on the continental shelf – the Gadidae (cods), clupeoids (sardines and anchovies), Salmonidae (salmons), Scombridae (tunas and mackerels), and Pleuronectidae (flounders) – only species belonging to the Pleuronectidae are commonly exploited in deep water. Many deepwater fisheries are based on entirely different orders, such as the Beryciformes, Zeiformes, and Scorpaeniformes. Differences at this taxonomic level indicate fundamental shifts in body plan and ecological strategy, as well as in evolutionary lineage.

1.2.3. Bank and seamount aggregating species

Many deepwater species aggregate on seamounts and banks, where they can be readily targeted and provide high yields per unit of effort. These aggregating species have evolved from distinct groups in the different major biogeographic provinces of the world ocean: orange roughy (*Hoplostethus atlanticus*) (Trachichthyidae) and the oreosomatids (Zeiformes) in the temperate South Pacific; alfonsino (*Beryx* spp.) (Berycidae) in the tropics and sub-tropics; Patagonian toothfish (*Dissostichus eleginoides*) (Nototheniidae) in the Subantarctic Southern Ocean; pelagic armorhead (*Pseudopentaceros wheeleri*) (Pentacerotidae) in the open North Pacific; and several species of *Sebastes* (Scorpaenidae) along the continental slope of the North Pacific and North Atlantic. These bank and seamount-aggregating species form a distinct guild based on common features of their body plan, proximate composition, physiology and metabolism, ecology, and life history (Koslow, 1996, 1997). They tend to be robust and deep-bodied in order to manoeuvre in the strong currents characteristic of this environment. The flesh is usually firm with a water content typically <80%, which contributes to their relatively high palatability and marketability. These fish generally do not migrate vertically. Rather, they depend on the flux of meso- and bathypelagic organisms past the seamount and on intercepting mesopelagic migrators on their downward migration, which enables them to maintain high population densities, despite the low productivity of the deep sea (Isaacs and Schwartzlose, 1965; Genin *et al.*, 1988; Tseitlin, 1985; Koslow, 1997). Many are exceptionally long-lived: orange roughy and oreosomatids apparently live to 100+ years (Tracey and Horn, 1999; Smith and Stewart, 1994) and deepwater *Sebastes* spp. to over 50 years (Chilton and Beamish, 1982; Campana *et al.*, 1990). Natural mortality rate (M) is thus exceptionally low, in the order of 0.05 or less. Growth is also very slow and maturity may be

delayed to >20 years, particularly in species like the orange roughy (and unlike *Sebastes* spp.), which develop as juveniles in deep water.

1.2.4 Slope and open seafloor-associated species

The other major group exploited in deep water belong to the Gadiformes, the most speciose order of deepwater fishes. The Macrouridae, in particular, generally dominate over relatively flat portions of the deep sea. These species are generalized predators and scavengers, feeding both in the water column and over the bottom (Haedrich and Henderson, 1974; Mauchline and Gordon, 1986). Several species have been aged to approximately 60 years and their growth rates are very low (Bergstad, 1990), although their life-history characteristics are generally not as extreme as the seamount-aggregating species. Morids (Moridae), cusk-eels (Brotulidae), and hakes (Merlucciidae) are more robust-bodied Gadiformes than the macrourids and are more active predators. Morids are generally benthopelagic, while cusk-eels are benthic. The hakes form a small but widely distributed family.

They are often the dominant piscivores over upper portions of the continental slope and typically migrate vertically into the upper waters at night to feed (Bulman and Blaber, 1986). Their productivity is thereby linked directly to the near-surface food web, and their life history is similar to that of shelf-dwelling cods: blue grandier (*Macrouronus novaezelandiae*), for example, matures at 4 to 7 years old and lives to a maximum of 25 years (Kenchington and Augustine, 1987). Other important continental slope species include the Greenland halibut (*Reinhardtius hippoglossoides*) (Pleuronectidae) and sablefish (*Anoplopoma fimbria*) (Anoplopomatidae). Sablefish is long-lived, but the juveniles grow rapidly in near-surface waters and they mature relatively early (Chilton and Beamish, 1982; Mason *et al., 1983)*.

1.2.5 Fishing crafts and gear

About 325,000 fishing crafts of various types, primarily of the small-scale variety, operate in and around the Indian EEZ. In India, Burma, Bangladesh, Maldives and north Sumatra, the number of non-mechanized craft far exceeds that of mechanized craft. The percentage of non-mechanized craft is 95% in India and Burma, 80% in the Maldives, 75% in Bangladesh, and 72% in the northern half of Sumatra. In Thailand and Malaysia, the mechanized fleet significantly exceeds the non-mechanized fleet which accounts for only 10-12% of the total

number. In Sri Lanka, the two types are almost equal with non-mechanized craft making up 51% of the fleet. Only Malaysia and Thailand exploit about 50% or more of their EEZs in the Bay of Bengal region. The number of fishing craft has increased significantly in Bangladesh and Burma in recent years; this trend is not visible in other countries.

A few types of traditional craft are common to some countries. Examples: the log rafts of Sri Lanka and India, and the pole-and-line crafts in the Maldives and India. On the whole, however, traditional crafts in different countries are not directly comparable in design and operational and fishing efficiency.

However, with advancements in technology the use of motorized vessels with High Speed Demersal Trawl (HSDT) and EXPO model trawl nets are catching pace.

1.2.6 Production highlights and trends

The annual production from the EEZs of neighbouring countries Sri Lanka, Bangladesh, Burma, Thailand, Malaysia and Maldives is approximately 2.2 million tonnes, while the production from international waters of the area—by far eastern nations—is in the region of 6,000 tonnes/annum, excluding sharks.

Percentage contributions to the total production, according to available figures, are: Malaysia 20%, Burma 20%, India 19%, Indonesia 15%, Sri Lanka 8.8%, Thailand 8.7%, Bangladesh 6.6%, Maldives 1.4%.

Only Sri Lanka and Bangladesh claim a steady increase in total production. The years of peak production for other countries were: Thailand (west coast) 1973, India (east coast) and Sumatra island 1975, Malaysia (west coast) 1980, Burma and Maldives 1981. The reliability of catch statistics for at least some of these countries is a matter for concern.

1.2.7 Fish species in EEZ of neighbouring countries

Over 215 demersal fish species, 65 pelagic species, 20 shrimp species and 40 cartilagenous species have been identified from the regions of the Indian EEZ. The grouping of species differs from country to country. Estimates of catch composition are reasonably good in some countries, incomplete in a few and totally lacking in the others. In Maldives, Sri Lanka and the west coast of Sumatra, the production of pelagics exceeds that of demersals, according to

available information. In other countries or EEZs, the production of demersals exceeds that of pelagics.

In recent years, ponyfishes on the east coast of India; small demersals (trash fish) and mackerels in Malaysia; and *Hilsa ilisha* in Bangladesh appear to show a significant increase in production. Shrimp production and catch rates show a declining trend in the project area, except in Sri Lanka and Bangladesh, which, however, are minor producers. The production of coastal surface tuna fluctuates in the Maldives and India, and shows a slight increase in Sri Lanka, Thailand and Sumatra. Since 1980, there has been no evidence of increased production of oceanic deep-swimming tunas. The production of small pelagics has declined in Sri Lanka, India (particularly the state of Tamil Nadu), Thailand and Indonesia, as also that of valuable demersals in Bangladesh, Thailand and Malaysia. The demersals of Indonesia declined sharply before the 1980 ban on trawling. An increase in the catches of threadfin breams *(Nemipterus* spp.) and bulls eye *(Priacanthus* spp.) is evident in Thai and Malaysian fisheries, because the trawl fishery has moved to deeper waters.

1.2.8 Status of exploited stocks and potential for development

Reliable estimates of catch in relation to effort are lacking in almost all the participating countries. Biological studies have been qualitative and insufficient for stock assessment. Correct species identification also presents a problem in some areas. In the Maldives, the main tuna stocks in the exploited range appear to have been intensively exploited; further increases may have to come from the unexploited range of its EEZ. However, production of other pelagics and demersals from the reef waters could go up. In Sri Lanka too, the production of large pelagics seems to show a trend similar to that in the Maldives; the status of small pelagics could not, however, be evaluated. As for demersals, the production of valuable demersals could possibly rise; that of small and less valuable demersals could rise significantly. On the east coast of India, major stocks appear to have been intensively exploited; a significant increase from exploited areas seems unlikely, a possible exception being the northern part. In Bangladesh, demersal production may be close to optimum yield levels, shrimp production may be close to the maximum potential or perhaps beyond it; the status of pelagics is rather vague. In Burma, recent surveys indicate the possibility of a 35% rise in production from the continental shelf area.

In Thailand and Malaysia, the major resources are already being heavily exploited. A rational increase in production from the exploited ranges does not seem possible. Possible exceptions: some crustaceans and molluscs. In the Indonesian waters of the Malacca Straits, demersal fish and shrimp stocks exceeded the MSY before the 1980 ban on trawling, but a 20% increase in the production of small pelagics over the 1980 level seems possible. On the west coast, a 40% increase in demersal production may be possible, but the prospects for small pelagics and shrimps in presently exploited areas are not bright.

1.2.9 Potential in unexploited ranges of the EEZs

Acoustic surveys and experimental trawling operations indicate substantial resources of deep sea fish, shrimp and lobsters in the unexploited ranges (80-350m) of almost all the EEZs. However, the economic viability of harvesting deep sea shrimps and lobsters is uncertain; so is the commercial value of deep sea fishes. Tunas and sharks constitute the main pelagic resource in the unexploited ranges of the EEZs, excluding that of Malaysia. Possibilities for expanding surface fishery for tunas are favourable in the EEZs of Maldives, Sri Lanka, India, Thailand and Indonesia (west coast of Sumatra). The oceanic long-line fishery for deep-swimming tunas in the Indian Ocean as a whole exceeded the MSY in the 1970s. Future entry into this fishery in the project area, therefore, depends on the reduction of fishing effort by the far eastern nations.

1.2.10 Management of marine fishery resources in the region

Management of marine resources is difficult at the national level; it is even more so at a multinational or regional level. Nonetheless, it is essential. Some of the problems in determining and applying management measures are lack of biological and economic information; poor linkage between research and statistical institutions; insufficient coordination; enforcement problems such as non-cooperative fishermen socio-political factors; difficulties of inspection; limited power vested in enforcement officers; and the cost of the whole process of implementation, inspection and legal action against erring fishermen.

Some of the management measures that one does encounter in the area are mesh-size regulation; allocation of fishing ranges according to craft size and type of fishing; ban on trawling; closed fishing seasons and areas.

1.2.11 The FSI Agenda

The Fisheries Survey of India has already surveyed the demersal resources in all the coastal sectors and the stocks are under advanced levels of exploitation. As the resources utilisation results in fluctuations of stock structure and stock densities and as responsible management and exploitation of the stocks are necessarily to be based on updated knowledge on the resource base, the institute continuously monitor the stocks in the inner continental shelf areas.

1.2.11.1 Deep sea resources survey

FSI has made considerable progress in the investigation of demersal fish stocks in the outer continental shelf. These areas support several un-conventional stocks that are scarcely exploited and some of these are the candidate stocks for further development of shelf fisheries in the country. Since the desired level of coverage is yet to be achieved in some of the sectors, FSI continues to undertake intensive trawl survey in these areas all around the mainland.

1.2.11.2 Neritic pelagic Resources survey

In view of the large-scale annual variability of pelagic stocks, their appraisal assumes significance for optimum utilisation and responsible management. Though FSI has made preliminary survey of neritic pelagic stocks by mid – water trawling and purse-seining in some sections of Indian coast, this is a major un-finished agenda the institute is taking up again.

1.2.11.3 Survey of continental slope resources

Trawl surveys have revealed availability of several stocks of deep sea crustaceans and finfishes in different sectors of the continental slope. Some of these stocks are of high economic value but are very sensitive requiring scientific and rational approach in their utilization and management. Trawl surveys are in progress to enable obtaining a complete picture on the resources composition, distribution and stock densities in the continental slope area all along the EEZ

1.2.11.4 Biological studies

Studies on size, rate of growth, age distribution, recruitment etc. are central aspects to provide prognosis on development of commercially important resources. Food and feeding habits and prey-predator relationships provide valuable insight to the ecological succession and interaction in the ecosystem. Information on reproductive biology is crucial for identifying some of the conservation and regulatory approaches. Considering such significance of biological information in scientific management of fishery resources, FSI continues to gather biological data on different stocks.

1.2.11.5 Andaman and Nicobar resource survey

The EEZ in and around the Andaman &Nicobar archipelago, forming about 30 per cent of the Indian EEZ, accounts for just one per cent of the fish production. Apparently the development prospects are enormous. FSI is currently undertaking survey of demersal resources in the deeper waters and the continental slope and longline survey for larger pelagic stocks within the EEZ.

1.2.11.6 Studies on Antarctic krill

Though the demand for fish and fish products is ever increasing, the production from the capture fishery has necessarily to be limited within the sustainable levels. In such a scenario one of the most promising options for augmenting marine fish production would be utilization of the huge biomass of krill and other harvestable resources in the Antarctic Ocean. Jointly with the DOD, Department of Science & Technology and other national and International institutes the FSI has already initiated studies on krill resources in the Indian Ocean sector of the Southern Ocean.

1.2.11.7 Eco-friendly and diversified fishing practices

In order to develop and promote technologies and fishing gear which will not add to physical and biological degradation of marine ecosystem and will minimize incidence of by-catch, FSI undertakes experimental fishing with eco-friendly and diversified fishing methods like squid jigging, trap fishing etc.

dispersal, coupled with larval retention along seamount chains (Parker and Tunnicliffe, 1994; Mullineaux and Mills, 1997). The high proportion of new species in the Tasmanian study indicates substantial differences in species composition between seamounts of Tasmania and New Zealand (cf. Probert *et al.*, 1997; Koslow and Gowlett-Holmes, 1998). Richer de Forges (1998) found only a 5% mean overlap in species between ridge systems at the same latitude and separated by only *about* 1000 kilometers in the Coral and Tasman Seas.

Concerns about the impacts of trawling on benthic seamount fauna led to one of the world's first deepwater marine reserves being established in 1995 over an area of 370 km^2 on the continental slope south of Tasmania. The reserve enclosed 14 seamounts in the vicinity of an orange roughy fishing ground. A study is currently underway to assess the impact of trawling on New Zealand seamounts. Given the high degree of endemism, adequate conservation will require a network of reserves both within areas of national jurisdiction and on the high seas. The size and distribution of the reserves should be based on a better understanding of the biogeography, reproductive strategies and ecology of the benthic fauna and is associated with deepwater fishes. In some fisheries, changes in fishing practice, such as switching from trawling to long-lining, should be considered.

1.2.13 Nutrition from Seafoods

Access to sufficient food of an adequate quality to maintain normal body composition and function throughout the life-cycle is fundamental to maintaining health. A source of protein is an essential element of a healthy diet, allowing both growth and maintenance of the 25 000 proteins encoded within the human genome, as well as other nitrogenous compounds, which together form the body's dynamic system of structural and functional elements that exchange nitrogen with the environment. The amount of protein that has to be consumed, as part of an otherwise nutritionally adequate diet, to achieve the desired structure and function is identified as the requirement.

Fish and seafood provide some of the leanest sources of protein and are one of the only natural sources of the omega 3 fatty acids – EPA and DHA. The American Heart Association recommends that everybody gets at least 2 servings of oily fish each week because research has proved time and again that both EPA and DHA support a healthy

cardiovascular system. The following chart shows which fish and seafood are the leanest sources of protein compared to fat content.

Fish & seafood	Serving size	Total calories	Protein (g)	Fat (g)
Crab (Alaska king)	One leg	130	26	2
Cod (Atlantic)	1 fillet	189	41	2
Cod (Pacific)	1 fillet	95	21	1
Flounder (sole, flatfish)	1 fillet	149	31	2
Lobster (Northern)	1 cup	142	30	1
Salmon (wild,Atlantic)	154g	346	40	21
Shrimp	22g	222	5	1
Squid	102g	40	5	2
Tuna (albacore)	172g	220	41	5
Tuna (bluefin)	302g	156	25	5

The innumerable health benefits of long chain n3 polyunsaturated fatty acids and the significance of a balanced combination of n3:n6 fatty acids have been discussed in the following chapters of this book. It may also be noted here that fresh water fish generally have lower levels of omega-3 PUFAs than marine fish. Therefore the need to hunt alternative sources of fishery resources becomes essential not only to feed the growing masses but also to conserve the dwindling coastal resources of the Indian nation. Very little literature is available regarding the biochemical compositions of deep sea creatures belonging to the Indian EEZ and hence it was decided to analyse the proximate composition of certain marine resources obtained during cruises on the FORV Sagar Sampada, analyse those resources with appreciable lipid content and thereby study their fatty acid and amino acid profiles.

1.3 MATERIALS AND METHODS

Deep sea exploratory trawling operations were carried out during cruise no. 241, 247 (Leg-I & II), 250 and 252 of FORV Sagar Sampada. Suitable demersal trawling grounds beyond 200m depths were identified using echo sounders EK 60 and EK 100 provided onboard. EXPO model fish trawl and HSDT (CV) were used for stock assessment studies. Gear performance at various depths was studied using Simrad Integrated Trawl Information (ITI) System. The various cruises conducted by the Ministry of Earth Sciences, New Delhi, in association with the Centre for Marine Living Resources and Ecology, Cochin and the Central Institute of Fisheries Technology, Cochin, have been shown in table 1a.

Table 1a. Participation of the team from the Central Institute of Fisheries Technology, Cochin in cruises

Cruise participated	Area covered	Personnel participated (Designation)
No. 241	Southwest coast of India	Shri U. Sreedhar (Chief Scientist), Shri G.V.S. Sudhakar (JRF) and Shri Mathen Mathew (JRF).
No.247 (Leg-I)	Northeast coast of India	Shri G.V.S. Sudhakar (JRF)
No. 247 (Leg-II)	Southeast coast of India	Shri U. Sreedhar (Chief Scientist)
No. 250	Southwest coast of India	Shri G.V.S. Sudhakar (JRF) and Shri Mathen Mathew (JRF).
No. 252	Andaman waters	Shri G.V.S. Sudhakar (JRF) and Shri Mathen Mathew (JRF).

Samples collected during these operations were identified to least taxon using standard keys and sorted out from the catch and when time limited, random sub samples were examined. Stocks were estimated for different gears, latitude wise and depth wise

along the continental slope of south-west, east coast of India and Andaman waters. Stock sizes are expressed as CPUE (kg/h).

Length frequency data was collected to study their age, growth rate, recruitment pattern and mortality rates. Fishes were dissected for their stomach contents and to identify the sex and stage of maturity. The stomach contents were quantified as percentage frequency occurrence (%FO). Gut contents were grouped into higher taxonomic categories for analysis. The food components were identified wherever up to the genus or species level, depending on the state of digestion.

1.3.1 Fishery resource survey of the continental slope

A total of 160 deep sea species has been identified during the four cruises. Southwest and Andaman coasts are more diversified compared to East coast. The number of species identified at various regions is given in the following table.

Table 1b. Number of species identified

	Southwest coast	East coast	Andaman coast
Teleostii	79	29	46
Elasmobranchi	10	3	5
Crustacea	14	5	6
Mollusc	6	1	9
Others	6	2	8
Total	115	40	74

1.3.2 Stock assessment and their biology

1.3.2.1 Stock size:

In the present investigation stocks were assessed to a total of 41 teleost species belonging to 29 families, 5 elasmobranchs belonging to 3 families, shrimps, crabs and squids. Shrimps consisted of *Arestius alckoci, Solenocera hextii and Heterocarpus gibbosus.* Crabs were mainly represented by *Charbdis smithi* and squids represented by *Simplectoteuthis oualniensis* and *Ancistrocheirus lesuerui..*

Estimated stock sizes at different latitudes and depths are furnished in table 1c. In the west coast maximum catch was observed at $11^0 - 12^0$ Lat (122.75 kg/h) followed by 14^0-15^0 Lat (78.78 kg/h) and $13^0 - 14^0$ Lat (58.44 kg/h). Along the east coast catches dominated at $19^0 - 20^0$ Lat (566.81 kg/h) shelf area (table 1d). Considering depth wise studies high stocks were observed at 101-200m (265.79kg/h) followed by 601-700m (130.78 kg/h) and 801-900m (107.69 kg/h) (table 1f). Whereas along the Andaman waters catches dominated at 300-400 m depths of 13^0 Lat. (Table 1e & 1g). Species belonging to Priacanthidae (16.26 kg/h) and Stromatidae (10.31 kg/h) dominated the deep sea catches (Fig. 1).

In the present investigation it was discovered that some species belonging to Trachichthydae, Macruridae, certain deep sea sharks, Ommasterephid and Loligonid squids are commercially being exploited by long lines, traps and squid jigs in the Indian waters.

1.3.2.2 Biology:

A total of 1150 individuals of 27 fish species were studied for their gut contents, in which only 260 individuals were observed with food and others were with empty stomachs. Average feeding intensities are given in Figure 2. *Nemichthys scolopasu* (75.56) followed by *Bathyuroconger brane (65.00)* showed high feeding intensities. *Synagrops janponicus* (4.25), *Hoplostethus mediterraneus* (42.00), *Psenopsis cyanea* (30.14) and *Eridacnis radcliffei* (26.51) showed medium feeding intensities, while all the other species collected exhibited ≤ 20 feeding intensities. % frequency of occurrence (FO) of different foods in deep sea species is represented in table 1j. Five groups of gut contents were observed in which crustaceans were the most frequently occurring group. These crustaceans consisted of *A. alckoci, Heterocarpus sp.*, crustacean appendages. The fish group mainly consisted of mesopelagic lantern fishes, fish spines, scales and other unidentified fish species. Among the molluscs, squids formed the major feed for the higher order fishes.

1.3.3 Harvesting technologies

Gear parameters were studied during cruise No. 241& 250 by using the Integrated Trawl Information (ITI) system. In the brief attempt it was observed that vertical opening of the net decreased with increasing depth. Gear parameters are given in the following table 1i.

Table1i. Gear parameters collected with ITI system

S.No.	Gear	Depth	Warp out(m)	Ship speed(kn)	Vertical opening (m)
1	EXPO	589	1080	3.2	1.0 (error)
2	HSDT (CV)	734.6	1485	3.5	2.0
3	EXPO	732	1478	3.5	2.3
4	HSDT (CV)	440	978	3.5	2.4
5	HSDT (CV)	750	1493	3.5	1.4

Gear performance was also measured in terms of CPUE (kg/h) (table 1h). The CPUE of HSDT (CV) (67.03kg/h) was observed to be better as compared to EXPO (49.47 kg/h) model fish trawl.

Table 1c. List of species assessed for their stock size

Teleosti		
	Alepocephalidae	*Alepocephalus bicolor*
		Talismania lonifilis
	Lophidae	*Lophiomus setigerns*
	Scyliorhimidae	*Apristurus investigatoris*
	Ateleopodidae	*Ateleopus indicus*
	Congridae	*Bathyuroconger braueri*
	Bathyclupeidae	*Bathycluepa elongata*
	Macrouridae	*Bathygadus melanobranchus*
		Coelorhynchus flabellispinis
		Coryphaenoides macrolophus
	Percophidae	*Bembrops caudimaculata*
	Toredinidae	*Benthobatis morsebyi*
	Bothidae	*Chascanopsetta lugubris*
	Chaunacidae	*Chaunax pictus*
	Serranidae	*Chelidoperca investigatoris*
	Chlorophthalmidae	*Chlorophthalamus bicornis*
	Nomeidae	*Cubiceps baxteri*

Table 1d. Abundance of deep sea fishery resources at different latitudes along the continental slope of south-west and east coast of India

		West coast							East coast			
	LATITUTDE	9⁰-10⁰	10⁰-11⁰	11⁰-12⁰	12⁰-13⁰	13⁰-14⁰	14⁰-15⁰	15⁰-16⁰	11⁰-12⁰(2)	16⁰-17⁰(1)	19⁰-20⁰(2)	18⁰-19⁰(1)
		CPUE (kg/h)										
Teleosti												
	Alepocephalidae	-	1.36	7.38	-	0.16	-	-	2.00	0.42	-	-
	Lophidae	1.56	-	-	-	-	-	-	0.25	-	-	-
	Scyliorhimidae	-	-	0.17	-	-	-	-	5.125	0.6	-	-
	Ateleopodidae	0.13	-	-	-		-	-	-	-	-	-
	Congridae	-	-	0.39	1.71	-	-	-	20.53	-	-	-
	Bathyclupeidae	-	-	0.06	-	-	-	-	0.26	-	-	-
	Macrouridae	-	9.32	2.94	0.07	0.26	-	0.35	2.80	-	-	-
	Percophidae	-	-	20.66	0.44	-	-	-	-	-	-	-
	Toredinidae	-	9.32	0.6	-	-	-	-	-	-	-	-
	Bothidae	-	-	1	-	-	-	-	-	-	-	-
	Chaunacidae	-	0.34	-	-	-	0.70	-	-	-	-	-
	Serranidae	-	-	5.24	1.47	0.80	-	-	-	-	-	-
	Chlorophthalmidae	0.1	-	-	-	-	-	-	-	-	-	-
	Nomeidae	-	-	-	-	-	0.08	-	-	-	-	-
	Cynoglossidae	-	-	3.57	0.11	0.28	-	-	-	-	-	-
	Scorpaenida	-	0.01	-	-	-	-	-	0.03	-	-	-
	Ophidiidae	0.30	-	4.40	0.88	34.43	54.5	3.5	9.43	0.12	-	-
	Trachichthyidae	-	0.20	0.60	-	-	-	-	-	-	-	-
	Gempylidae	1.85	-	-	0.01	0.02	0.05	0.01	0.43	-	-	-
	Neoscopelidae	0.29	0.01	-	-	-	-	-	-	-	-	-
	Oneirodidae	-	-	0.06	-	-	-	-	-	-	-	-
	Moridae	-	-	-	-	-	2.13	-	-	-	-	-
	Priacanthidae	-	-	-	-	0.40	-	-	-	-	178.46	-

	Stromateidae	0.06	-	18.09	4.87	-	0.40	-	-	-	90.01	-
	Triglidae	-	-	9.21	0.03	-	-	-	-	-	4.22	-
	Muraenidae	-	2.68	3.87	1.00	-	2.11	5.42	56.2	5.54	-	0.38
	Acropomatidae	4.31	-	-	-	0.04	-	-	-	-	-	-
	Uranoscopidae	-	-	11	1.10	-	-	-	-	-	-	-
	Zeidae	0.19	-	-	-	-	-	-	-	-	-	-
Elasmobranchii												
	Squalidae	-	-	3.80	0.08	0.36	-	0.42	1.8	-	-	-
	Proscylliidae	0.66	-	0.80	0.41	-	2.42	2.24	2.13	-	-	-
	Rhinochimaeridae	-	1.46	3.60	-	-	4.50	-	-	-	-	-
Crustacea												
	Shrimp	-	0.16	0.5	0.18	0.26	0.11	0.15	0.55	0.72	-	16.00
	Crabs	-	-	-	-	-	6.3	-	7.42	-	-	-
Mollusc												
	Squid	1.26	-	0.29	0.19	-	1.50	-	-	-	1.90	1.60
Discard		0.00	0.00	8.00	0.00	19.33	9.07	9.10	22.66	9.72	22.30	7.05
Total CPUE		**10.71**	**24.86**	**106.23**	**12.55**	**56.34**	**83.87**	**21.19**	**131.62**	**17.12**	**296.89**	**25.03**

Table 1e. CPUE at different latitudes along the Andaman waters

	Latitude	13^0	12^0	11^0
		CPUE (kg/h)		
Teleostii				
	Alepocephalus bicolor	0.05	0.05	2.1
	Argyropelecus hemigymnus	0.01	0.30	-
	Astronesthes martensii	0.12	-	-
	Astronesthis sp	-	0.03	0.2
	Bathyclupae elongata	0.20	-	-
	Bathypterois dubius	0.01	0.96	-
	Bembrops caudimaculata	0.44	0.90	-
	Brotualtenea crassa	0.24	-	-
	Brotulenatenea sp	-	-	0.2
	Centrophorus sp	-	1.51	8
	Chanux pictus	0.42	0.05	0.7
	Chanux sp.	-	-	-
	Chauliodus sp.	0.08	1.28	-
	Chlorophthalamus bicornis	0.33	29.41	-
	Chlorophthalamus sp.	19.18	0.14	-
	Coelorinchus braveri	0.38	2.75	-
	Coraphenoides sp.	0.21	-	0.4
	Cryprosaras couesii	0.01	-	-
	Cubiceps sp	-	1.57	1.2
	Cubiceps sp	1.78	-	0.2
	Cyclothone sp.	0.07	6.55	-
	Diaphus sp.	1.18	-	0.05
	Dicrolene multifilis	0.22	0.06	1.2
	Dicrolene sp.	-	-	0.6
	Dicrolene tristis	-	0.06	0.5
	Dicrolene sp.	-	1.32	-
	Epigonus pandinois	1.43	4.25	0.20
	Eteleopus barnardi	2.32	-	5.80

Flatfish (Penonectidae)	0.01	-	-
Flatfish (Pleuromectidae)	0.14	-	-
Halieutaea sp.	-	-	-
Idiacanthus fasciola	-	-	-
Lamprogramus like	1.69	-	-
Lophius sp	0.11	-	1.20
Macroramphosus uradoi	0.22	-	-
Macrurus sp.	0.03	-	-
Malacosteus niger	0.01	-	-
Malacosteus sp.	0.01	-	-
Melanocetus sp.(angler)	-	-	0.60
Nemichthys scolofaceus	0.06	-	-
Neoharriota pinnata	-	-	10.00
Neopenula orientalis	-	0.14	0.25
Neoscopulus microchir	0.18	-	1.30
Nettastomatidae	-	-	0.30
Owstonia webri	0.14	-	-
Partriacanthodes retrospinis	0.11	1.80	-
Peristedion barberi	-	0.03	0.15
Peristedion investigatoris	0.01	0.83	-
Peristedion miniatum	0.09	-	-
Peristedion webari	0.60	-	-
Polymixia nobilis	0.84	86.17	-
Priacanthus hamrur	10.53	0.80	0.10
Psenopsis cynea	-	0.46	0.10
Pterigotrigal sp.	-	-	-
Ray	2.81	1.05	-
Rexia sp.	0.04	1.16	-
Seatarches guenteri	1.16	-	-
Setarches congimanus	0.01	-	0.80
Thyrsitops sp.	0.28	-	-
Trichiurus lepturus	-	-	0.50
Trichiurus sp.	-	0.28	-
Tydemania sp.	0.22	-	-
Apristerus sp.	-	-	0.20

Elasmobranchs		-	-	-
	Eridacnis radclifii	0.28	-	0.50
	Shark	-	3.00	25.00
	Sting ray	-	-	-
	Benthobatis sp.	-	-	1.00
Crustacea		-	0.31	-
	Shrimp	2.94	-	3.00
	Crab	0.52	-	-
	Panularis sp.	0.56	-	-
	Pleurulus sewali	2.81	-	-
Mollusc		-	7.69	-
	Ancestrocheirus sp	0.21	4.00	-
	Squids	0.10	1.00	-
	Octopus sp.	0.02	6.41	0.08
	Nautilus sp.	0.10	-	-
Others		-	-	-
	Starfish	0.02	-	-
	Seaurchin	0.20	2.00	-
	Glass sponge	0.00	-	-
	Soft corals	0.05	-	-
	Tunicates	0.60	0.50	-
	Sea anemone	0.02	0.90	0.65
	Jelly fish	-	1.00	-
Trash fish		0.28	0.50	2.50
Discard		0.16	171.22	1.00

Table 1f. Abundance of deep sea fishery resources at different depths along the continental slope of south-west and east coast of India

DEPTH (m)	101-200	201-300	301-400	401-500	501-600	601-700	701-800	801-900	901-1000	1001-1100
						CPUE(kg/h)				
Teleosti										
Alepocephalidae	-	-	-	-	-	5.92	0.04	3.32	-	-
Lophidae	-	1.26	-	-	-	0.22	-	-	-	-
Scyliorhimidae	-	-	-	-	-	3.68	1.16	-	-	-
Ateleopodidae	-	-	0.07	-	-	-	-	-	-	-
Congridae	-	-	-	-	1.17	12.16	-	3.94	-	1.27
Bathyclupeidae	-	-	-	-	-	0.18	0.13	-	-	-
Macrouridae	-	-	-	-	-	4.94	5.81	1.59	-	0.53
Percophidae	-	17.29	-	-	-	-	-	-	-	-
Toredinidae	-	-	-	-	-	1.65	6.41	-	-	-
Bothidae	-	0.84	-	-	-	-	-	-	-	-
Chaunacidae	-	-	-	-	0.58	-	0.22	-	-	-
Serranidae	-	6.14	-	-	-	-	-	-	-	-
Chlorophthalmidae	-	0.003	0.06	-	-	-	-	-	-	-
Nomeidae	-	-	-	-	0.13	-	-	-	-	-
Cynoglossidae	0.93	2.35	-	-	-	-	-	-	-	-
Scorpaenida	-	-	-	-	-	0.01	-	-	-	-
Ophidiidae	-	-	3.73	-	21.86	19.78	5.98	36.43	-	0.2
Trachichthyidae	-	-	-	-	-	0.51	0.13	-	-	-
Gempylidae	-	0.01	1.8	-	-	0.21	-	-	-	-
Neoscopelidae	-	-	0.26	-	-	-	0.006	-	-	-
Oneirodidae	-	-	-	-	-	0.05	-	-	-	-
Moridae	-	-	1.6	-	0.06	0.09	-	-	-	-

Priacanthidae	146.33	0.01	-	-	-	-	-	-	-	-
Stromateidae	76.6	15.45	0.135	-	0.24	0.4	-	-	-	-
Triglidae	4.95	6.01	-	-	-	-	-	-	-	-
Muraenidae	-	0.04	-	-	4.95	49.23	5.22	3.45	0.26	
Acropomatidae	0.03	-	3.6	-	-	-	-	-	-	-
Uranoscopidae	-	9.76	-	-	0.14	-	-	-	-	-
Zeidae	-	-	0.21	-	-	-	-	-	-	-
Elasmobranchii										-
Squalidae	-	-	-	-	-	4.77	0.24	0.27	-	-
Proscylliidae	0.185	0.115	1.4	-	1.35	2.9	1.2	-	-	-
Rhinochimaeridae	-	-	-	-	3.75	3	1.1	-	-	-
Crustacea									-	-
Shrimp	3.05	5.98	-	-	3.3	2.3	0.62	-	-	-
Crabs	-	0.125	-	-	0.2	0.19	2.62	8.1	-	-
Mollusc										-
Squid	-	-	2.66	-	2.25	0.24	-	-	-	0.38
Discard	15.44	19.01	10.7	-	9.62	10.74	6.04	12.03	3.12	1.04
Total CPUE	**247.515**	**84.393**	**26.225**	-	**49.6**	**123.17**	**36.926**	**69.13**	**3.38**	**3.42**

Table 1g. CPUE at various depths along the Andaman waters

Depth (m)	300	400	500	600
	CPUE (kg/h)			
Alepocephalus bicolor	-	-	1.05	0.24
Argyropelecus hemigymnus	0.02	-	0.01	-
Astronesthes martensii	0.18	-	-	-
Astronesthis sp	0.04	-	-	-
Bathyclupae elongata	0.21	-	-	-
Bathypterois dubius	-	-	-	0.05
Bembrops caudimaculata	0.61	0.10	-	-
Brotualtenea crassa	0.42	-	-	-
Brotulenatenea sp	0.04	-	-	-
Centrophorus sp	-	-	4.00	-
Chanux pictus	0.51	1.20	0.30	-
Chanux sp.	0.01	-	-	-
Chauliodus sp.	-	-	0.16	0.08
Chlorophthalamus bicornis	0.58	-	-	-
Chlorophthalamus sp.	25.06	-	-	-
Coelorinchus braveri	0.31	0.50	-	-
Coraphenoides sp.	0.70	0.10	0.20	0.20
Cryprosaras couesii	0.00	-	0.04	-
Cubiceps sp	0.00	-	0.60	-
Cubiceps sp)	1.81	1.36	0.10	0.05
Cyclothone sp.	0.00	-	0.18	-
Diaphus sp.	2.43	0.28	0.03	-
Dicrolene multifilis	0.14	0.27	0.60	0.12
Dicrolene tristis	0.01	-	0.30	-
Dicrolene sp.	-	-	0.25	-
Dicrolenesp.	0.01	-	-	-
Epigonus pandinois	1.53	0.82	0.10	-
Eteleopus barnardi	3.17	-	2.90	-
Flatfish (Penonectidae)	0.01	-	-	-
Flatfish (Pleuromectidae)	0.14	-	-	-
Halieutaea sp.	-	-	0.01	-
Idiacanthus fasciola	-	-	0.01	-
Lamprogramus like	1.69	-	-	-
Lophius sp	0.11	-	0.60	-
Macroramphosus uradoi	0.06	0.80	-	-
Macrurus sp.	-	0.15	-	-
Malacosteus niger	-	-	0.03	0.01
Malacosteus sp.	-	-	0.02	-
Melanocetus sp.	-	-	0.30	-
Nemichthys scolofaceus	-	-	0.06	0.20
Neoharriota pinnata	-	-	5.00	-
Neopenula orientalis	-	-	0.13	-
Neoscopulus microchir	0.16	0.26	0.65	-
Nettastomatidae	-	-	0.15	-
Owstonia webri	0.14	-	-	-
Partriacanthodes retrospinis	0.11	-	-	-
Peristedion barberi	0.36	-	0.08	-
Peristedion investigatoris	0.01	-	-	0.05
Peristedion miniatum	0.23	0.12	-	-

	Peristedion webari	0.60	-	-	-
	Polymixia nobilis	0.84	-	-	-
	Priacanthus hamrur	27.34	2.10	0.05	-
	Psenopsis cynea	0.16	-	0.05	-
	Pterigotrigal sp.	0.09	-	-	-
	Ray	2.81	-	-	-
	Rexia sp.	0.25	-	-	-
	Seatarches guenteri	0.97	2.10	-	-
	Setarches congimanus	-	-	0.43	-
	Thyrsitops sp.	0.28	-	-	-
	Trichiurus lepturus	-	-	0.25	-
	Trichiurus sp.	-	-	0.01	-
	Tydemania sp.	0.17	0.52	-	-
	Apristerus sp.	-	-	0.10	-
Elasmobranchs		-	-	-	-
	Eridacnis radclifii	0.28	-	0.25	-
	Shark	5.00	-	-	-
	Sting ray	0.60	-	-	-
	Benthobatis sp.	-	-	0.50	-
Crustacea		-	-	-	-
	Shrimp	1.95	5.00	1.63	-
	Crab	0.52	-	-	-
	Panularis sp.	0.56	-	-	-
	Pleurulus sewali	2.81	-	-	-
Mollusc		-	-	-	-
	Ancestrocheirus sp	1.63	-	0.30	-
	Squids	0.80	-	0.25	-
	Octopus sp.	0.20	-	0.09	-
	Nautilus sp.	1.38	-	-	-
Others		-	-	-	-
	Starfish	-	-	-	0.10
	Seaurchin	-	-	-	1.00
	Glass sponge	0.40	-	-	
	Soft corals	-	-	-	0.25
	Tunicates	-	-	-	3.00
	Sea anemone	0.12	-	0.33	-
	Jelly fish	0.18	-	-	-
Trash fish		0.40	1.41	0.75	-
Discard		0.10	0.78	0.50	-

Table 1h. CPUE (kg/h) for different gears

Name of the speceis	CPUE (kg/h)	
	EXPO	HSDT(CV)
Alepocephalus bicolor	1.73	0.42
Alepocephalus blanfordi	0.90	0.00
Angler fish	0.19	0.00
Apristurus investigatoris	0.04	0.00
Aristeus sp. (shrimp)	0.53	0.00
Ateleopus indicus	0.00	0.05
Bathuroconger braueri	0.70	0.00
Bathyclupea elongata	0.02	0.08
Bathygadus melanobranchus	0.08	0.00
Bembrops caudimaculata	0.00	8.87
Benthobatis morsebyi	0.45	3.88
Beryx splendens	0.00	0.83
Centrophorus lucitanicus	0.47	0.00
Centrophorus sp.	0.33	0.00
Champsodon sp.	0.00	0.01
Charybdis smithi	0.00	0.00
Chascanopsetta lugubris	0.00	0.42
Chauliodus sp.	0.05	0.00
Chaunax pictus	0.02	0.14
Chelidoperca investigatoris	0.00	2.44
Chlorophthalmus bicornis	0.00	0.01
Coelorhynchus flabellispinis	0.00	0.17
Coryphaenoides macrolophus	0.79	3.47
Crab	0.50	0.00
Cryptopsaras couesii	0.00	0.00
Cubiceps sp.	0.03	0.00
Cuttle fish	0.59	0.00
Cynoglossus carpenteri	0.00	1.63
Dicrolene sp.	0.09	0.00
Echinorhinus brucus	1.32	0.00
Ectreposebastes imus	0.00	0.00
Eel	0.01	0.02
Eridacnis radcliffei	1.47	0.36
Etmopterus pusillus	0.18	0.00
Glyptophidium argenteum	0.44	0.00
Glyptophidium lucidum	0.00	0.04
Hepthocara simum	0.03	0.00
Heterocarpus sp.	0.00	0.82
Hoplostethus mediterraneus	0.15	0.08
Hypopleuron caninum	0.51	0.07
Isopod	0.07	0.00
Jelly fish	0.00	0.26
Lamprogrammus exutus	5.71	0.00
Lamprogramus niger	0.03	0.00

Leptoteuthes sp.	0.00	0.10
Lophiomus setigerus	0.00	0.55
Luciobrotula bartschi	1.62	0.00
Melamphidae	0.00	0.00
Myctophid	0.00	0.56
Narcetes sp.	0.75	0.00
Nemichthys sp.	0.01	0.00
Neobythites sp.	0.18	0.00
Neoharriota pinnata	2.30	0.61
Neopinnula orientalis	0.02	0.62
Neoscopelus microchir	0.00	0.10
Nephropsis stewarti	0.02	0.00
Normichthys sp	0.02	0.00
Octopus	1.00	0.00
Oneirodes kreffti	0.01	0.00
Ophidiform	0.46	0.00
Parapandalus sp.	0.02	1.28
Phrynichthys wedli	0.00	0.00
Physiculus roseus	0.04	1.42
Prawns	0.00	0.97
Priacanthus hamrur	0.00	0.01
Psenopsis cyanea	0.17	10.32
Psettina sp)	0.00	0.04
Pterygotrigla hemisticta	0.00	3.85
Rays	0.00	1.25
Rexea promethoides	0.00	0.04
Rouleina ap.	0.21	0.00
Saurenchelys taeniola	3.40	0.42
Sciona sp.	0.01	0.00
Scopelengys tristis	0.00	0.00
Sharks	0.00	2.50
Shrimp	0.67	2.55
Spider crabs	0.05	0.08
Squid	2.48	0.61
Stemenosudis sp.	0.00	0.08
Stomiformes	0.01	0.11
Synagrops sp.	0.00	1.09
Synagrops philippinensis	0.00	0.43
Synapobranchus sp	0.11	0.01
Talismaniasp.	0.03	0.00
Uranoscopus sp	0.03	7.17
Xenomystax trucidens	0.75	0.83
Zenopsis conchifer	0.00	0.07
Discards	17.72	3.75
Total CPUE (kg/h)	**49.47**	**67.03**

Figure 1: Relative abundance (CPUE kg/h) of deep sea fishery resources at different depths along the continental slope of south-west and east coast of India

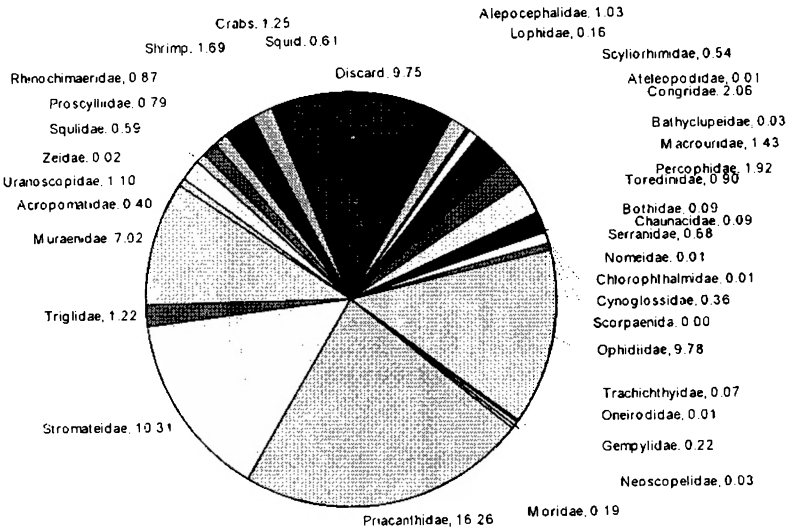
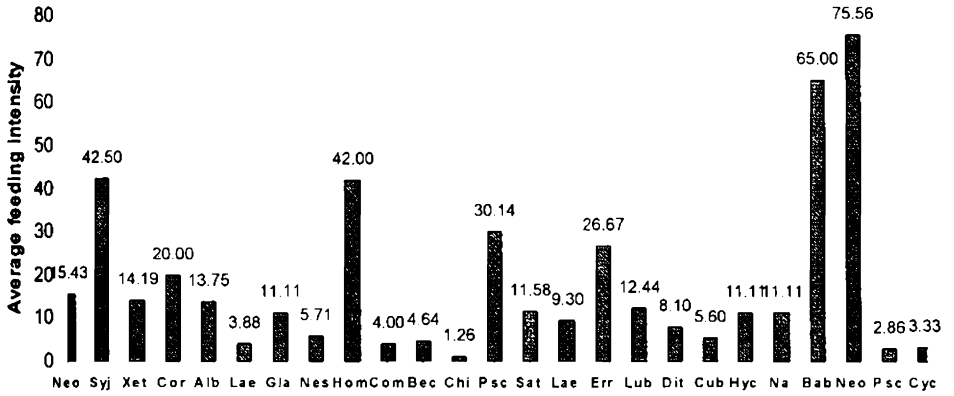


Figure 2:Feeding intensity of defferent species of deepsea fishes



Species: Neo: *Neopenula orinetalis*, Syj: *Synagrops janponicus*. Xet: *Xenomystax trucidens*, Cor: *Coloconger raniceps*, Alb: *Alepocephalus bicolor*, Lae: *Lamprogrammus exutus*, Gla: *Glytophedium argenteum*, Nes: *Neobythetis steatiticus*, Hom: *Hoplostethus mediterraneus*, Com: *Coryphaenoides macrolophus*, Bec: *Bembrops caudimaculata*, Chi: *Chelidoperca investigatoris*, Psc: *Psenopsis cyanea*, Sat: *Saurenchelys taeniola*, Lae: *Lamprogramus exutus*, Err: *Eridacnis radiclifei*, Lub: *Luciobrotula bartschi*, Dit: *Dicrolene tristis*, Cub: *Cubiceps baxteri*, Hyc: *Hypopleuran cnium*, Na: *Narcetus sp.*,Ba: *Bathyuroconger* sp., Ne: *Nemichthys sp.*, Ps: *Psettodes erumei*, Cyc: *Cynoglossus carpentari.*

Table 1j. Percentage frequency of occurrence (FO) of different food items in deep sea fishes

S.No.	Species	N	Fish	Crustaceans	Mollusc	Semi digested food
1	*Alepocephalus bicolor*	7	14.29	28.57	0.00	28.57
2	*Bathyuroconger braueri*	5	100.00	0.00	0.00	0.00
3	*Bathyuroconger sp.*	2	100.00	0.00	0.00	0.00
4	*Bembrops caudimaculata*	6	16.67	33.33	0.00	50.00
6	*Chelidoperca investigatoris*	6	16.67	33.33	0.00	50.00
7	*Coloconger raniceps*	7	30.00	0.00	20.00	50.00
8	*Coryphaenoides macrolophus*	10	30.00	20.00	0.00	50.00
9	*Cubiceps baxteri*	4	0.00	0.00	0.00	100.00
10	*Dicrolene tristis*	8	0.00	37.50	0.00	12.50
11	*Eridacnis radiclefi*	60	11.67	13.33	28.33	46.67
12	*Glyptophidium argenteum*	8	0.00	62.50	0.00	37.50
13	*Hoplostethus mediterraneus*	6	16.67	83.33	0.00	0.00
14	*Hypopleuran cnium*	1	0.00	100.00	0.00	0.00
15	*Lamprogrammus niger*	6	50.00	0.00	16.67	33.33
16	*Lamprogramus exutus*	11	27.27	18.18	9.09	45.45
17	*Luciobrotula bartschi*	13	0.00	84.62	0.00	15.38
18	*Bathytroctes calcaeratus*	5	0.00	0.00	0.00	100.00
19	*Nemichthys sp.*	9	0.00	44.44	0.00	55.56
20	*Neobytheris steatiticus*	2	0.00	100.00	0.00	0.00
21	*Neopenula orientalis*	6	16.67	0.00	50.00	33.33
22	*Psenopsis cyanea*	35	0.00	73.53	0.00	26.47
23	*Psettodes erumei*	1	0.00	0.00	0.00	100.00
24	*Raulenia sp.*	2	0.00	0.00	0.00	100.00
25	*Saurenchelys taeneloa*	7	42.86	28.57	0.00	28.57
26	*Synagrops japonicus*	16	0.00	0.00	81.25	18.75
27	*Xenomystax trucidens*	17	5.88	23.53	0.00	41.18

1.3.4 Biochemical Analyses

1.3.4.1 Proximate Composition

Proximate compositions of different marine species were carried out following the Association of the Official Analytical Chemists (AOAC, 1984) methods. All the specimens collected were defrosted, the average length and weight of the specimens recorded, using a standard ruler and a beam balance, respectively. The meat tissues of each specimen were minced in a Waring blender and a portion of it was taken for the analyses. (The liver tissues of the shark specimens were collected and studied separately). The moisture content of the fish was determined by drying the meat in an oven at 110°C for 4h. The total Nitrogen content was determined using the Kjeldahl method; the quantity of protein calculated as 6.25 x N (method 7015, AOAC, 1984). The crude fat determined using the Soxhlet apparatus and the ash content determined by dry ashing in a furnace oven at 615°C for 6h.

All chemicals and reagents used were of analytical grade and purchased from Merck, India.

1.3.4.2 Determination of moisture (AOAC, 1975)

20-30 g sample was weighed into a dried and preweighed clean petridish on a Sartorius balance. Dishes were placed in a hot air oven at 100+1°C for 6 hours. Dishes were cooled in a desiccator and weighed. This was repeated until a constant weight was obtained. Moisture is expressed as percentage.

1.3.4.3 Determination of ash content. (AOAC, 1975)

About 1-2 g of sample was transferred into a previously heated, cooled and weighed silica crucible. The sample was carbonized by burning at low red heat. Then the crucible was placed in a muffle furnace at 550°C for about four hours until a white ash was obtained. Crucibles were weighed after cooling in a desiccator. Ash content is expressed as percentage.

1.3.4.4 Determination of crude protein (AOAC, 1975)

a) Digestion of the sample

0.5-1 g of the well-minced fish sample was transferred to a kjeldahl of 100 ml capacity. A few glass beads, a pinch of digestion mixture (8 parts of K_2SO_4 and one part of $CuSO_4$) and 15 ml concentrated Sulphuric acid were also added. It was digested over a burner until the solution turns colourless. To the digested and cooled solution, distilled water was added in small quantities with shaking and cooling until the addition of water did not generate heat. It was transferred quantitatively into a 100 ml standard flask and made up.

b) Distillation

2 ml of made-up solution was transferred to the reaction chamber of the micro Kjeldahl distillation apparatus. Two drops of phenolphthalein indicator and 40%sodium hydroxide were added till the indicator changes to pink. Distillation was done for 4 minutes and ammonia liberated was absorbed into 2% boric acid containing a drop of Tashiro's indicator. The amount of ammonia liberated was

determined by titrating with N/50 sulphuric acid. Crude protein percentage was determined by multiplying 6.25 with percentage total nitrogen.

1.3.4.5 Determination of crude fat (AOAC, 1975)

About 2-3 g of accurately weighed moisture free sample was taken in a thimble plugged with cotton and was extracted with petroleum ether (40-60 °C boiling point) in a Soxhlet apparatus for about 16 hours at a condensation rate of 5-6 drops per second. Excess solvent was evaporated and the fat was dried at 100° C to a constant weight. The crude fat was calculated and expressed as percentage.

1.3.4.6 Mode of determination of amino acid & fatty acid profile

The amino acid profile was determined using a modified method of Ishida *et al.*,(1981). Briefly, about 0.1 g sample was accurately weighed into 20ml rimmed test tubes, 10 ml 6N HCl added, flushed with nitrogen and sealed, refluxed for 24 h at 110^{0}C in a hot air oven. The test tube was broken carefully and the contents were filtered through a Whatman filter paper into a 150 ml flat bottomed flask, the contents were water washed thoroughly and reduced to minimum volume by flash evaporation, made upto a known volume using 0.1N HCl, filtered through 0.45um filter cartridges and then injected into HPLC for the analysis of the amino acids. The detailed specifications of the column, the instrument, the program, the buffer systems used and the mode of analysis of amino acids have been discussed in detail in the 5th chapter of this book. Amino acid standard (Sigma chemical Co., St. Louis, USA) was also run to calculate the concentration of amino acids in the sample (Refer Plate I.2). The amount of each amino acid was expressed as g/100g nitrogen. The mode of analysis of fatty acids by using Gas Chromatograph clubbed with Flame ionisation detector has been mentioned in the 2nd chapter of this book.

1.4 RESULTS AND DISCUSSION

The proximate composition of over 20 different deep sea fishery resources from the Indian Ocean and the Arabian Sea were studied. These included the cusk eels *Lamprogrammus niger*, the snake mackerels *Neopinnula orientalis*, *Synopsis cyanea*, the ray finned fishes *Hoplostethus mediterraneus*, *Alipocephalus blanfordii*, *Bathytroctes squamosus*, the deep sea shark *Echinorhinus brucus*, the narrow nosed chimaera *Neoharriotta raleighana*, the conger *Bathyuroconger braueri*, the rose shrimp *Aristeus alcockii*, the eels *Luciobrotula corethromycter*, *Xenomystax trucidens* Fairy basslets *Chelidoperca investigatoris*,the angler fish *Lophiomus setigerus*,the ray finned fishes *Bembrops caudimaculata*, the Black-spotted gurnard *Pterygotrigla hemisticta*, Sea Anemone *Cribrinopsis fernaldi*, Octopus *Octopus vulgaris*, Cuttle fish/Squid (brown) *Sepia officinalis*, Graceful fig shell *Ficus gracilus* (meat). The percentage of moisture, protein, fat and ash contents of the deep sea creatures have been tabulated in Table 1k 3. The moisture content was found to be almost 80% for the ray finned fish *Hoplostethus mediterraneus*, the shark *Echinorhinus brucus*, as well as for other species of *Neoharriotta raleighana*, *Alipocephalus blanfordii*, *Bathyuroconger braueri*, *Lophiomus setigerus*, *Sepia officinalis and Ficus gracilus*. High lipid contents were obtained for *Echinorhinus brucus* (67% liver lipids), *Neoharriotta raleighana* (69%), *Neopinnula orientalis*, *Synopsis cyanea*, *Bathytroctes squamosus* and *Aristeus sp.* These species also recorded higher iodine values and higher amounts of non saponifiable matter (table 1m) in their oils. The common names, depth and region of collection of these species have been shown in table 1l.

Works related to the proximate composition of deep sea creatures is scarce, though a very few workers have carried out the biochemical compositions of certain deep sea species. For eg. The species of the slope and open sea slopes *Coryphaenoides rupestris* and *Anolopoma fimbria* have been found to contain greater fractions of moisture content in their muscle tissues (85 & 79% respectively) (Krzynowek and Murphy, 1987; Crabtree, 1995)

Since the bioactive potentials of marine oils are numerous and a rapidly developing field today, their activities in liver oils of certain sharks and chimaeras were studied further and have been presented in the successive chapters. The protein contents were found to be as high as 23% in the ray finned fish *Bembrops*

caudimaculata to as low as 8% in *octopus vulgaris*. High mineral contents were observed for the sea anemone *cribrinopsis fernaldi* and the graceful fig mollusk *ficus gracilus* at 2.7 and 2.8 % (w/w) levels respectively.

With respect to the amino acid profile as shown in table 1n it was noted that significantly high amounts of histidine, tyrosine, leucine and proline were observed in the tissues of *N.raleighana* numerous workers have shown that leucine, isoleucine, valine, lysine, threonine, tryptophan, methionine, phenylalanine and histidine are the indispensable amino acids. Histidine is considered to be an indispensable amino

Table 1l. Common names, depth, region of collection of deep sea creatures

No.	Species	Common name	Depth (m)	Region (off-shore)
1	*Aristeus alcockii*	*Arabian red shrimp*	*913*	*Condapore*
2	*Bathytroctes squamosus*	*Deepscale slickhead*	*916*	*Kasargode*
3	*Echinorhinus brucus*	*Bramble shark*	*673*	*Honavar*
4	*Neoharriotta raleighana*	*Narrownose chimaera*	*536*	*Gangavali*
5	*Neopinnula orientalis*	*Gemfish*	*587*	*Calicut*
6	*Psenopsis cyanea*	*Indian Ruff*	*235*	*Kannore*

Table 1m. Lipid Analyses

Species	NSM % total lipid	Iodine values	Phospholipids (g/100g tissue)
Echinorhinus brucus liver	62.00	213.49	0.64
Neoharriotta raleighana liver	78.00	270.61	0.73
Neopinnula orientalis	35.64	120.36	0.62
Synopsis cyanea	19.41	121.33	0.40
Bathytroctes squamosus	12.96	132.99	0.38
Aristeus sp.	61.27	158.42	0.79
Anemone sp.	43.16	190.09	1.18

acid because of the detrimental effects on haemoglobin concentrations that have been observed (Kriengsinyos, 2002) when individuals are fed histidine-free diets.

The deep sea shrimp A.*alcockii* was found to possess significantly high amounts of arginine and glutamic acid while the snake mackeral *P.cyanea* contained significantly high amounts of threonine and leucine. The threonine requirement is particularly nutritionally important, since it has been suggested that, after the sulfur amino acids, it is the second rate-limiting amino acid in the maintenance requirement (Syed and Hegsted, 1970; Fuller, *et al.*,1989) probably because it accounts for the largest single component of the ileal loss into the large bowel (Wang TC and Fuller MF, 1994; Millward *et al., 1994*). It is also present at low concentrations in cereals.

Table 1n. Amino acid composition of marine creatures

Amino Acids (g/100g N_2)	*Echinorhinus brucus*	*Neoharriota raleighana*	*Psenopsis cyanea*	*Neopinnula orientalis*	*Bathytroctes squamosus*	*Aristeus alcockii*
Asp	7.31	8.66	10.65	9.52	10.73	17.85
Thr	4.51	5.50	17.62	5.77	7.66	1.36
Ser	3.62	2.22	3.12	3.54	4.08	5.59
Glu	9.16	12.16	16.54	16.31	15.62	33.00
Pro	3.05	1.08	2.02	3.23	5.14	5.53
Gly	4.52	5.48	4.30	3.89	7.35	6.34
Ala	6.36	6.23	9.83	5.37	7.60	9.40
Cys	4.41	6.30	6.10	5.33	3.64	4.08
Val	2.30	2.33	2.77	3.27	4.47	4.64
Met	4.32	5.81	5.34	4.96	8.50	5.11
Ile	0.00	0.00	3.35	1.63	4.65	5.44
Leu	4.95	6.19	6.08	5.84	1.25	0.89
Tyr	7.14	8.68	3.07	8.32	4.60	5.97
Phe	0.57	0 68	0.63	0.79	0.00	0.00
His	2.72	4.29	2.02	2.38	2.06	2.59
Lys	0.81	1.16	0.87	1.08	1.12	1.64
Arg	3.82	4.97	3.60	4.32	4.43	7.95

Of the total sulfur amino acids, methionine and cysteine, the former is nutritionally indispensable while the latter, as a metabolic product of methionine catabolism, is dependent on there being sufficient methionine to supply the needs for both amino acids. They are important nutritionally since their concentrations are marginal in legume proteins, although they are equally abundant in cereal and animal proteins. Although their occurrence in proteins is less abundant than other

amino acids, they are important metabolically to the extent that their relative requirement for maintenance is probably higher than that for human growth. For this reason it is believed that the obligatory nitrogen losses occur at a rate determined by the need to mobilize tissue protein to supply the sulfur amino acid requirements. In the present study, significantly high amounts of the essential amino acid methionione were observed in the muscle tissues of the slick head *B.squamosus*.

It has been learnt that of the aromatic amino acids, phenylalanine and tyrosine, the former is nutritionally indispensable while the latter, as a metabolic product of phenylalanine catabolism, is dependent on there being sufficient phenylalanine to supply the needs for both amino acids. Thus studies have either measured the total requirement for phenylalanine plus tyrosine, by giving diets lacking, or very low in, tyrosine, or examined the ability of tyrosine intake to lower the apparent requirement for phenylalanine. The total aromatic amino acid requirement is set at 25 mg/kg per day, which is close to the midpoint of a range of requirement estimates all of which have some considerable uncertainty.

Whereas the occurrence of tryptophan in proteins is generally less than many other amino acids, it is nutritionally important since it is a precursor for important metabolites such as serotonin and nicotinamide, in the latter case giving it vitamin-like properties through its ability to replace dietary niacin. Its content is low in cereals, especially maize, where it may be the nutritionally limiting amino acid in some varieties. The value of tryptophan requirement is set at 4 mg/kg per day, based on an average of values derived from a variety of approaches, each yielding results close to this value.

Evidence has been accumulated from human studies that diets with a higher proportion of protein are beneficial for the heart (Leverton, 1956, Rose WC, 1955). evidence has accumulated from human studies that diets with a higher proportion of protein are beneficial for the heart (Leverton, 1956, Rose WC, 1955)

Liver of *E.brucus* and *N.raleighana* contained 70% lipids which possessed a high fraction of the non-saponifiable matter (78%) (table 1m) and unsaturated fatty acids. Liver oils of *N.raleighana* contained 46% saturated fatty acids, while the lipids of *S.cyanea* and *B.squamosus* contained 40 and 38% saturated fatty acids respectively (table 1p). Palmitic and myristic acids were the dominant saturated fatty acids in most of the deep sea fishery resources analysed. Significantly high amounts

of oleic acid were observed in the oils of *P.cyanea, B.squamosus and A.alcockii.* Among the PUFAs analysed, n-3 PUFA (EPA, DHA) formed the dominant class of fatty acids in *E.brucus* and *N.raleighana* liver oils (table 1o). The PUFAs formed more than 50% of the total fatty acids in liver oils of *E.brucus* and nearly 30% of the total fatty acids in *N.raleighana liver oils*. n-3:n-6 fatty acid ratio was significantly high in *P.cyanea* and *B.squamosus*. Several workers have confirmed the effects of taking a diet rich in n3 to n6 fatty acids since n3 fatty acids helps in lowering the inflammatory mediators and act as an anti lipidemic agent whereas the metabolites of n6 are proinflammatory and raises the production of cytokines and inflammatory prostaglandin series during various disorders in the body.

Table 1o. Fatty Acid Composition of certain deep sea creatures

Fatty acids	*E.brucus*	*N.raleighana*	*N.orientalis*	*P.cyanea*	*B.squamosus*	*A.alcockii*
C14:0	2.36	5.72	1.92	4.06	3.97	2.64
C15:0	0.37	1.98	0.28	0.71	1	0.65
C16:0	14.79	24.06	10.82	26.49	25.58	23.72
C16:1	3.51	13.84	22.81	3.16	10.3	6.11
C17:0	0.54			0.73	0.4	0.62
C17:1	2.01			0.55	0.95	
C18:0	8.27	8.69	3.77	8.21	3.56	5.15
C18:1n9	12.13	4.66	41.2	43	42.6	43.54
	0.26					
C18:2n6	9.24	2.46	4.32	0.74	0.73	4.72
C18:3n3	0.89		2.8			
C18:3n6	2.23	4.3				
C20:0	0.19	2.42	2.32	0.5	3.71	4.23
C20:1	0.55	3.24		2.02		
C20:3n3	5.17		1.78	1.43		
C20:5n3	16.27	7.22	2.18	2.56	5.15	2.41
C22:0	2					
C22:6n3	18.1	15.63	5.81	3.85	2	4.33
C23:0	1.13	1.37				
C24:0		2.4				
others	2.15	2.14		2.71	1.74	1.87
Σn3	40.43	22.85	12.57	7.84	7.15	6.74
Σn6	11.47	6.76	4.32	0.74	0.73	4.72
n3/n6	**3.524847**	**3.380178**	**2.909722**	**10.59459**	**9.794521**	**1.427966**
SFA	29.65	46.64	19.11	40.7	38.22	37.01
MUFA	18.2	21.74	64.01	48.73	53.85	49.65
PUFA	51.9	29.61	16.89	8.58	7.88	11.46
PUFA/SFA	**1.750422**	**0.634863**	**0.88383**	**0.210811**	**0.206175**	**0.309646**

Table 1k. Proximate Composition of Deep Sea Creatures (% Composition)						
No.	Scientific Name	Common Name	Water	Protein	Fat	Ash
1	*Lamprogrammus niger*	Cusk eels	85.78	13.72	0.52	0.91
2	*Neopinnula orientalis*	Sackfish/ snake mackerel	63.13	14.84	20.38	0.75
3	*Hoplostethus mediterrraneus*	Ray-finned fishes	80.67	17.03	0.77	1.05
4	*Echinorhinus brucus* (meat)	Bramble shark	80.37	17.81	1.08	1.21
5	*Echinorhinus brucus* (liver)	Bramble shark	13.45	10.22	64.86	10.82
6	*Neoharriotta raleighana* (meat)	Narrow-nose Chimaera shark	81.41	15.75	1.16	1.35
7	*Neoharriotta raleighana* (liver)	Narrow-nose Chimaera shark	11.85	8.52	69.98	8.80
8	*Alipocephalus blanfordii*	Slickheads/ ray-finned fishes	82.14	15.54	2.36	0.66
9	*Bathytroctes squamosus*	Slickheads/ ray-finned fishes	64.19	16.52	17.29	0.59
10	*Aristeus sp.*	Rose shrimp	72.99	18.46	5.57	1.08
11	*Bathyuroconger braueri*	Large-toothed conger	81.27	17.50	0.74	0.90
12	*Synopsis cyanea*	Sackfish/ snake mackerel	54.85	17.52	26.20	0.99
13	*Luciobrotula corethromycter*	Cusk eels	78.20	18.37	0.27	1.46
14	*Chelidoperca investigatoris*	Fairy basslets	76.58	20.57	0.50	1.85
15	*Lophiomus setigerus*	Angler fish	81.05	16.42	0.17	0.96
16	*Bembrops caudimaculata*	Ray-finned fishes	73.49	23.88	0.78	1.00
17	*Pterygotrigla hemisticta*	Black-spotted gurnard	75.93	18.67	3.32	1.07
18	*Xenomystax trucidens*	Eel	76.94	19.52	1.45	1.61
19	*Cribrinopsis fernaldi*	Sea Anemone	82.50	9.84	5.59	2.70
20	*Octopus vulgaris*	Octopus	90.98	8.68	0.49	1.47
21	*Sepia officinalis*	Cuttle fish/Squid (brown)	82.39	15.73	1.10	1.34
22	*Ficus gracilus* (meat)	Graceful fig shell	80.02	16.21	0.27	2.88

1.5 SUMMARY AND CONCLUSION

The seas of the Indian EEZ shelter wide varieties of creatures in the deeper zones and many of the fishery resources that dwell in these regions are not known to man. Research in this area is beneficial not only to understand the treasure contained within the deep seas but also to analyse the various biological and pharmacological benefits associated with their bioactives. A data bank with regards to the fishery resources of the Indian EEZ must be created in order to understand our resources in a deeper way and if possible preserve our wealth in the coming generations.

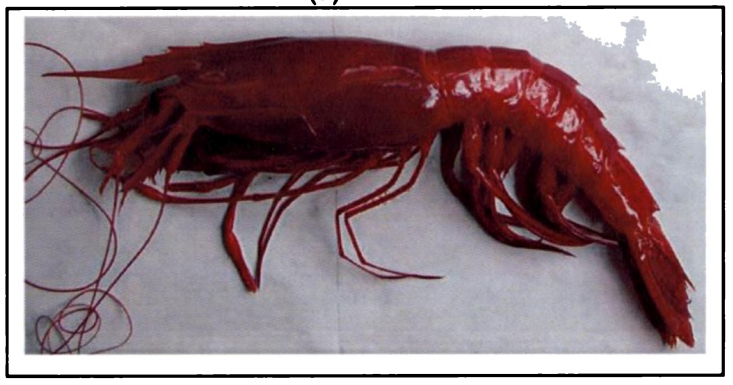
The immense potentials possessed by deep sea fish have caught the imagination of scientists all over. Though these creatures tend to look fierce, most, but not all, are quite small. Their fierce appearance comes from their adaptations that allow them to eat anything that comes their way in the sparsely populated waters.

In the present investigation the biochemical composition w.r.t. the moisture, protein, lipid and ash content of over 20 different deep sea marine resources were analysed. Six different creatures were further analysed for the amino acid and fatty acid profiles. It was observed that these species possess bioactive proteins and lipids which could make them serve as alternative food resources for the nation and even as better aids to the pharmaceutical industry.

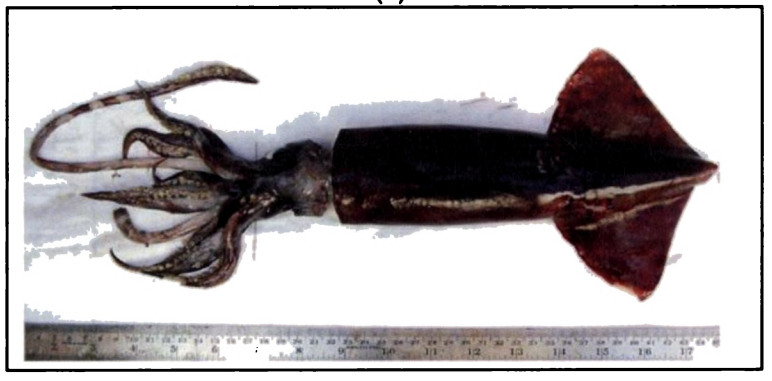
However the use of these fishes as potential food resources deserves further investigation since many of these species may contain toxic metabolites and a thorough scrutiny must be conducted before use in food or medicine.

PLATE I.1 Marine fishery resources of the Indian Exclusive Economic Zone

(a)



(b)



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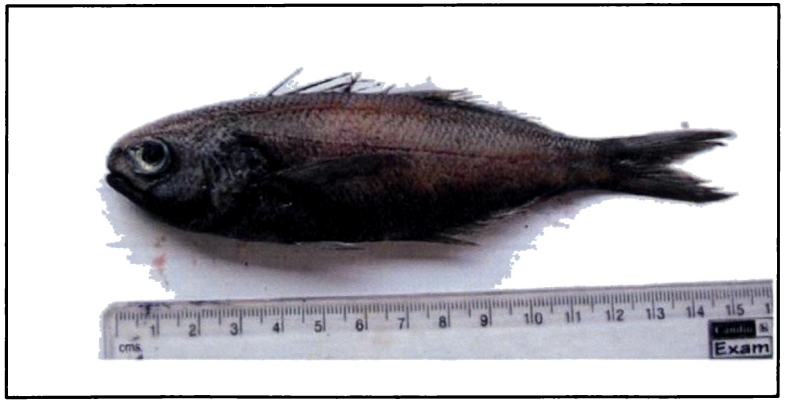
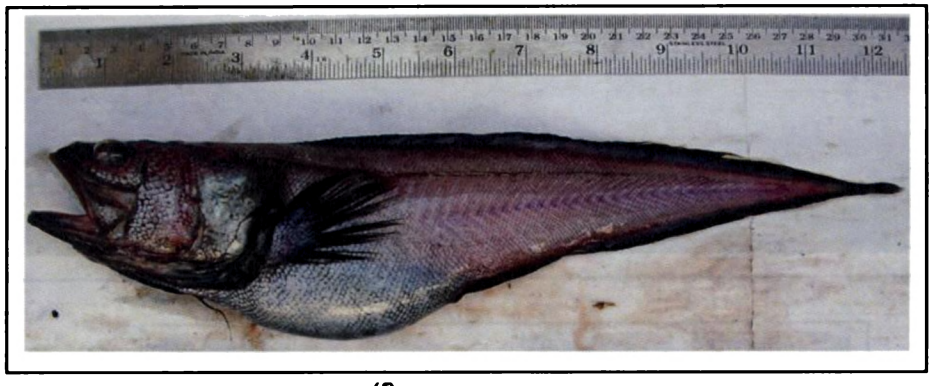


(d)

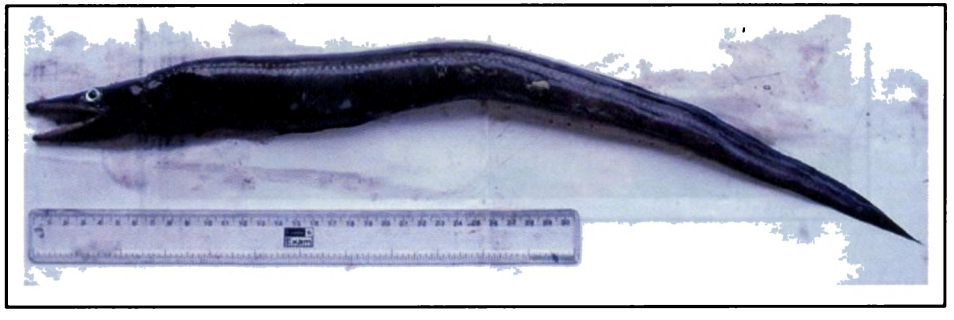
(a)Deep sea rose shrimp (b)squid (c)Sackfish (d) ray-finned fish

PLATE I.1 Marine fishery resources of the Indian Exclusive Economic Zone (Contd.)

(e)

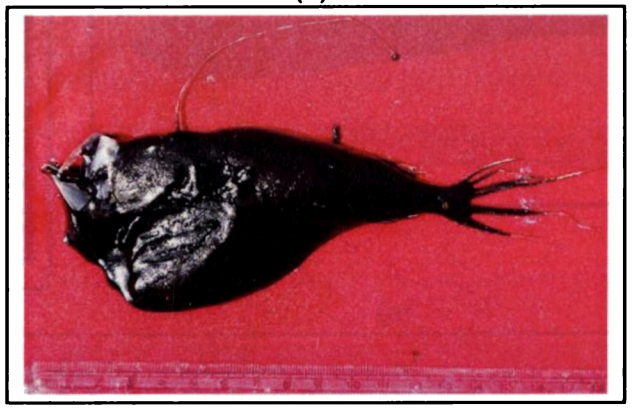


(f)

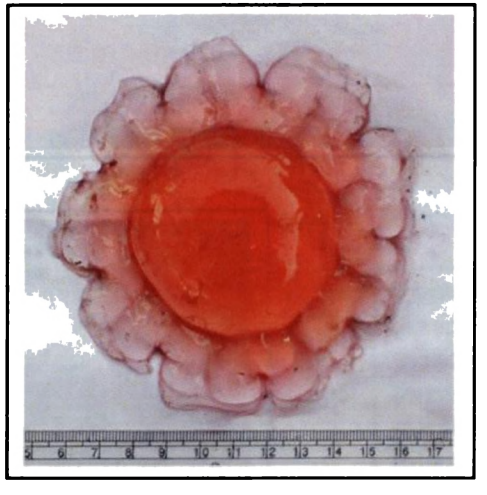


(g)

(h)



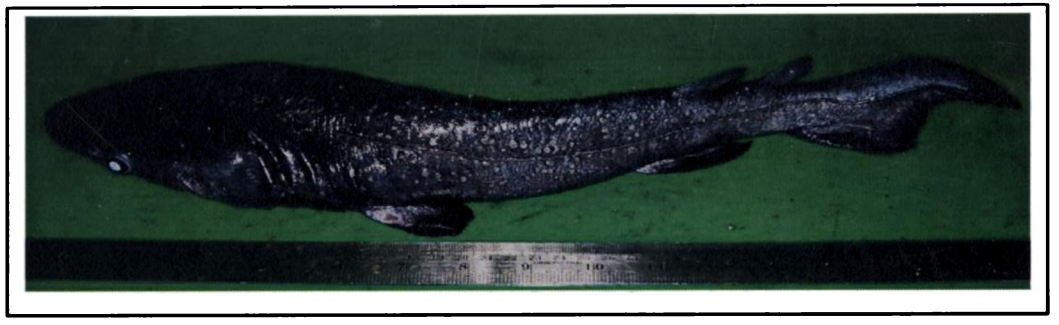
(i)



(e)cusk eels (f)large toothed conger (g) slick-head/ray-finned fish (h) angler fish (i)jelly fish

PLATE I.1 Marine fishery resources of the Indian Exclusive Economic Zone (Contd.)

(j)

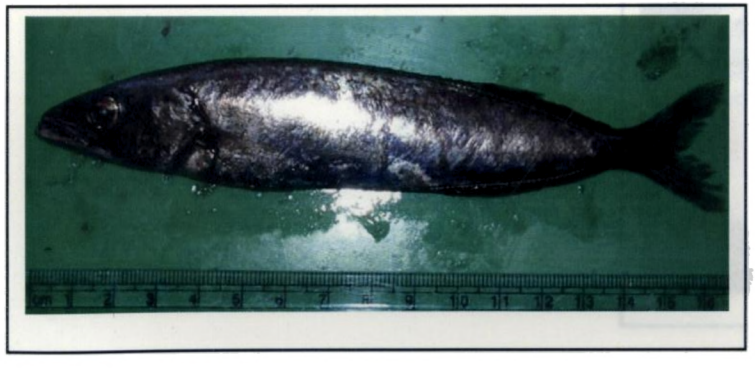


(m)

(k)

(n)

(l)

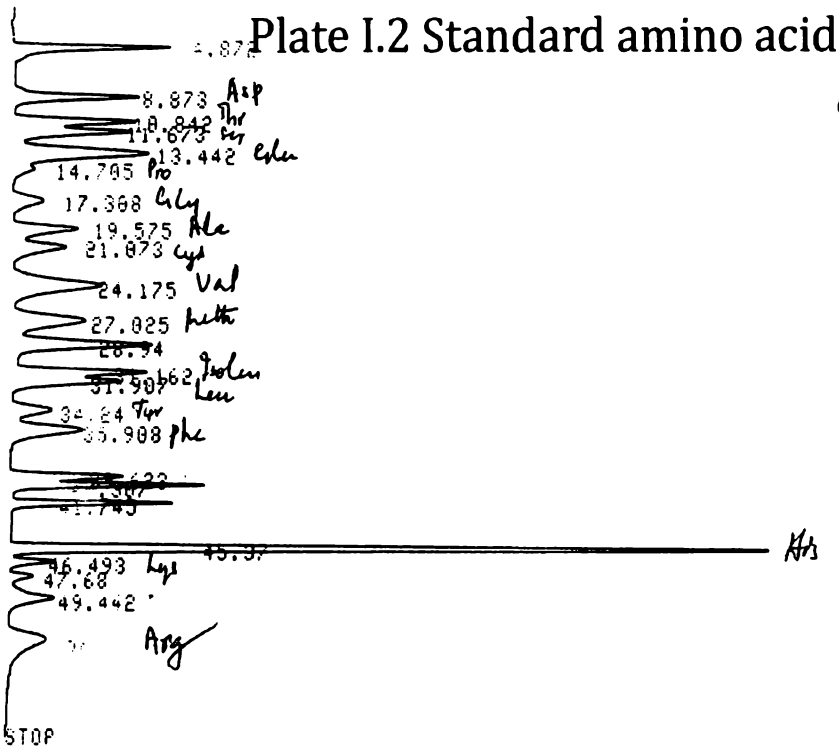


(o)

(j)bramble shark
(k)red snapper
(l) Indian Ruff
(m)myctophid
(n)catshark
(o)octopus

Stock assessment and sample analysis on board the FORV *Sagar Sampada*

Plate I.2 Standard amino acid profile



CHROMATOGRAM 1 MEMORIZED

CHROMATOPAC C-R6A
FILE NO 0
DATA NO 5516
FILE
METHOD
SAMPLE

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	4.872	6045				
2	8.873	5524	Asp	1	0.0033	ASP
3	10.842	5622	Thr	2	0.0025	THR
4	11.673	5995	V Ser	3	0.0027	SER
5	13.442	13372	V Glu	4	0.0061	GLU
6	14.705	7220	V Pro	5	0.0036	PRO
7	17.308	2401	Gly			
8	19.575	4092	V Ala	6	0.0026	GLY
9	21.873	3938	V	7	0.0029	ALA
10	24.175	8714	V Val	8	0.0069	CYS
11	27.025	6627	V Met	9	0.0032	VAL
12	28.94	8554	V	9	0.0041	VAL
13	31.162	6546	V	10	0.0038	MET
14	31.907	5688	V Leu	11	0.0025	ILE
15	34.24	2910	V Tyr	12	0.0014	LEU
16	35.908	5551	V Phe	13	0.0079	TYR
17	39.623	4959				
18	40.307	6276	V			
19	41.745	5056	V			
20	45.37	28151	His	15	0.0075	HIS
21	46.493	1752	V Lys	16	0.0002	LYS
22	47.68	1057	V	16	0.0001	LYS
23	49.442	3450	V	17	0.0005	ARG
24	52.77	4719	Arg	18	0.0005	ARG
	TOTAL	145219			0.052	

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Chapter 2.

Biochemical analyses of liver oils of selected sharks & chimaeras of the Indian EEZ

Chapter 2. Biochemical analyses of liver oils of selected sharks & chimaeras of the Indian EEZ

2.1 INTRODUCTION

Sharks, chimaeras and other elasmobranch resources are of great commercial importance the world over, apart from being a significant link in the marine ecology. The annual average landings of sharks, skates and rays during 1996-2006 were 60,866 t of which sharks constituted 60.1% (36,592 t) (CMFRI statistics, 1996-2006). Though a harvest potential of 1,85,000 t of elasmobranchs has been indicated for the Indian EEZ, they have not been fully exploited. Sixty-five species of shark have been sighted in Indian waters and over 20 of these, of the Carcharhinidae and Sphyrnidae families, contribute to the fishery. Tamil Nadu, Gujarat, Maharashtra, Kerala, Karnataka and Andra Pradesh supply around 85% of the shark landings in India. Despite the commercial importance, no serious attempts have so far been made at any targeted exploration of this valuable resource.

There are several types of gear that take sharks as incidental catch; the most important among them being trawl nets and gill nets (FAO, 1999). There is no detailed information on the landings of sharks by gear type but data available on shark production by mechanized boats at major fishing centres show that trawl nets account for 60% of the shark landings and gill nets account for 38%. Purse seine in Cochin and Mangalore and hook-and-line in Cochin and Bombay take a very small fraction of the catch.

Most of the sharks and chimaeras are usually obtained as by-catches during trawling operations from the open seas and find limited applications in the fisheries sector except for a few, being used for their fins or body oils. The sharks selected in our study *Apristurus indicus*, *Centrophorus scalpratus*, *Centroselachus crepidater* and the chimaeras *Neoharriotta raleighana* and *Harriotta pinnata* are presumably taken by bottom trawlers off the Indian coasts and find limited commercial applications. Therefore collection of such potential specimens for further research including molecular studies is essential. Information on the by-catches of sharks and chimaeras from the Indian EEZ (and other chondrichthyans) is urgently required and the conservation status of these species should be reassessed without delay when such information is obtained. Though many species constitute the shark fishery along Tamil Nadu and Kerala coast only a few comprise the regular fishery and the

species dominance in the fishery differs from gear to gear depending on the area, depth and mode of operation of the gear concerned.

Interest in the potential health benefits of lipids from deep sea fishes have emerged since the 1950s. Previous studies have reported benefits of cod liver oil on diabetes (Stene 2001), hypercholesterolemia (Brox *et al.*,2001), and arthritis (Gruenwald *et al.*,2002). An early finding was that the Eskimo of northern populations had a low incidence of heart disease despite high fat intake. It was found that the deepwater fish the Eskimo consumed are abundant with n-3 long-chain fatty acids (Calder, 2006). These early observations led to an increase in research examining the beneficial and/or preventative effects of lipid bioactives on numerous debilitating and common conditions, including cardiovascular disease (CVD), rheumatoid arthritis and asthma, among others.

Shark liver oil is typically obtained from sharks that are caught as a by-product of deep-sea fishing, making a valuable remedy from a natural resource that would have otherwise gone waste. The liver oils from deep sea sharks and chimaeras, which typically inhabit the cold, non-polluted waters of the sea, have been found to possess life-enhancing compounds including alkylglycerol (or alkoxyglycerol, AKG, Glycerol Ether Lipid), squalene, and natural trace elements in addition to fat soluble vitamins and long chain n3 polyunsaturated fatty acids. Alkylglycerols contained in shark liver oils may have anti-cancer properties. The antilipidemic hydrocarbon squalene, the antioxidant vitamin E and the membrane stabilizing n3 PUFAs contained in these oils help strengthen and regenerate the immune system while benefiting many other functions and organs of the body. Therefore it is rightfully promoted as a complementary or alternative form of treatment for cancer and other inflammatory diseases.

Numerous bio-analytical techniques are available today to assess and estimate the bioactive components present in fish oils. The thin layer chromatography clubbed with the flame ionisation detector (TLC-FID) is used to separate fish oils to their classes since it can separate them based upon their wide polarity due to various alkyl chains. Moreover, the TLC-FID method can simultaneously determine the hydrocarbon content, the triacyl glycerols, monoalkyl glycerols, fat vitamins, free fatty acids etc. contained in the fish oils. The different classes of fatty acids present in the fish oils can be determined using a Gas

Chromatograph with a flame ionisation detector (GC-FID). Better resolutions and accurate quantifications of the lipid components may be further achieved by the use of a high performance liquid chromatograph clubbed with an evaporative light scattering detector (HPLC-ELSD). This method specially yields in improved separation performance for neutral lipid classes which are highly variable and abundant in marine oils.

Giving all these aspects a thought and with an intention of understanding the lipid components of sharks and chimaeras obtained from our deep sea expeditions along the Indian EEZ, the major objectives of the study were framed as below.

1. To identify sharks and chimaeras with potential lipid bioactives, from the Indian EEZ which were abundantly caught as bycatches during trawling operations along the south west and south east coasts of the Indian EEZ.
2. To calculate and compare the hepatosomatic indices of the selected sharks & chimaeras of the Indian EEZ.
3. To estimate the major components of the liver oils of these sharks and chimaeras that contributes to their pharmacological behavior.
4. To conduct biochemical characterisation of liver oils of *Apristurus indicus*, *Centrophorus scalpratus*, *Centroselachus crepidater*, *Neoharriotta raleighana* and *Harriotta pinnata* obtained during cruises onboard the FORV *Sagar Sampada* using TLC-FID, GC-FID and HPLC-ELSD.

2.2 REVIEW OF LITERATURE

2.2.1 Elasmobranch resources of the Indian EEZ

The annual production of elasmobranchs in India is around 70 000 tonnes, over 4% of total marine fish landings (Dholakia *et al.*,2004). Sharks account for between 60 and 70% of this. Tamil Nadu, Gujarat, Maharashtra, Kerala, Karnataka and Andra Pradesh supply around 85% of the shark landings in India. Sixty-five species of shark have been sighted in Indian waters and over 20 of these, of the Carcharhinidae and Sphyrnidae families, contribute to the fishery. Sharks are of great commercial importance the world over, apart from being a significant link in the marine ecology. In India the present annual shark production is around 45,500 tonnes, obtained as a by-catch from a variety of gears. Despite the commercial importance, no serious attempts have so far been made at any targeted exploitation of this valuable resource. Information on the composition of the species of shark landings is very scarce apart from the gross catch statistics.

There are several types of gear that take sharks as incidental catch; the most important among them are trawl net and gill net (FAO 2000). There is no detailed information on the landings of sharks by gear type but data available on shark production by mechanized boats at major fishing centres show that trawl nets account for 60% of the shark landings and gill nets account for 38%. Purse seine in Cochin and Mangalore and hook-and-line in Cochin and Bombay take a very small fraction of the catch. The official sources also indicate that the fishery is limited to the 50–70m depth zone where the sharks are fished as a bycatch by many multispecies gears like trawls and the drift gillnets which are used all along the Indian coast. The shark fishery takes a number of smaller species, e.g. spade-nose shark (*Scoliodon laticaudus*), milkdog shark (*Rhizoprionodon acutus*), *R. oligolinx*, *Carcharhinus sorrah*, *C. dussumeiri*, C. *brevipinna* and *C. macloti*. Apart from these, juveniles of large species, such as *C. melanopterus*, *C. limbatus*, *C. plumbeus*, *Sphyrna zygaena* and a few others also are caught. The occurrence of many of these species is highly seasonal and their landings vary from centre to centre during the year.

In the past there was no organised shark fishery in the country and the sharks were caught incidentally and formed only a bycatch of the gears then used. During the 1950s and 1960s the shark fishery was more or less neglected and the resource

was not adequately studied for the reason that the shark flesh was less preferred as an edible meat owing to its pungent odour caused by the presence of trimethylamine (Anthoni et al.,1990). In those days in the absence of any demand for shark meat, all fishermen did was to remove the fins and throw the maimed sharks back into the sea. However, in later years shark meat gained popularity, both in domestic and international market, in part because of the increase in the demand for seafood in general. The high value fetched by the fins, liver oil, cartilage and skin also boosted the demand for shark that encouraged many to undertake shark fishing. This trend accelerated with the entry of sophisticated fishing trawlers into the fisheries and increasing export demand for shark products. Sharks emerged as a valuable catch and consequently fishermen went after sharks equipped with different gears exclusively to exploit sharks. As fishing for sharks gained momentum in recent years India emerged as a major shark producing country. Much of the trade is, however, still restricted to the west coast of India (Varma 2002).

Biological information and size composition of the various species of sharks exploited is scanty except for a limited number of studies. This is mostly due to the highly seasonal and erratic occurrences of most of the species in the bycatches taken by the various gears. Further, the high cost of sampling makes it difficult to carry out detailed studies on their biology.

2.2.2 Status of Chondrichthyan fishes in India

Despite the widespread recognition of the vulnerability of deepwater chondrichthyan fishes (sharks, batoids and holocephalans) to overfishing and their potential inability to recover from depletion, there is a lack of a concise overview of the present status of knowledge concerning the biodiversity and life history of this group. Deepwater chondrichthyans have been defined as those sharks, rays and holocephalans whose distribution is predominantly at, are restricted to, or spend the majority of their lifecycle at, depths below 200m (Dholakia, A.D., 2004). This depth is generally recognised as the continental and insular shelf edge, and therefore, deepwater species are those occurring on or over the continental and insular slopes and beyond, including the abyssal plains and oceanic seamounts. Of the global chondrichthyan fauna (1193 species), 581 species are considered to be deepwater (48.7% of the global total). The deepwater fauna is divided between 278 sharks

(55.8% of global), 257 batoids (39.8% of global) and 46 holocephalans (93.9% of global).

The bulk of the deepwater shark fauna is attributable to the squaloid dogfishes (Order Squaliformes) and scyliorhinid catsharks (Order Carcharhiniformes, Family Scyliorhinidae), together comprising 84.5% of deepsea sharks. Three families of skates (Arhynchobatidae, Rajidae and Anacanthobatidae) dominate the deepwater batoid fauna, together comprising 89.9% of deepsea batoids.

The total number of known species is ever increasing as exploratory and taxonomic work ensues. Undescribed taxa, those new or recently identified species yet to be formally treated by science, represent over one fifth (21%) of all known deepwater chondrichthyans and the systematics and inter-relationships of several groups of deepsea chondrichthyans remains unresolved. This high proportion not only highlights the overall lack of knowledge of the deepsea fauna at even the most basic (i.e. taxonomic) level, but also that the deepsea chondrichthyan fauna is far from fully documented.

Chondrichthyans are generally considered to be K-selected species, displaying conservative life history parameters such as relatively slow growth, late age at maturity, low fecundity and low natural mortality, resulting in limited reproductive output. These characteristics place them at risk of overexploitation and population depletion, with an inability to recover from reduced population levels once depleted. An understanding of the biological parameters of a species is important to accurately assess its productivity and thus make inferences concerning its vulnerability to fisheries.

For the vast majority of deepwater chondrichthyans, details of their life history characteristics are lacking and many groups remain very poorly-known. However, reported world landings of chondrichthyan fishes are a gross underestimate. They do not include the vast quantities caught as bycatch which are almost entirely undocumented and totally unregulated. Bycatch alone may represent 50% of the actual world cartilaginous fish catch (Bonfil 1994).

2.2.3 The Catch Statistics

There have traditionally been important fisheries for elasmobranchs in India with a relatively steady growth up to the mid 70's, followed by a period of stability during

most of the 80's, then a tremendous increase in catches in 1987 resulting in India becoming one of the top three elasmobranch producers in the last ten years. Indian production of sharks and rays represents 8.78% of the world elasmobranch catches! Still, because of large inland yields, elasmobranch resources comprised only 1.72% of total national catches in 1987–1991. Catch results are not given by species or families in the statistics and the composition of catches is only known by FAO areas. Approximately equal amounts (about 26,000 t/yr) were obtained from both FAO areas for the period 1977–1991. Catches from the west coast were slightly larger than those of the east coast during 1977–1991.

During 1983–1985 sharks comprised 55% of the elasmobranch catch of the country (Dholakia, A.D., 2004.). The main fishing areas in order of importance were Gujarat, Maharashtra, Kerala, Andhra Pradesh, Karnataka and Tamil Nadu and important fishing grounds for sharks are reported for Ashikode, Kerala Province (Anon. 1983). Sharks catches are incidental to other fisheries in India and are mainly taken with longlines, which vary in design by region, and are also as by catch of trawlers using disco nets off Ratnagiri (Maharashtra), with bottom set gillnets in Porto Novo (Tamil Nadu) and by shrimp trawlers of Kerala (Kulkorni and Sharangdher 1990). Rays are caught with bottom set gillnets in Gujarat, northwest India and Cudalore and are abundant on the outer shelf and slope off Kerala and Karnatakta (Devadoss 1978; Kunjipalu and Kuttappan 1978; Sudarsan et al. 1988). Devadoss (1984) indicates that batoids comprise 10% of by catches in Calicut; 90% of the by catch comes from trawlers, 8% from gillnets and 2% from hook and lines. Both sharks and rays are abundant in Lakshadweep and form important by catches in trawl fisheries in Krishnapatnam (Swaminath et al. 1985; James 1988).

Dahlgren (1992) noted that directed fisheries for sharks are developing on a seasonal basis on the east coast of India. About 500 vessels, both sail - powered and motorized, fish for sharks with bottom or drift longlines of the coasts of Orissa Andhra Pradesh and Tamil Nadu. Bottom longlines are usually set in waters 80–150m deep and occasionally as deep as 500m for bull sharks and tiger sharks. The longlines have up to 400 hooks and the meat is usually salted on board during the trip. In Orissa alone, about 200 boats are engaged in drift longlining on a seasonal basis (December - March). The most common species caught by drift longlines are silky sharks and scalloped hammerhead sharks.

Catch composition data are not readily available but the multispecies nature of these fisheries is evident from the literature. Appukuttan and Nair (1988) reported that more than 20 species of sharks (mainly carcharhinids and sphyrnids) are commonly caught. Their data for Pamban and Kilakkarai show that *Rhizoprionodon acutus, R. oligolinx, Carcharhinus limbatus, C. sorrah, C. hemiodon, Sphyrna lewini and Eusphyra blochii* are the most important species. Other species caught are *C. melanopterus* and *Scoliodon laticaudus* (Devadoss 1988). Important batoids are: *Dicerobatis eregoodoo, Rhynchobatus djiddensis, Rhinobatus granulatus, Himantura uarnak, H.bleekeri, Dasyatis sephen, D.jenkinsii, Aetobatus narinari, A. flagellum, Aetomylus nichofii* and *Mobula diabolus* (Devadoss 1978, 1983; Kunjipalu and Kuttappan 1978).

Local assessments of the state of the fisheries for elasmobranchs exist (Krishnamoorthi *et al.* 1986, Devadoss *et al.* 1988, Sudarsan *et al.* 1988), but no overall studies exist (Appukuttan and Nair 1988). Devadoss (1978) reports that ray resources off Calicut were apparently overfished by 1980 while according to Reuben *et al.* (1988) shark and ray resources of Northeast India were still underexploited in 1985. Devadoss *et al.* (1988) did local assessments using Schaefer's model and made suggestions for effort changes for the different areas. The present situation needs careful monitoring. There appears to be a high level of catches of elasmobranchs in India (peak of 73 500t in 1988) and it is unlikely that such large yields are sustainable over a long periods. The collapse of the neighbouring Pakistani elasmobranch fisheries in 1983 could indicate future catch reductions for the Indian elasmobranch fisheries.

2.2.4 The fisheries

The annual average landings of sharks, skates and rays during 1996-2006 were 60,866 t of which sharks constituted 60.1% (36,592 t) (CMFRI statistics, 1996-2006). Though a harvest potential of 1,85,000 t of elasmobranchs has been indicated for the Indian EEZ, they are not fully exploited. Catches in the exploratory surveys by the Government of India tuna longliners indicate that the pelagic sharks constitute 42% in the Arabian Sea, 36% in the Bay of Bengal, 43% in the Andaman Sea and 31% in equatorial areas. In Kerala the sharks, chimaeras, skates and rays formed an average catch of 8000t/yr accounting for nearly 2% of the total landings of Kerala.

They formed 13% of the total elasmobranch catches at the all India level. In 1975, landings were 10000t, which is the highest so far recorded in Kerala. The minimum was 4900t in 1987. During 1994–97 the shark landings in Kerala varied from 1647t in 1997 to 3781t in 1994 with an average of 2600.3t which formed 0.5% of the total marine fish production by Kerala.

2.2.5 Species composition

About 70 species of sharks occur in Indian seas of which about 22 species have only limited occurrence and value; around 12 are moderately abundant though not frequently caught and only six are major species (Table 2) in the fishery. The most common and abundantly fished shark is *Scoliodon laticaudus* followed by *Rhizoprionodon acutus*. Among the requiem sharks, *Carcharhinus sorrah*, *C. limbatus* and *C. melanopterus* and the hammerhead shark *Sphyrna lewini* are common and reach a maximum length of one to 2.5m. Other sharks which occur moderately in the catches are the grey sharks, *C. macloti*, *C. hemiodon*, *C. dussumieri*, *C. Sealei*, *Loxodon macrorhinus* and *Rhizoprionodon oligolinx*. These sharks grow up to a metre in length. The snaggle-tooth shark (*Hemipristis elongatus*), mako shark (*Isurus oxyrinchus*), and the tiger shark (*Galeocerdo cuvier*) are larger sharks and grow to more than 2m. The hammerhead sharks, *Sphyrna mokarran* and *Eusphyra blochii* grow up to 2m also occur in the catch.

About 68% of the sharks landed are along the west coast. *Scoliodon laticaudus* is the dominant species in the catch (83.3%) along the Gujarat and Maharashtra coasts followed by *Carcharhinus* spp. (13%), *Rhizoprionodon* spp. (2%) and the tiger shark along with other minor groups constitute the rest of the catch. On the southwest coast (Kerala, Karnataka and Goa) the grey sharks (*Carcharhinus* spp.) and the dogfishes (*Centrophorus* spp.) form 56.1% of the major catch of 56.1% followed by the hammerhead sharks (*Sphyrna lewini*, *S. mokarran*) - 26.5%, *S. laticaudus* - 3% and other *Carcharhinus* shark and hammerhead sharks form 14.4%. On the east coast (Chennai region) the major grey sharks contribute 59.4% of the shark fishery followed by the hammerheads (*Sphyrna lewini* and *Eusphyra blochi*) - 23%, *Rhizoprionodon* spp. - 15%. The other sharks which include the tiger shark *Galeocerdo cuvieri*, *Isurus oxyrhinchus*, *C. Sealei*, *Hemipristis elongatus*,

Chiloschyllium griseum comprise 2.5% of the value (Devadoss 1988). The chimaeras contribute almost 18% of the total fish catch along the south west coast. In Tamil Nadu and Kerala as many as 30 to 40 species of sharks belonging to 15 genera occur along the Tamil Nadu and Kerala coasts. Five species of skates and 15 species of rays belonging to three and seven genera respectively also occur in the fishery. However, only a few species constitute the commercial fishery.

The following species of sharks have been observed in the shark fishery along the Tamil Nadu and Kerala coast: *Alopias vulpinus, Apristurus indicus, Stegostoma fasciatum, S. obesus, Chiloscyllium indicus, C. griseum, Rhincodon typus, Galeocerdo cuvieri, Scoliodon laticaudus, S. walbeehmii, Rhizoprionodon oligolinx, R. acutus, Eulamia eliioti, Sphyrna blochii, S. zygaena, S. tudes, S. lewini, Carcharhinus sorrah, C. dussumieri, C. gangeticus, C. limbatus, C. longimanus, C. melanopterus, C. brevipinna, Centrophorus molluccensis, C. scalpratus, C. crepidater, Echinorhinus brucus, Hemipristis elongatus, Loxodon macrorhinus. Scoliodon laticaudus, Rhizoprionodon acutus, Carcharhinus sorrah, C. limbatus, C. melanopteraus, Sphyrna blochii, S. zygaena, S. tudes, Laxodon macrorhinus* and *Stegostoma fasciatum*. Among the skates, *Pristis microdon, P. cuspidatus, Rhinobatus granulatus, djiddensis, R. obtusus, R. armatus*, and *Raja mamillidens* are recorded in the fishery. Rays recorded in the fishery are *Dasyatis bleekeri, D. kuhlii, D. zugei, D.uarnak, D. sephen, D. imbricata, Aetobatus flagellum, A. narinari, Rhinoptera javanica, Manta birostris, Narcine indica, Tygon zuge* and *Gymnra poecilura*. Apart from sharks, rays and skates the chimaeras (*Neoharriotta raleighana, Harriotta pinnatta*) are also an important resource among the marine fishes caught from the Arabian Sea and the Indian Ocean.

2.2.6 Distribution of the fishery

Shark fishery is multispecies and no species is dominant throughout the entire coast of India. Neither a single species, nor a group of species, dominates in the different states. *Scoliodon* dominates the fishery in the Gujarat and Bombay regions and Grey sharks and hammerhead sharks dominate the catch in Kerala and Karnataka states. The whale shark (*Rhincodon typus*) has become the target fishery using harpoons at Veraval on the Gujarat coast at some other places, *Carcharhinus* spp. are targetted and are hunted for their liver and fins.

Though many species constitute the shark fishery along Tamil Nadu and Kerala coast only a few comprise the regular fishery and the species dominance in the fishery differs from gear to gear depending on the area, depth and mode of operation of the gear concerned (FAO, 1999). Mostly the drift gillnet with larger mesh size (140-200mm) and the hook and line gear, especially that with larger hooks, exploit the shark resource more effectively than the other gears. The large mesh gillnets and hook and line units land bigger sharks such as the *Carcharhinus sorrah, C. melanoptera, Laxodon macrorhinus, Sphyraena tudes, S. blochii, Galeocerdo cuvieri,* etc. The trawl net and the gillnets with smaller mesh size (40-90mm) land smaller sized sharks such as *Scoliodon laticaudus, Chiloscyllium indicus*, etc. Along the southeastern coast of Tamil Nadu *C. sorrah, L. macrorhinus* constitute the major part of the shark landings and along the northeastern coast *Rhizoprionodon acutus* and *S. acutus* form the major portion of the landings. Studies on the biology and stock assessment of these species reveal that *C. sorrah* is exposed to higher fishing pressure and the females are more severely fished than the males along the Gulf of Mannar. Similarly *R. acutus* is exposed to higher fishing pressure (Krishnamoorthy and Jagdish 1986) along the Madras coast.

A closer look at the pattern of catches shows that pelagic sharks are more abundant on the West Coast. A different pattern emerges on the east coast where even though sharks contributed 32% to the total sharks caught the share of the batoid fishes taken constituted 53% of the total elasmobranch catch in India (Devadoss et al. 1997). Sudarsan et al. (1988) identified the existence of potentially rich grounds for pelagic sharks off the Gulf of Mannar, (on the East Coast in the state of Tamil Nadu). The incidence of non-conventional species of shark like the bramble shark as shown by their catch by deep sea water trawlers operating off Tuticorin in the Gulf of Mannar is an encouraging feature. The present fishing area (which is mainly exploited by trawlers) falls within a narrow coastal zone up to the 70m isobath and the knowledge of sharks in this area is based on the catches within this zone. The projected potential Indian yield for sharks and rays is about 0.18 million tonnes of which the share for sharks is 0.12 million tonnes (Sudarsan *et al.* 1988). At the present level of fishing a large gap exists between the projected potential yield and the actual catch in the offshore waters. It has been also reported that the total potential of the EEZ of India is estimated at 4 470 000t. Of these about 2 260 000t or

50.6% lies within the 50m isobath, around 38% lies within 200m and 11% beyond 200m.

Generally speaking, explotation of elasmobranchs at present along the Indian coast fishing is unbalanced. Some regions are excessively exploited and some are totally unexploited. Kerala comes under the former category with a high level of exploitation. There is scope for expanding the commercial exploitation of sharks (Chen and Yuan, 2006), but this needs to be done carefully as discussed below.

Availability of food is largely the limiting factor for any fishery including sharks. On the west coast along Kerala and Karnataka states the sharks fishery is at its peak when mackerel and oil sardines appear in shoals. That the pelagic sharks hunt the fast moving pelagic fishes is evident by the exclusive presence of mackerel and oil sardine in their stomachs during this period. The distribution of *S. laticaudus* in large concentrations along Gujarat and Bombay coasts is also due to the availability of preferred food. Demersal sharks are abundant in this region and forage on bottom living fauna. The juveniles also inhabit the bottom and are caught by bottom trawls. The large adults, with greater mobility, come to the surface where they are taken by driftgill net gear. The large *Scoliodons* prefer pelagic fishes while the juveniles feed on the bottom fauna such as crabs, squilla, small prawn, etc. (Chen and Yuan, 2006).

Kerala: This state's coastline of 590km is almost one-tenth of Indian's total coastline. Sharks are landed in 222 landing centres along the Kerala coast. Important fisheries ports are Cochin, Sakthikulangara, Munambam, Azheekal, Ponnani, Beypore, Vizhinjam, Quilandi and Azhikkode. The major gears which catch sharks along the Kerala coast are hook and line, drift/set gillnets and trawls. In hook and line fisheries, elasmobranchs form 22% of the catch. Other associated species are catfish (31%), carangids (24%), tunnies and mackerels (5%), and seerfish (2%). The hook and line fishery is prevalent mostly in Trivandrum and Quilon districts and to some extent in Kozhikode. In drift gillnetting elasmobranchs constituted 26% of the catch; the other resource groups include tunnies (25%), catfish (18%), seerfish (14%) and Pomfrets (3%).

2.2.7 Distribution of landings

The distribution of elasmobranchs in both the Arabian Sea and the Bay of Bengal is not uniform. Gujarat state contributes a little over 50% of the total catch from the west coast and Maharashtra and Gujarat share 81% of the shark catches on the west coast and 55% of all Indian shark catch. The east coast contributed 11000t constituting 32% in the total sharks caught. Tamil Nadu and Andhra Pradesh together account for 78% of the total sharks fished along the east coast and 25% of the total sharks caught in Indian coastal waters. Even though sharks appear to be distributed all along the coast, there are places where particular species or groups of sharks are present in large numbers. As stated earlier, *S. laticaudus* and *R. acutus* are found in large sharks, which are highly pelagic, are more prevalent along Kerala, Karnataka and Tamil Nadu coasts. The pelagic sharks, *C. limbatus*, *C. sorrah* and *C. melanopterus* start appearing in the fishery along Tamil Nadu coast from April until September coinciding with the mackerel and sardine fishery (Sudarshan *et al.*,1988).

2.2.8 Species selected for the study

The elasmobranch resources *Centrophorus scalpratus*, *Centroselachus crepidater*, *Apristurus indicus*, *Neoharriotta raleighana* and *Harriotta pinnata* were obtained during cruises onboard the FORV Sagar Sampada from along the south west and south east coasts of the Indian EEZ. None of these species have been recorded in the "vulnerable" and "near threatened" species as categorized within the IUCN red list.

1) ***Centrophorus scalpratus***[a,b] McCulloch, 1915

Kingdom: Animalia

Phylum: Chordata

Class: Chondrichthyes

Order: Squaliformes

Family: Centrophoridae

Red list category and Criteria: Data deficient

Synonyms : *Centrophorus moluccensis* Bleeker, 1860; *Atractophorus armatus* Gilchrist, 1922.

FAO Names : Smallfin gulper shark; Endeavour dogfish

Marine fishing areas:

Indian Ocean – western; Indian Ocean – eastern; Pacific – northwest; Pacific – southwest; Pacific – western central

Field Marks:

No anal fin, two dorsal fins with large spines, bladelike unicuspidate teeth in upper and lower jaws, with lowers much larger than uppers, a moderately long snout, moderate-sized first dorsal fin and very small second dorsal, blocklike sessile-crowned, wide-spaced, cuspidate lateral denticles, and rear tips of pectoral fins narrowly angular and greatly elongated.

Diagnostic Features:

Snout moderately long and parabolic, preoral snout greater than mouth to pectoral origins; upper anterolateral teeth with semierect or oblique cusps. First dorsal fin fairly high and short; second dorsal very small, half height of first dorsal or less, with base less than 1/2 to nearly 3/5 length of first dorsal base, and spine origin well behind rear tips of pelvic fins; distance from first dorsal insertion to origin of second dorsal spine greater than distance from tip of snout to pectoral insertions in adults; free rear tips of pectoral fins formed into narrow, angular and greatly elongated lobes that reach well beyond the level of first dorsal spine, inner margins equal or longer than distance from second dorsal spine to caudal origin; caudal fin with a deeply notched post ventral margin in adults. Lateral trunk denticles not overlapping each other, block like, with crowns sessile on bases and no pedicels, crowns broad, squared or vertically rhomboidal in adults, with a strong main cusp and no lateral cusps on their posterior edges.

Geographical Distribution:

Western Indian Ocean: South Africa, southern Mozambique. Western Pacific: Japan (Okinawa), Indonesia (Ambon), Australia (Victoria), New Hebrides, New Caledonia.

Habitat and Biology:

A common deepwater dogfish of the outer continental shelves and upper slopes, thrives on or near the bottom at depths from 128 to 823 m. Ovoviviparous, number of young two per litter. Full term fetuses were found in summer off South Africa. Feeds primarily on bony fish, including lanternfish, bramids, carangids, worm-eels, bonito, hairtails, oilfishes, as well as other dogfish sharks, squid, octopi, shrimp, and even tunicates.

Size: Attains maximum at about 100 cm; males maturing between 69 and 73 cm and reaching 86 cm; females maturing above 89 cm and reaching upto 98-100 cm; size at birth is about 31 to 37 cm.

Interest to Fisheries: Presumably taken by bottom trawlers off South Africa, India and Australia. Potentially important for its abundance off the coasts of South Africa and southern Mozambique. The shark finds limited commercial applications to the fisheries sector and is mainly used as fish meal for its meat, fins and liver oil.

2) ***Centrophorus crepidater/Centroscymnus crepidater***[a,b] Barbosa du Bocage & de brito Capello, 1864

Kingdom: Animalia

Phylum: Chordata

Class: Chondrichthyes

Order: Squaliformes

Family: Somniosidae

Synonyms: *Centroselachus crepidater*, Barbosa du Bocage & de brito Capello, 1864, *Centrophorus jonsonnii* Saemundsson, 1922.

FAO Names : Long nose velvet dogfish, Golden dogfish,

Red list category and Criteria: Least Concern

Geographical distribution

A fairly common but poorly studied species with a wide but patchy distribution. Occurs in the eastern Atlantic (Iceland to southern Africa), Indian Ocean (Aldabra Islands and India), eastern Pacific (northern Chile) and the western Pacific, from New Zealand and southern Australia, on or near the bottom of continental and insular shelves in depths of 270-1,300 m. Locally from Sydney (New South Wales) to Perth (Western Australia), including Tasmania and the southern seamounts. Native to Angola; Australia (New South Wales, South Australia, Tasmania, Victoria, Western Australia); Benin; Cameroon; Chile; Congo; Côte d'Ivoire; Equatorial Guinea; France; Gabon; Gambia; Ghana; Guinea; Guinea-Bissau; Iceland; India; Ireland; Liberia; Mauritania; Morocco; Namibia; New Zealand; Nigeria; Portugal; Senegal; Seychelles (Aldabra); Sierra Leone; South Africa; Spain (Canary Is.); Togo; United Kingdom; Western Sahara

Marine fishing areas

Atlantic – northeast; Atlantic – southeast; Atlantic – eastern central; Indian Ocean – western; Indian Ocean – eastern; Pacific – southwest

Habitat and ecology

Demersal on the slope in depths of 270 to 1,300 m; off Australia most common in 780 to 1,100 m. Feeds mainly on fish and cephalopods. In the Rockall Trough in the northeastern Atlantic, the diet was dominated by squid and micronektonic fish including myctophids. This species would appear to feed clear of the seabed on benthopelagic organisms (Mauchline and Gordon 1983). The lack of a seasonal pattern to reproduction, with females breeding throughout the year, means that the gestation period is currently unknown. Litter sizes average six with a range from 3 to 9. Annual fecundity is unknown. The productivity of this species appears to be low, with age at maturity in Australia of 15 years at 64 cm (males) and 22 years at 82 cm (females), and longevity of around 60 years (S. Irvine, pers.comm.). The maximum size of specimens in Australia is 105 cm (Daley *et al.* 2002).

Size

The male attains a maximum size of 130 cm with a reported age of 54 years.

Field marks

Snout greatly elongated, preoral length about equal to distance from mouth to pectoral fin origins. Upper labial furrows greatly elongated, their lengths greater than distance between their anterior ends

Diagnostic features

Dorsal spines (total): 2; Anal spines: 0. Black or blackish brown in color, dorsal fins with very small fin spines, very long snout, greatly elongated labial furrows that nearly encircle mouth, lanceolate upper teeth and bladelike lower teeth with moderately long, oblique cusps, fairly slender body that does not taper abruptly from pectoral region, moderately large lateral trunk denticles with partly smooth, oval, cuspidate crowns in adults and subadults.

Interest to fisheries

Mainly a bycatch species taken by trawl and hook, although with some limited targeting for its flesh and oil. Catches in Australia have been increasing in the last few years with relaxation of mercury laws and fishers looking for non- quota species in the South East Trawl Fishery. Biomass surveys extending over 10 years in New Zealand show an increasing trend, but may be confounded by the use of different

vessels. The productivity of this species appears to be low, with age at maturity in Australia of 15 years (males) and 22 years (females), and longevity of around 60 years, thus further increases in catches should be viewed with concern. However, the species is currently still abundant and a Near Threatened assessment cannot be justified at this time, although the situation should be monitored carefully.

3) ***Apristurus indicus***[a,b] Brauer, 1906

Kingdom: Animalia

Phylum: Chordata

Class: Chondrichthyes

Order: Carcharhiniformes

Family: Scyliorhinidae

Synonyms: *Scylliorhinus indicus*

FAO Names: smallbelly catshark

Red list category and Criteria: Not evaluated

Marine fishing areas: Western Indian Ocean, Gulf of Aden, Oman, Southeast Atlantic Ocean

Size: 34 – 45cm TL male/unsexed

Depths: 700 – 1800 m

Biology: found on continental slopes, Oviparous

Interest to fisheries: probably caught with bottom trawls, Not evaluated

4) ***Neoharriotta raleighana***[a,b] Goode & Bean, 1895

Kingdom: Animalia

Phylum: Chordata

Class: Chondrichthyes

Order: Chimaeriformes

Family: Rhinochimaeridae

Synonyms : *Anteliochimaera chaetirhamphus*, Tanaka 1909; *Harriotta curtissjamesi* Townsend & Nichols 1925; *Harriotta opisthoptera* Deng, Xiong & Zhan 1983.

FAO Names : Bentnose Rabbitfish, Long-nosed Chimaera, Narrow-nose Chimaera

Red list category and Criteria: Least Concern

Marine fishing areas: The fish is native to the oceans of the Atlantic – eastern central; Atlantic – northeast; Atlantic – northwest; Atlantic – southeast; Atlantic – southwest; Indian Ocean – eastern; Pacific – southwest; Pacific – northwest; Pacific eastern central; Pacific – southeast

Diagnostic features

Dorsal spines (total): 1; Anal spines: 0; Anal soft rays: 0. A longnose chimaera with a rather long, narrow, depressed snout, a small eye situated above or behind the mouth, a rather long first dorsal fin and spine, knobby tooth plates, and caudal fin lanceolate with no tubercles on upper edge but with a long terminal filament. Dark brown or blackish in color. No separate anal fin. Claspers are rod like, rather slender, unbranched, with tip somewhat swollen. Jugular and oral canals arising separately from orbital, with a short interspace; angular (maxillary) canal joining suborbital about 2/7 of distance from front level of eye toward tip of snout.

Geographical distribution

Range appears to be widespread and worldwide (although not widely recorded in the Indian Ocean at present), with the largest numbers recorded from the western Pacific and northern Atlantic. Nothing is known of population structure, although molecular evidence may support regional populations. Other species of chimaeroids appear to have wide ranges (e.g., *R. atlantica* and *R. pacifica*), but *H. raleighana* is the only chimaeroid that may be global in its distribution.

Habitat & biology

Found on the continental slope and ocean floor. Appears to feed mainly on shellfish and crustaceans. Maximum length 120 cm without tail filament

Size: 120 cm OT male/unsexed; (Ref. 26346); 102.5 cm TL (female)

Environment

Bathydemersal; marine; depth range 200-2600 m.

Interest to fisheries

This species appears to be the only chimaeroid with a widespread, global distribution. Occurs in deep waters of the continental slopes in depths of 380 to 2,600 m in both the Atlantic and Pacific Oceans. Also occurs in the Indian Ocean (off southern Australia). They seem to be somewhat common in the Northern Atlantic, Northwest Pacific and Southwest Pacific, however, very little is known about the biology of this species. They are oviparous but nothing is known of spawning and

reproduction and very few juveniles have been collected. As with many other chimaeroids adults and juveniles may occupy different habitats. Known to be captured in deepwater research trawls and as bycatch in deepwater commercial trawls. Data from the South Tasman Rise Trawl Fishery (south of Tasmania, Australia) indicates that this species is a negligible component of bycatch. Increased deepwater trawl fisheries could pose a potential threat to habitats and populations in the future. At present this species appears to be widespread geographically and bathymetrically and relatively abundant with no immediate threats to the population and is thus classified as least concern. However, bycatch data from other fisheries and the monitoring of expanding deepwater fisheries are required.

5) ***Harriotta pinnata***[a,b] Schnakenbeck 1931

Kingdom: Animalia

Phylum: Chordata

Class: Chondrichthyes

Order: Chimaeriformes

Family: Rhinochimaeridae

FAO Names: Siclefin chimaera

Synonyms : *Neoharriotta pinnata* Schnakenbeck 1931

Marine fishing areas: the fish is native to Atlantic – southeast; Atlantic – eastern central; Indian ocean - eastern

Diagnostic features

A longnose chimaera with a narrow, slightly flattened snout, and blunt-edged, ridged tooth plates; pectoral fins short and broad, anal fin large and curved, and caudal fin with no tubercles on upper edge but with a short terminal filament (Ref. 5578). Dark brown in color

Geographical distribution

The occurrence of the species is distributed across the Eastern Atlantic: Cape Blanc, Mauritania to Walvis Bay, Namibia (Ref. 4444). Western Indian Ocean: Arabian Sea

Habitat & biology

Very poorly known species. Occupies a relatively shallower shelf and slope habitat than other rhinochimaerids at depths of 200 to 470 m. Few adult specimens have

been collected, but males and females appear to reach sexual maturity at about 50 to 60 cm body length (BDL), as all specimens greater than 60 cm BDL are sexually mature. Oviparous, likely exhibiting similar reproductive patterns to other chimaeroids. Diet is unknown but probably consists primarily of a variety of benthic invertebrates.

Size: 130 cm TL male/unsexed

Environment: Bathydemersal; marine; depth range 150 - 700 m, usually 200 - 470 m

Red list category and Criteria: Data deficient

Interest to fisheries

Neoharriotta pinnata appears to be widespread in the Atlantic off the coast of Western Africa and in the Arabian Sea off the south west coast of India. Although widely reported, only a relatively small number of voucher specimens have been collected and the species does not appear to be common. Not known to be targeted in any commercial fishery or utilized in any way; however, its occurrence at depths ranging from 200 to 700 m puts this species within range of most deepwater trawling operations and it is likely collected as bycatch. Nothing is known of the biology of this species, particularly reproduction, population structure, habitat and ecology and it is recommended that further studies be conducted. In particular, collection of specimen data (locality, size, sex, reproductive state) from each capture would enhance our understanding of geographic range and basic population structure of this species. Collection of additional specimens for further research including molecular studies is also essential. Information on the bycatch of this species (and other chondrichthyans) is urgently required and the conservation status of this species should be reassessed without delay when such information is obtained.

Over the decade, the increasing use of fish oil for various ailments has promoted worldwide research and analyses on the bioactive potentials of marine lipids. Marine lipids and those extracted from livers of sharks inhabiting waters beyond 500 m depth and of the nutraceutical and pharmacological values they posess has been the topic of discussion in major lipid conventions and seminars. Scientists today are on the verge of discovery of major bioactives from shark liver oils that could serve as powerful anti-inflammatory and anti-tumour agents.

2.2.9 Shark liver oils – an overview

Shark liver oil is extracted from the livers of deep-water sharks which typically inhabit the cold, non-polluted waters of the sea. Raw shark liver oil that is minimally processed contains life-enhancing compounds like alkylglycerol (or Alkoxyglycerol, AKG, Glycerol Ether Lipid), squalene, and natural trace elements in addition to fat soluble vitamins A, D and E and long chain n3 poly-unsaturated fatty acids (Brunel *et al.*,2005). Alkylglycerols contained in shark liver oils may have anti-cancer properties. Shark liver oil is typically obtained from sharks that are caught as a by-product of deep-sea fishing, making a valuable remedy from a natural resource that would have otherwise gone waste. This oil is known to help strengthen and regenerate the immune system while benefiting many other functions and organs of the body. Therefore it is rightfully promoted as a complementary or alternative form of treatment for cancer and other inflammatory diseases.

Shark liver oil is widely used alongside conventional cancer treatment in northern Europe and is sold as a dietary supplement in the United States. Available scientific evidence does not support claims that shark liver oil supplements are effective against cancer in humans (Calder 2006). Recent research has focused on certain components of shark liver oil (alkylglycerols, squalamine, and squalene). Early laboratory studies suggest that they may have anti-tumor effects in animals, but their effects in humans are not yet known. Clinical trials are currently under way.

2.2.10 Biochemical constituents of shark liver oils

a) Fat vitamins

Shark liver oil is promoted as a dietary supplement as it contains fat vitamins A, D and E used to boost the immune system, fight off infections, heal wounds, treat cancer and lessen the side effects of conventional cancer treatment. Shark liver oils are rich in antioxidants like vitamin E (Devaraj and Jialal 2000) which reduce inflammation by decreasing C-reactive protein levels and by blocking the activity of TNF-α (tumour necrosis factor-alpha) series 2-prostaglandins (PGE-2) and cyclooxygenases (James *et al.* 2003). Antioxidants are well known to alleviate the inflammatory processes mediated by allergic substances. They also curb inflammation by quenching hazardous molecules called free radicals, which stimulate inflammation (Vittala and Newhouse 2004).

In the pharmaceutical industry, vitamins are used in supplement preparations such as tablets or capsules. Vitamins are also used in the cosmetics industry in skin care, hair care and oral hygiene products. Vitamins have been added to skin care products to boost the skin's antioxidant or anti-inflammatory response. They also function as immune system strengtheners, clarifiers or wrinkle reducers.

The major fat soluble vitamins present in fish oils are vitamin A and vitamin E.

b) Vitamin A

A fat-soluble vitamin occurs in two principal forms in nature: retinol and certain carotenoids. Retinol is found only in animal sources, in foods such as fish, meat, eggs and full-fat milk. In plant foods, vitamin A can be obtained from a family of substances called carotenoids that are found in brightly coloured fruit and vegetables, and leafy green vegetables. The best known form of carotenoid is β-carotene (pro-vitamin A).

c) Vitamin E

Vitamin E is the major lipid-soluble antioxidant in the cell antioxidant defence system and is exclusively obtained from the diet (Schneider 2005). The four tocopherol homologues (*d*-a-, *d*-b-, *d*-g-, and *d*-d-) have a saturated 16-carbon phytyl side chain, whereas the tocotrienols homologues (*d*-a-, *d*-b-, *d*-g-, and *d*-d-) have three double bonds on the side chain. There is also a widely available synthetic form, *dl*-a-tocopherol, prepared by coupling trimethylhydroquinone with isophytol. Vitamin E is an example of a phenolic antioxidant. Such molecules readily donate the hydrogen from the hydroxyl (-OH) group on the ring structure to free radicals, which then become unreactive. On donating the hydrogen, the phenolic compound itself becomes a relatively unreactive free radical because the unpaired electron on the oxygen atom is usually delocalised into the aromatic ring structure thereby increasing its stability (Bello *et al.*,2005).

The major biologic role of vitamin E is to protect PUFAs and other components of cell membranes and low-density lipoprotein (LDL) from oxidation by free radicals. Vitamin E is located primarily within the phospholipid bilayer of cell membranes. It is particularly effective in preventing lipid peroxidation, a series of chemical reactions involving the oxidative deterioration of PUFAs. Elevated levels of lipid peroxidation products are associated with numerous diseases and clinical conditions (Ibrahim *et al.*,1999). Although vitamin E is primarily located in cell and

organelle membranes where it can exert its maximum protective effect, its concentration may only be one molecule for every 2000 phospholipid molecules. This suggests that after its reaction with free radicals it is rapidly regenerated, possibly by other antioxidants (Hsu *et al.*,2001).

There are many signs of vitamin E deficiency in animals most of which are related to damage to cell membranes and leakage of cell contents to external fluids. Disorders provoked, for example, by traces of peroxidized PUFAs in the diets of animals with low vitamin E status are cardiac or skeletal myopathies, neuropathies, and liver necrosis (Suzuki *et al.*,1999). Muscle and neurological problems are also a consequence of human vitamin E deficiency (Bieri *et al.*,1984). Early diagnostic signs of deficiency include leakage of muscle enzymes such as creatine kinase and pyruvate kinase into plasma, increased levels of lipid peroxidation products in plasma, and increased erythrocyte haemolysis. Several animal models (Cho and Choi, 1994) suggest that increasing intakes of vitamin E inhibit the progression of vascular disease by preventing the oxidation of LDL. Evidence suggests that oxidized lipoprotein is a key event in the development of the atheromatous plaque which may ultimately occlude the blood vessel (Suzuki *et al.*,1999).

It is suggested that when the main PUFA in the diet is linoleic acid, a *d*-a-tocopherol-PUFA ratio of 0.4 (expressed as mg tocopherol per g PUFA) is adequate for adult humans, and the ratio has been recommended in the United Kingdom for infant formulas (Li *et al.*, 1999). Use of this ratio to calculate the vitamin E requirements of men and women with energy intakes of 2550 and 1940 kcal/day containing PUFA at 6 percent of the energy intake (approximately 17 and 13 g, respectively) (Horwitt MK, 2001) produced values of 7 and 5 mg/day of a-TEs, respectively. In both the United States and the United Kingdom, median intakes of a-TE are in excess of these amounts and the a-tocopherol-PUFA ratio is approximately 0.6, which is well above the 0.4 ratio which would be considered adequate. The Nutrition Working Group of the International Life Sciences Institute Europe has suggested an intake of 12 mg a-tocopherol for a daily intake of 14 g PUFAs to compensate for the high consumption of soya oil in certain countries where over 50 percent of vitamin E intake is accounted for by the less biologically active g form.

d) Alkylglycerols

Alkylglycerols were discovered by two Japanese scientists in 1922 (Tsujimoto M, Toyama, 1922). They are naturally occurring esters, which are chemicals formed by the combination of a fatty acid and an alcohol molecule. The most common alcohols in these compounds are batyl alcohol, chimyl alcohol, and selachyl alcohol. Hallgren and Larsson studied the occurrence of alkylglycerols in humans, cattle and sharks. When they occur in nature, the alkylglycerols are found esterified with fatty acids. In animals and humans the alkylglycerol-esters are found in red blood cells, the spleen, liver and especially, the bone marrow. They are also involved in the production of white blood cells in the bone marrow. They appear to be as essential to white blood cell production as iron is to red blood cell production. Alkylglycerols also occur naturally in mother's milk. There are ten times more alkylglycerols in human milk than in cow's milk. But the most abundant source of alkylglycerols is the liver oil of certain sharks (Pelton and Overholser).

Early research with leukemia patients showed that taking alkylglycerols during the course of radiation therapy may reduce and even prevent leukopenia and thrombocytopenia. Another study demonstrated that women who took alkylglycerols prophylactically starting eight days before the beginning of radiation therapy had lower mortality rates and greater survival than women who consumed them during the course of radiation therapy. Comparison with controls showed that the prophylactic administration of alkylglycerols before starting radiation also slowed tumor growth.

Radiation therapy can produce a wide range of tissue damage and injury. Several studies have been published showing that the number of radiation-induced injuries is substantially lower in patients treated with alkylglycerols. (Ko *et al.* 2002; Pedrono *et al.* 2004; Arita *et al.* 2005) The more advanced tumors (stages IIB-IV) regressed toward less advanced stages. Alkylglycerols have also been shown to cause a regression of tumor growth in studies on mice in a laboratory environment. (Deniau *et al.*,2009)

It has been suggested that these alkylglycerols fight cancer by killing tumor cells indirectly. Proponents claim they activate the immune system in two ways: by stimulating immune system cells called macrophages, which consume invading germs and damaged cells; and by inhibiting protein kinase C, which is a key regulator of cell growth. Proponents also claim that alkylglycerols reduce the side

effects of chemotherapy and radiation treatment, supposedly because of their ability to protect cell membranes. Because of their supposed immune-boosting effects, alkylglycerols are also claimed to help against colds, flu, chronic infections, asthma, psoriasis, arthritis and AIDS. Since macrophages are also important in wound healing, alkylglycerols are said to have healing effects. These claims have not been studied in controlled clinical trials. Shark liver oils comprise mainly of 1-O-alkylglycerols which constitute about 10-30% of the unsaponifiable matter of the oils (Hallgren and Larsson 1962). These alkylglycerols or AKGs are indeed responsible for reducing pain or inflammation in the body (Pedrono *et al.* 2004). The exact mechanism by which they function has not been fully understood but it has been proposed that they work by either inhibiting the synthesis, release or action of inflammatory mediators, namely histamine, serotonin and prostaglandins that might be involved in inflammation. It has been reported that naturally occurring AKGs have potent biological activities on various cells or systems (Devaraj and Jialal 2000).

e) Squalene

Squalene is a remarkable nutrient produced in our body and is also found in nature. Chemically, squalene is a polyunsaturated aliphatic hydrocarbon of low density, which can produce oxygen by combining with water. It belongs to a class of antioxidants called isoprenoids. An isoprenoid is a cell-friendly molecule that neutralizes the harmful effects of excessive free radicals in the body. Squalene is a pure isoprenoid wherein it is not attached to any other molecule. It has six isoprene units, which provides stability in its function as an antioxidant (Kohno *et al.,* 1995; Ko *et al.,* 2002). The stability of the isoprenoid molecule determines its effectiveness in combating cell damaging free radicals. The more stable the isoprenoid, the more free radicals are neutralized, and the more cells are protected. Scientific research and clinical trials have shown that squalene is safe as a dietary supplement in food and in capsules and no untoward incidents have been reported in the use of squalene. Japanese people have been using squalene for centuries and have attributed their strength and health to this substance.

Squalene is usually bound to sterol-carrier proteins in hepatocytes (Scallen *et al.,* 1971). In 1926, squalene was first proposed as a precursor of cholesterol (Channon, 1926; Helibron *et al.,* 1926). Squalene is synthesized from acetate and is

metabolized to cholesterol in liver (Langdon and Bloch, 1953; Tchen and Bloch, 1957; Srikantaiah *et al.*, 1976). The endogenous synthesis of squalene begins with the production of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA). The initial reduction of HMG CoA (a niacin-dependent reaction) results in the formation of mevalonate. The enzyme involved in this reduction, HMG CoA reductase, has been the target of a class of cholesterol-lowering drugs. Mevalonate is then phosphorylated in three stages (via magnesium-dependent enzymes) and finally decarboxylated to form delta 3-isopentenyl diphosphate, the donor molecule for all polyprenyl compounds. Successive additions of prenyl groups result in the formation of the 15-carbon farnesyl diphosphate (Langdon and Bloch, 1953; Tchen and Bloch, 1957; Srikantaiah *et al.*, 1976). Two molecules of farnesyl diphosphate are then enzymatically joined and reduced (niacin dependent) resulting in the formation of squalene (Goodman and Popjak, 1960; Bloch, 1965; Radisky and Poulter, 2000). After its biosynthesis, squalene can be transported to other areas of the body for incorporation into tissues or it can be further metabolized, resulting in the eventual formation of cholesterol (Tchen and Bloch, 1957; Bloch, 1965; Srikantaiah *et al.*, 1976) and its steroid metabolites.

In addition to the contribution to cholesterol biosynthesis, a portion of the hepatic squalene pool is secreted via bile. In fact, the squalene levels are much higher in bile than in plasma, and in subjects on squalene-free diets, squalene appears also in feces (Liu *et al.*, 1976). In rats, approximately 31% and 34% of absorbed ^{3}H-labeled squalene were excreted as fecal neutral steroids and bile acids, respectively (Tilvis and Miettinen, 1983b). In humans, squalene feeding elevated fecal excretion of neutral steroids and bile acids by 31% and 20%, respectively (Strandberg *et al.*, 1990).

Squalene helps to clean, purify, and detoxify the blood from toxins, facilitating circulation. It cleanses the gastrointestinal tract and kidneys, causing better bowel movement and urination. Many diseases are cured if the blood is purified, by supplementing squalene (Gregory and Kelly, 1999). Experimental evidence suggests that squalene can act as a "sink" for highly lipophilic xenobiotic assisting with their elimination from the body. Since it is a nonpolar substance, it appears to have the highest affinity for unionized drugs. Squalene can be used as an alternative to paraffin to enhance the elimination of [14C] hexachlorobenzene (HCB) and

organochlorine xenobiotic (Richter *et al.,* 1982). Dietary treatment with squalene is as effective as paraffin in markedly enhancing fecal excretion of HCB. The amount of HCB excreted with feces is about three times higher and the half-life of HCB elimination from the body is markedly lower (mean 34-38 days as compared to 110 days for controls) in squalene-treated animals (Richter and Schafer, 1982a).

Sharks inhabiting waters beyond 600m depth are believed to possess reasonably high content of this hydrocarbon (Ko *et al.* 2002), the antioxidant with potent pharmaceutical values. Its role as an antilipidemic agent and membrane stabilizer has been reported (Qureshi *et al.* 1996). Because some early studies have shown that squalamine can slow the growth of tumor blood vessels, proponents claim it may help to treat cancer, either alone or combined with chemotherapy. It is also being studied for use against macular degeneration, an eye condition that results in loss of vision. Squalene has been promoted as having cell-protecting abilities, which may reduce the side effects of chemotherapy.

f) n-3 PUFA

Fish oil is a rich source of long-chain n-3 PUFA, which has been shown to reduce symptoms in rheumatoid arthritis (RA) and other inflammatory disorders (Belluzzi *et al.*,1996), to increase the interval between relapses in Crohn's disease, and to reduce progression to renal failure in IgA nephropathy. Dietary n-3 PUFAs have also been shown to reduce cardiovascular risk factors and events, especially sudden cardiac death (Watkins *et al.*,2007). Reduction in the latter has been shown to correlate with erythrocyte EPA (20:5n-3) levels. Cleland, L.G. *et al.* (2003) showed that fish oil supplementation exerted anti-inflammatory effects (partly) by displacing arachidonic acid (AA) from the pool of highly unsaturated fatty acids in the *sn*-2 position of membrane phospholipids, from which it is released by phospholipase A2 to provide substrate for eicosanoid-forming enzymes (Boudrault *et al.*,2009). EPA acts as an alternate substrate and inhibitor of AA metabolism.

The proposed mechanisms for health benefits of n-3 fatty acids appear to be related to the incorporation of the fatty acids into membrane phospholipids. This results in increasing the production of series 3 eicosanoids, prostaglandin I3, thromboxane A3, and series 5 leukotriene B5 via the cyclooxygenase and lipoxygenase pathways. Eicosanoids, produced by both n-6 and n-3 fatty acids, are involved in the regulation

of inflammation, platelet aggregation, and vasoconstriction/dilation. Both EPA and n-6 arachidonic acid (ARA) (C20:4) compete for the common cyclooxygenase and lipoxygenase enzymes; thus the n-6:n-3 fatty acid ratio seems to be a determining factor for the outcome of the enzymatic pathways. Compared to EPA, ARA produces more potent inflammatory and pro-aggregatory eicosanoids. This is particularly important when considering the abundance of n-6 fatty acids and the scarcity of n-3 fatty acids in our diets (Boudrault *et al.*,2009).

Depending on the commercial preparation, shark liver oil may also be rich in omega-3 fatty acids. Shark liver oils contain high proportions of γ-linolenic acid (Zurier *et al.* 1996), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (James *et al.* 2003). It has been shown that these long chain n-3 PUFAs (James *et al.* 2003) lower the incidence of inflammatory diseases such as asthma and arthritis (Shahidi and Senanayake 2006). These dietary fatty acids are known to reduce the levels of arachidonic acid metabolites and lower the formation of proinflammatory compounds, like prostaglandins and leukotrienes, by blocking their activity (Olivera 2004). Early studies reviewed by Stamp *et al.* (2005) and Calder (2006) attributed the anti-inflammatory effects of fish oils to competition with arachidonic acid for production of inflammatory eicosanoids. Anti-inflammatory effects of EPA and DHA have been studied by several workers (Arita *et al.* 2005; Lukiw *et al.* 2005; Hudert *et al.* 2006). EPA and DHA contained in fish oils provide nutrients needed to build anti-inflammatory prostaglandin series 1 and 3 (Simopoulos 1991). An immunomodulatory effect of these latter fatty acids is suggested by epidemiological studies which show that populations such as Greenland Eskimos, who consume large quantities of marine mammal and fish oils which are rich in eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids, have a very low incidence of inflammatory and autoimmune disorders (4). Furthermore, a number of clinical studies reported that fish oil supplementation has some beneficial effects in rheumatoid arthritis, psoriasis, lupus, and inflammatory bowel disease and prolongs the survival of grafts (Calder, 1999). The potential clinical use of oils rich in n-6 or n-3 PUFA has given rise to a number of investigations of the effects of fatty acids and dietary oils upon immune cell functions.

Parks *et al.* 1992, showed that fish oil feeding reduced plasma and liver cholesterol levels in African green monkeys fed on a high fat diet. The reduced level

of cholesterol absorption in the blood was attributed to the increased content of n-3 fatty acids in the diet. The mixed micelles in the intestine that contain n-3 fatty acids may not solubilize the dietary cholesterol as efficiently as for absorption. Studies in human beings have shown that oleinate reduces total plasma cholesterol and LDL cholesterol concentrations with no effect on HDL cholesterol (Mattson and Grundy 1985).

g) Sterols

Cholesterol is the major sterol found in the lipids of the deep sea sharks. It is a waxy steroid metabolite found in the cell membranes and transported in the blood plasma of all animals (Leah 2009). It is an essential structural component of mammalian cell membranes, where it is required to establish proper membrane permeability and fluidity. In addition, cholesterol is an important component for the manufacture of bile acids, steroid hormones, and several fat-soluble vitamins. Cholesterol is the principal sterol synthesized by animals, but small quantities are synthesized in other eukaryotes, such as plants and fungi.

h) Regulation of cholesterol synthesis

Biosynthesis of cholesterol is directly regulated by the cholesterol levels present, though the homeostatic mechanisms involved are only partly understood. A higher intake from food leads to a net decrease in endogenous production, whereas lower intake from food has the opposite effect. The main regulatory mechanism is the sensing of intracellular cholesterol in the endoplasmic reticulum by the protein SREBP (sterol regulatory element-binding protein 1 and 2) (Espenshade 2007). In the presence of cholesterol, SREBP is bound to two other proteins: SCAP (SREBP-cleavage-activating protein) and Insig1. When cholesterol levels fall, Insig-1 dissociates from the SREBP-SCAP complex, allowing the complex to migrate to the Golgi apparatus, where SREBP is cleaved by S1P and S2P (site-1 and -2 protease), two enzymes that are activated by SCAP when cholesterol levels are low. The cleaved SREBP then migrates to the nucleus and acts as a transcription factor to bind to the SRE (sterol regulatory element), which stimulates the transcription of many genes. Among these are the LDL receptor and HMG-CoA reductase. The former scavenges circulating LDL from the bloodstream, whereas HMG-CoA

reductase leads to an increase of endogenous production of cholesterol (Brown and Goldstein, 1997). A large part of this signaling pathway was clarified by Dr. Michael S. Brown and Dr. Joseph L. Goldstein in the 1970s.

Cholesterol synthesis can be turned off when cholesterol levels are high, as well. HMG CoA reductase contains both a cytosolic domain (responsible for its catalytic function) and a membrane domain. The membrane domain functions to sense signals for its degradation. Increasing concentrations of cholesterol (and other sterols) cause a change in this domain's oligomerization state, which makes it more susceptible to destruction by the proteosome. This enzyme's activity can also be reduced by phosphorylation by an AMP-activated protein kinase. Because this kinase is activated by AMP, which is produced when ATP is hydrolyzed, it follows that cholesterol synthesis is halted when ATP levels are low

Since cholesterol is insoluble in blood, it is transported in the circulatory system within lipoproteins, complex spherical particles which have an exterior composed of amphiphilic proteins and lipids whose outward-facing surfaces are water-soluble and inward-facing surfaces are lipid-soluble; triglycerides and cholesterol esters are carried internally. Phospholipids and cholesterol, being amphipathic, are transported in the surface monolayer of the lipoprotein particle.

In addition to providing a soluble means for transporting cholesterol through the blood, lipoproteins have cell-targeting signals that direct the lipids they carry to certain tissues. For this reason, there are several types of lipoproteins within blood called, in order of increasing density, chylomicrons, very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). The more cholesterol and less protein a lipoprotein has the less dense it is. The cholesterol within all the various lipoproteins is identical, although some cholesterol is carried as the "free" alcohol and some is carried as fatty acyl esters referred to as cholesterol esters. However, the different lipoproteins contain apolipoproteins, which serve as ligands for specific receptors on cell membranes. In this way, the lipoprotein particles are molecular addresses that determine the start- and endpoints for cholesterol transport.

Synthesis of the LDL receptor is regulated by SREBP, the same regulatory protein as was used to control synthesis of cholesterol de novo in response to cholesterol presence in the cell. When the cell has abundant cholesterol, LDL

receptor synthesis is blocked so that new cholesterol in the form of LDL molecules cannot be taken up. On the converse, more LDL receptors are made when the cell is deficient in cholesterol. When this system is deregulated, many LDL molecules appear in the blood without receptors on the peripheral tissues. These LDL molecules are oxidized and taken up by macrophages, which become engorged and form foam cells. These cells often become trapped in the walls of blood vessels and contribute to artherosclerotic plaque formation. These plaques are the main causes of heart attacks, strokes, and other serious medical problems, leading to the association of so-called LDL cholesterol (actually a lipoprotein) with "bad" cholesterol (Tymoczko *et al.*,2002). Also, HDL particles are thought to transport cholesterol back to the liver for excretion or to other tissues that use cholesterol to synthesize hormones in a process known as reverse cholesterol transport (RCT) (Lewis and Rader, 2005) Having large numbers of large HDL particles correlates with better health outcomes (Gordon *et al.*,1989). In contrast, having small numbers of large HDL particles is independently associated with atheromatous disease progression within the arteries.

2.2.11 Cholesterol levels in deep sea sharks

The cholesterol levels in salmon sharks have been reported to range from 1.6% in females to 3.4% in males (Jayasinghe *et al.*, 2003). Bordier *et al.*, 1996 reported the cholesterol levels in liver oils of the deep sea shark *Centrophorus squamosus* to be 4.5% of the total lipids.

2.2.12 The hepatosomatic index of fishery resources

Sharks have no swim bladder and their large livers saturated with oil maintain their buoyancy in water. Deep sea sharks such as gulper shark (*Centrophorus granulosus*), smallfin gulper shark (*Centrophorus scalpratus*), basking shark and tope shark are the major species targeted for this purpose, as they contain a higher yield of oil. Batista and Nunes (1992) report that the size and weight of a shark's liver varies by species and season. The weight of the liver of some shark species constitutes almost one fifth of its weight. Weight tends to increase with size as the larger the shark, the greater the relative weight of the liver. The ratio of liver weight to total body weight of some species is as follows:

Kitefin (*Dalatias licha*)	19.2%
Tiger (*Galeocerdo cuvieri*)	17.5%
Salmon (*Lamna ditropis*)	12.0%
Thresher (*Alopias pelagicus*)	10.0%
Soupfin (*Galeorhinus japonicus*)	2.9%

Jayasinghe *et al.*, 2003 repported the hepatosomatic indices (HSI) of salmon sharks to be 6.8% in males and 7.3-8.0% in females. However the average HSI for the black dogfish or squalid shark *Centroscyllium fabricii* was 20% for both males and females. The hepatosomatic indices of holocephalian fishes was examined by Oguri (1977). Compared to those in teleostean fishes , the index was remarkably high in the fishes studied nearly 16.6% for *Hydrolagus colliei*. He even recorded the high amounts of fat droplets in the liver tissues.

2.2.13 Chromatographic techniques for the determination of lipid classes

In recent years, enormous strides have been made in the development of methods for the analysis of lipids using high-performance liquid chromatography (HPLC). It is almost certainly true to say that this technique has replaced preparative gas chromatography entirely. In addition, it could be claimed that there is no type of lipid separation for which thin-layer chromatography (TLC) was once favoured that cannot now be done by HPLC (Christie, 2003). The latter offers great versatility in that it can be used in the adsorption, reversed-phase, ion exchange and silver ion modes. It operates at room temperature, so is particularly suited to molecules containing thermally labile functional groups. A host of bonded-phases, offering varying selectivities in specific analyses, are available commercially and many have yet to be properly explored in lipid applications.

With TLC in the adsorption mode (silica gel), the principle application in lipid analysis is for the separation of different lipid classes from animal and plant tissues. It is a relatively easy matter to resolve each of the main simple lipids from a tissue in one step, i.e. cholesterol esters, triglycerides, free fatty acids, cholesterol and diacylglycerols, using mobile phases consisting of a mixture of hexane and diethyl ether, with a little formic acid to ensure that the free acids migrate successfully. Complex lipids such as phospholipids and glycosphingolipids will remain at the origin, and they can then be quantified as if they were a single lipid class (Christie,

2003). In the analysis of plasma lipids from clinical experiments, such separations often afford adequate information for diagnostic purposes, and there are many other circumstances where this type of separation is sufficient. It is perhaps one of the disadvantages of HPLC that there is no analogous method for the determination of complex lipids as a single entity. High-performance TLC makes use of silica gel of a very uniform and small particle size, permitting excellent separations with comparatively short elution times. For example, most of the important lipid classes in clinical samples can be achieved by one-dimensional TLC in a single chromatographic run.

TLC offers considerable versatility and precision in lipid analysis with relatively low capital costs.

2.2.14 The TLC-FID using Iatroscan^{c} MK-6s

Analysis of the various lipid components in a system (crude oil, carbon products, marine oils and sediments, foods etc.) may be analysed using a Thin layer chromatograph clubbed with a flame ionisation detector, in a single run. This principle is made use of the Iatroscan MK-6s instrument. Here A specially designed Chromarod (a quartz rod coated with a thin layer of silica or alumina on which the sample is developed and separated), is advanced at a constant speed through the flame of the FID, the substances are ionized through energy obtained from the hydrogen flame. Effected by the electric field applied to the poles of the FID the ions generate electric current with an intensity proportional to the amount of each organic substance entering the flame. The analysis with IATROSCAN MK-6s is favorable in maintenance and price, because of the low consumption of solvents and the reusable Chromarods.

The separation is made with the TLC Method on so called Chromarods and the Detection with a Flame Ionization Detector (FID). With an additional FP-Detector it is possible to analyze sulphur and phosphorus, too (MK-6).The Analysis is made with different complementary accessories.

The spotting of the sample is made with a specially developed Application System (Autospotter or Full-Automatic-Sample Spotter) on the 10 Chromarods lying in the Rod Holder. The Chromarods are developed in a special Development Tank. After the separation of the substances the Holder is put in the IATROSCAN. The Chromarods are scanned through the Hydrogen flame. The collector, which is placed

above the flame is generating an analogue signal, which is evaluated with a PC and the SES ChromStar Software.

Numerous workers have made use of the TLC-FID analytical technique for the detection and quantification of various components. Cebolla *et. al.* (1995) , Karlsen *et al.*,(1991) and Sol *et al.* (1985), have used this technique for the analysis of crude hydrocarbons - saturates, aromatics, resins and asphaltenes (SARA) constituents in crude oils and solvent extracts. Itoh *et al* 1985 separated the methanolysis products of neutral sphingolipid and archaebacterial neutral glycolipid on Chromarods S-II (silica gel) with a double developing system. The lipid constituents separated on the rods were scanned automatically with a hydrogen flame ionization detector (Iatroscan). The molar ratios of the constituents determined by this system were very close to the theoretical values of the lipid. Reiffova *et al.*, (2003) used the technique for the separation of oligosaccharides. Chromatography was performed on Chromarods S III with two different mobile phases, ethyl acetate-formic acid-water and butanol-ethanol-water. Pretreatment of the biological samples was minimal.

2.2.15 The HPLC with an evaporative light scattering detector (HPLC-ELSD)[d]

Lipid analysts were initially slow to come to terms with the potential of high-performance liquid chromatography (HPLC), largely because of the non-availability of a sensitive universal detector. In contrast the flame-ionisation detector, commonly used in gas chromatography (GC), is highly sensitive and exhibits a linear response over a wide range of sample sizes. Transport-flame ionisation detectors for HPLC have always looked promising, but have never been a commercial success. The evaporative light scattering detector has the advantages of being simple yet versatile in the commercial analysis of samples.

With this instrument, the solvent emerging from the end of the column is evaporated in a stream of air in a heated chamber; the solute does not evaporate, but is nebulized and passes in the form of minute droplets through a light beam, which is reflected and refracted. The amount of scattered light is measured and bears a relationship to the concentration of material that is eluting.

There are no special wavelength requirements for the light source, and in some commercial instruments, it is simply a projector lamp. Such a detector can be considered to be universal in its applicability, in that it will respond to any solute that

does not evaporate before passing through the light beam. The instrument gives excellent results under gradient elution conditions, and it is simple and rugged in use. The sensitivity is comparable to that of a refractive index detector, but the evaporative light-scattering detector is not affected by changes in the mobile phase or small variations in the room temperature or in the flow rate of the mobile phase, as is the former.

Once the instrument is warmed up and is running, there is little base-line drift during continuous operation even with abrupt changes in solvent composition. Most organic solvents, including acetone and chloroform, for example, can be used, and these can contain up to 20% water and small amounts of ionic species even. The minimum detection limit is dependent to a certain extent on the nature of the mobile phase, the nature of the sample, and the specific instrument, but it is certainly less than one microgram.

There has also been some debate about the efficacy of the evaporative light-scattering detector in quantification. However, Herslof and Kindmark (1996) obtained good reproducibility for the relative proportions of different molecular species in analyses of the triacylglycerols of soybean oil. When the technique is used in research with triacylglycerols differing widely in composition, the best approach to quantification consists in collecting fractions and adding an odd-chain fatty acid as an internal standard prior to transesterification and GC analysis, *i.e.* the technique long used with thin-layer chromatography. The fatty acid composition and the amount of each fraction are thereby obtained simultaneously.

Graeve and Janssen (2009) in their study on the separation of lipid classes of marine zooplanktons presented an improved HPLC method, which allowed better resolution and quantification of a broad range of lipid classes with special regard to neutral lipids. Marine zooplankton species often produce high amounts of exceptional lipids, especially at high latitudes, in order to cope with the harsh environmental conditions and strong seasonality in food supply. Major neutral lipid classes analysed in their study were wax esters, triacylglycerols, diacylglycerol ethers, free fatty alcohols and sterols. Neutral and polar lipids were separated and identified on a monolithic silica column (Chromolith®Performance-Si) using high performance liquid chromatography (HPLC) with an evaporative light scattering detector (ELSD). Their method resolved a broad spectrum of lipids, varying in

polarity from squalene to lysophosphatidylcholine in a single run. The total run time was 35 min including column re-equilibration. The calibration was made at levels of 0.1–60 μg lipid/injection, but a 10–15-fold greater amount could have been injected if single lipid classes had to be separated, e.g. for further determination of individual fatty acids. The method was applied to representative Arctic zooplankton species (copepods, pteropods, euphausiids and ctenophores) that were known to biosynthesize in particular neutral lipids like diacylglycerol ethers and free fatty alcohols.

Homan and Anderson (1998) achieved rapid separation and quantitation of combined neutral and polar lipid classes by the HPLC-ELSD method. In their study, Substitution of acetone for 2-propanol in a portion of the solvent gradient program yielded consistent resolution of diacylglycerol and cholesterol without sacrificing baseline resolution of the remaining major lipid classes. Moreover, previously noted instabilities in triacylglycerol retention time are eliminated. The introduction of acetone also enabled a 20% reduction in flow-rate without an increase in total run time. As a further modification of the mobile phase composition, acetic acid and ethanolamine were substituted for the serine–ethylamine combination that was originally shown to improve column performance. The combination of acetic acid and ethanolamine yielded the same result but the increased volatility of these solutes over serine resulted in decreased baseline noise. Finally, 1,2-hexadecanediol was introduced as an internal standard that well suited for their method. The chromatographic performance obtained with the modifications was demonstrated in compositional analyses of lipid extracts from rat liver, heart, kidney and brain.

2.3 MATERIALS AND METHODS

All the elasmobranch resources *Apristurus indicus*, *Centrophorus scalpratus*, *Centroselachus crepidater*, *Neoharriotta raleighana* and *Harriotta pinnata* were caught during Cruises 250 and 252 on the Fisheries Oceanic Research Vessel (FORV) Sagar Sampada from beyond 600 m depth along the southwest and eastern coasts of India. Expo model trawl nets were used to catch these deep sea fishery resources. The liver was separated from these fishes, the total lengths and body weights of each fish were recorded; the corresponding liver weights were also noted down to calculate the ratio between the body and liver weights or the hepatosomatic index (HSI). The fish along with their respective livers were immediately frozen at -20^{0}C onboard the vessel; brought to the laboratory for further analyses. The catch details of the five different elasmobranch resources, whose liver oils were analyzed for their bioactives, are shown in Table 2a. All chemicals and reagents used were obtained from Merck (Darmstadt, Germany). The chemical standards used for the calibration and standardisation were purchased from Sigma-Aldrich Chemical Inc. (St. Louis, MO, USA).

2.3.1 Extraction of liver oils

Accurately weighed liver of each fish was subjected to lipid extraction by the method of Folch *et al.* (1957). Briefly, minced liver was homogenized in a 2:1 (v/v) mixture of chloroform-methanol and filtered. 20% water was added to this mixture and the layers were allowed to separate overnight. The aqueous layer was discarded the following day and the total solvent extract was concentrated (i.e. solvents were removed *in vacuo*) using rotary evaporation at 40°C. The oil was made up to a known volume in chloroform and stored in amber-coloured bottles under nitrogen at -20°C. A portion of the oil was saponified (Hallgren and Larsson 1962), in a mixture of 150% potassium hydroxide (w/v) and absolute ethanol for 2 h in a water bath at 75°C under an inert atmosphere of nitrogen. The resulting mixture was extracted with ether, water-washed, dried over anhydrous sodium sulphate and finally condensed to a known volume. A small portion of the ether layer was air dried to estimate the fraction of the non-saponifiable matter (NSM) present in the oils.

2.3.2 Analysis of lipid components using TLC-FID

Aliquots of the ether extract or the diluted crude liver oil were analyzed using an Iatroscan MK-6s (M/s. Mitsubishi Kagaku Iatron Inc. Tokyo, Japan) employing the TLC-FID method, to determine the abundances of individual lipid classes (hydrocarbons, alkoxyglycerols, triacylglycerols, fatty acids) (Bakes and Nichols 1995). Samples were applied in triplicates to silica gel SIII chromarods (5 µm particle size) using 1 µl disposable micropipettes. Chromarods were developed in a glass tank lined with pre-extracted filter paper. The solvent system used for the lipid separation was chloroform-methanol-water-ammonia (47:23:4:0.25 v/v/v/v), a mobile phase separating polar lipids. A second non-polar solvent system of hexane-diethyl ether (60:15 v/v) was also used to resolve the non-polar lipid components. After development, the chromarods were oven dried and analyzed immediately to minimize adsorption of atmospheric contaminants. The flame ionization detector (FID) was calibrated for each compound class (squalene, monopalmitoyl-rac-glycerol, di-oleoyl-rac-glycerol, cholesterol, oleic acid, tripalmitin, α-tocopherol, retinol, phosphatidylcholine). The peaks obtained via a Chromatocorder were quantified and tabulated. The standards were prepared in five different concentrations ranging from 0.1- 2 ug/ml and 1 ul of each standard was spotted on chromarods to determine the coefficient of linearity.

2.3.3 Analysis of lipid components using HPLC-ELSD

It is well established that separation in normal phase chromatography is based on the polar groups of the molecule regardless the non-polar side chain. This fact permits one to separate lipid classes regardless the number of carbon atoms and degree of saturation of the compound. However, the whole structure of the molecule contributes on the separation. Although the effect of alkyl groups to the retention is limited, it should be also taken into account. In addition, steric effects have also influence on the retention making possible the separation of *cis* and *trans* isomers. Hence, this type of chromatography is preferred when commercial mixtures of oils have to be analyzed because of the presence of complex mixture of chemical species.

The quantification of the different lipid classes of squalene, cholesterol palmitate, a-tocopherol, tripalmitin and monopalmitoyl-rac-glycerol in the liver oils of the 5 elasmobranchs was further confirmed with the help of HPLC-ELSD technique. The

lipid separation was accomplished by normal phase HPLC, using quarternary gradient pumps (Merck Hitachi L-2700 Germany). 20ul volume of sample was injected into the column using a 20ul sample loop. The Chromatographic column was a LiChrosphere Si60 Nor Ph. The flow rate was 1.0ml/min and the column temperature was maintained at 35^0C during all runs. An evaporative light scattering detector (Alltech 2000 ES USA) was used for the detection and nitrogen was used for the gas. The detector temperature was 45^0C and the air pressure was 2.0 bar. The photomultiplier sensitivity was adjusted to the mean value of the total photomultiplier range (gain 7). The detector signal was recorded and integrated by a personal computer and a software program (GynkoSoft Chromatography Data System, version 4.22, Gynkotek, Munich, Germany).

The lipid classes were separated by three different solvent and gradient systems. A combination Gradient is shown in Table 2e. Isooctane with 0.5% of MTBE enables the separation of squalene, cholesterol as their corresponding esters, and mono-alkyl glycerols in a time of analysis of 50 min.

2.3.4 Analysis of fatty acids using GC-FID

Aliquots of the ether extract of the liver oil (three replicates) were methylated using BF_3-methanol and the resulting fatty acid methyl esters (FAME) (Davidson and Cliff 2002) were injected into the Trace GC Ultra gas chromatograph (M/s. Thermo Electron Corporation, Milan, Italy) equipped with Perkin Elmer Elite 225, 50% cyanopropyl phenyl – 50% methyl capillary column (30 m × 0.25 mm i.d.), a flame ionization detector (FID) and a split/splitless injector. Nitrogen was the carrier gas. Briefly, the aliquots were injected in splitless mode at an oven temperature of 110°C. After 4 min the oven temperature was raised to 240°C at 2.7°C/min. Peaks were analyzed and quantified using Chromcard software, with the help of running authentic standards.

2.3.5 Statistical analysis

Data obtained from three replicates of the liver oil sample were subjected to descriptive statistics using SPSS 16.0 Software Package and the values were expressed as mean ± SD.

2.4 RESULTS

The sharks and chimaeras obtained for the study from the south west coasts of India namely *Apristurus indicus*, *Centrophorus scalpratus*, *Centroselachus crepidater* and the chimaeras *Neoharriotta raleighana* and *Harriotta pinnata* were taken by bottom trawlers off the Indian coasts (Plate II.1, Table 2a). These species formed the dominant catches among the elasmobranchs analysed during our expeditions but however they find limited commercial applications. Therefore the need to understand and utilise such potential specimens for research including molecular studies is essential.

The hepatosomatic indices of the elasmobranchs were studied and tabulated as in Table 2b. The body to liver weight ratio of all the species studied was approximately 20% as observed by several other workers. The weight of the liver of some shark species constitutes almost one fifth of its weight. Weight tends to increase with size as the larger the shark, the greater the relative weight of the liver.

The lipid composition of oils extracted from the liver of four species of deep sea sharks found in southern Indian waters was determined. The oils of NR, CS and CC recorded high NSM content of 80, 73 and 60 %, respectively (Table 2c). However, Al oil had the lowest content of NSM (25%) among the four oils examined. Alkylglycerols and hydrocarbons (HCs), predominantly the isoprenoid squalene, were the major components of the NSM. HC content varied significantly among the species analyzed. Oils of Al species recorded the lowest amount of squalene at 20.1 %, while that of CS was 67.4 %. Oils of CC and NR species contained 52 and 62 % squalene, respectively. AKGs comprising of both mono- and di-alkoxy-glycerols were present in all shark species at levels between 12.2 and 21.1 %. Polar lipids were either present in low abundance (<2%) or were not detected in the extracted oils.

Triacylglycerols composition ranged from 2-4% of total lipids in liver of the selected elasmobranchs. Cholesterol was observed at almost 5% levels in NR, CS and Al and at half its amount in CC. Significantly high amounts of monoacylglycerols (7% total lipid) were observed in the livers of the *Neoharriotta* sp. Vitamin E at 2% lipid levels of liver were observed in both the *Neoharriotta sp.* and the *Centrophorus sp.* Traces of Vitamin A were also recorded in the selected species. However the lipids of *Apristurus sp* recorded high levels of saponifiable matter (68% total lipids)

Table 2a. Details of shark species collected during cruises on the FORV *Sagar Sampada*

No.	Name	Region	Lat N	Long E	Depth (m)
1.	*Apristurus indicus* (AI) Small-belly catfish	Azhikkal	$12^0$04	$74^0$16	735
2.	*Centrophorus scalpratus* (CS) Endeavour dogfish	Diglipur	$13^0$21	$93^0$07	695
3.	*Centrosymnus crepidater* (CC) Deep sea dogfish	Kasargode	$12^0$25	$74^0$07	740
4.	*Neoharriotta raleighana* (NR) Long-nosed ratfish	Alleppey	$09^0$17	$75^0$38	724
5.	*Harriotta pinnata* Sickle-finned chimaera	Alleppey	$09^0$17	$75^0$38	724

Table 2b. Fork lengths, body & liver weights and hepatosomatic indices of sharks and chimaeras of the Indian EEZ

Sharks/chimaeras (N=3)	Total length (cm)	Body weights (kg)	Liver weights (kg)	Hepatosomatic index (%)
C. scalpratus	160.6 ± 5.3	416.2 ± 2.3	83.1 ± 1.6	20.1
C. crepidater	127.5 ± 4.6	318.6 ± 1.1	72.2 ± 1.4	22.7
A .indicus	52.3 ± 4.7	99.2 ± 1.3	16.1 ± 1.2	16.3
N. raleighana	120.3 ± 2.7	312.4 ± 1.9	71.7 ± 1.4	23.1
H. pinnata	134.5 ± 6.1	281.4 ± 1.2	55.8 ± 1.5	19.8

Results are mean±SD for n=3 determinations.

Table 2c. Characterisation of liver lipids of elasmobranch resources of the Indian EEZ

	Neoharriotta raleighana (NR)	*Centrosymnus crepidater* (CC)
Crude fat content (w/w)	69.28 ± 1.83	77.43 ± 2.85
Non-saponifiable matter	80.01 ± 2.03	60.7 ± 3.60
Hydrocarbon (squalene)	59.43 ± 2.52	52.71 ± 1.29
Triacylglycerols	3.89 ± 3.07	2.01 ± 1.37
Diacylglyceryl ether	3.46 ± 4.23	1.24 ± 1.94
Monoacylglyceryl ether	7.61 ± 3.80	2.42 ± 1.20
Sterol (cholesterol)	5.73 ± 2.15	2.73 ± 1.05
Vitamin E (tocopherol)	2.55 ± 0.59	0.35 ± 0.32
Vitamin A (retinol)	0.96 ± 0.88	0.27 ± 0.13
Saponifiable matter	22.86 ± 0.45	28.26 ± 2.45
Free fatty acids	0.85 ± 0.20	0.87 ± 0.16
Total fatty acids	21.18 ± 4.85	26.63 ± 2.35
Polar lipids	0.83 ± 0.06	0.76 ± 0.36

Lipid composition of liver oil of	*Neoharriotta pinnata.*	*Centrophorus scalpratus* (CS)	*Apristurus indicus* (AI)
Crude fat content (w/w)	69.28 ± 3.33	79.46 ± 2.27	70.58 ± 1.87
Lipid composition (as % total lipid)			
Non-saponifiable matter	78.01 ± 3.43	73.6 ± 1.50	30.90 ± 0.70
Hydrocarbon (squalene)	52.43 ± 8.29	60.67 ± 0.84	18.10 ± 0.35
Triacylglycerols	3.89 ± 3.07	2.65 ± 1.02	4.43 ± 1.37
Diacylglyceryl ether	3.46 ± 4.23	1.46 ± 0.23	3.46 ± 4.23
Monoacylglyceryl ether	10.61 ± 3.80	2.62 ± 1.53	0.84 ± 1.80
Sterol (cholesterol)	5.73 ± 2.15	5.73 ± 1.17	4.73 ± 1.24
Vitamin E (tocopherol)	2.55 ± 0.59	2.74 ± 0.15	0.55 ± 0.59
Vitamin A (retinol)	0.96 ± 0.88	0.34 ± 0.10	0.52 ± 0.31
Saponifiable matter	22.86 ± 0.45	24.26 ± 0.45	68.83 ± 0.45
Free fatty acids	0.85 ± 0.20	0.28 ± 0.11	1.94 ± 0.25
Total fatty acids	21.18 ± 4.85	23.09± 1.63	66.35 ± 2.05
Polar lipids	0.83 ± 0.06	0.89 ± 0.23	0.54± 0.16

Results are mean % of three replicates of the liver oil ± S.D.

Table 2d. Total fatty acid composition of liver oils from deep-sea sharks collected from Indian waters*

Fatty Acid	Percentage composition (as % total fatty acid)				
	A. indicus	*C. crepidater*	*C. sculpratus*	*N. raleighana*	*N.pinnata*
14:0	0.65	1.17	0.21	1.72	0.72
14:1	0.82	0.16	1.30	0.30	0.28
15:0	1.00	1.00	0.06	1.98	0.78
15:1	0.70	0.11	0.54	0.10	0.12
16:0	14.15	12.43	10.82	12.36	11.36
16:1	2.89	4.00	3.34	4.71	4.85
17:0	0.15	0.35	0.16	0.30	0.22
17:1	2.93	4.98	3.82	3.10	2.85
18:0	9.79	5.65	4.17	3.60	4.60
18:1(n-9)	16.34	24.11	33.43	27.68	29.68
18:2(n-6)	1.39	ND	1.68	2.46	2.16
20:0	0.21	0.47	0.31	0.40	0.50
20:1	23.85	15.99	15.87	11.35	12.05
20:3(n-3)	7.15	5.82	8.75	5.20	5.11
20:5(n-3)	0.62	0.88	1.87	4.53	5.03
22:0	0.32	0.37	0.28	0.67	1.87
22:1	10.17	14.01	7.46	6.22	6.92
22:6(n-3)	3.88	4.08	3.10	10.03	9.11
24:1	1.50	2.42	1.83	2.40	2.93
Others	1.50	1.99	1.01	0.88	0.68
Total	100.00	100.00	100.00	100.00	100.00
Sum Saturates	26.26	21.43	18.01	21.03	19.03
Sum monounsaturates	59.20	65.79	68.59	49.96	57.87
Sum polyunsaturates	13.04	10.79	15.40	21.92	22.22
Total Fatty Acid (mg/g)	687.82	282.71	242.34	220.10	211.80

* All values are expressed as percentage of the total fatty acids unless otherwise stated.
GC results are subject to an error of ± 1 %.
ND = not detected

Table 2e. Gradient mobile phase composition (%)

Time (min)	Percent Solvent		
	Isooctane	MTBE[a]	Isopropanol
0	99.5	0.5	0
3	99.5	0.5	0
3.01	98.5	1.5	0
20	89	11	0
30	75	25	0
30.01	71	27	2
50	71	27	2
50.01	75	25	0
60	75	25	0
70	99.5	0.5	0

[a] MTBE contained 0.01% (v/v) of formic acid.

compared to the others. The total free fatty acids were found to be at less than 1% levels in all the species evaluated.

The fatty acid (FA) and total fatty acid content of the four species of shark are given in Table 2d. The total fatty acids ranged from 22 to 28 % of the total lipid in NR, CS and CC species whereas it was as high as 68 % in the liver oils of AI species. The predominant fatty acids in all species were the monounsaturates (MUFA) ranging from 55 to 67 % with 18:1(n-9), 20:1 and 22:1 being the major fatty acids. Polyunsaturated fatty acid (PUFA) levels varied from 10 to 22 % of the total fatty acid content in oils.

2.5 DISCUSSION

The composition of liver oils from the elasmobranchs *Apristurus indicus*, *Centrophorus scalpratus*, *Centroselachus crepidater* and the chimaera *Neoharriotta raleighana* have not been previously documented. These sharks chimaeras are frequently caught in deep-sea fishery trawls along the southwest coast of India. The liver oil of these speceis was found to contain high levels of the HC squalene which is typical of liver oils from deep-water elasmobranchs inhabiting water depths between 600 and 1000 m (Bakes and Nichols 1995). Triacylglycerols, di- and monoacyl glyceryl ethers together formed 18% of the liver oils of *N. pinnata*. According to Deprez *et al.* (1990), the levels of these specific lipids have been found to vary from 18% in certain species of dogfish sharks (*Centrophorus scalpratus*) to as high as 90% in Plunket (*Somniosus pacificus*) and sleeper sharks (*Centroscymnus plunketi*). The role of specific lipids and hydrocarbons as buoyancy regulators in the liver of deep sea sharks has been documented (Malins and Barone 1970; Phleger and Grigor 1990) and it is apparent that different sharks regulate liver lipid composition to maintain buoyancy. The levels of squalene, triacylglycerols, di- and monoacyl glyceryl ethers and the unusually high amounts of tocopherol in the liver oils of chimaera could also be affected by the dietary intake of specific components and seasonal variations (Kayama *et al.* 1971; Hayashi and Takagi 1981). Phleger and Grigor (1990) showed that *Hoplostethus atlanticus* found at similar depths to these deep-sea chimaeras use lipid deposits to control buoyancy.

The total fatty acid content obtained from the liver oil of *N. pinnata* was 211 mg/g. Previous reports by Buranudeen and Richards-Rajadurai (1986) confirmed the variations in the total fatty acid content in certain *Centrophorus* species (possessing high squalene contents in their liver oils), to range from 95–600 mg/g. The fatty acids in the liver oils of *N. pinnata* were mainly the mono- and poly-unsaturated types. The role of EPA and DHA in lipid fluidity has been previously documented (Russell 1990) and the high levels of DHA in chimaeras and sharks may complement the levels of AKGs in them and play a role in maintaining their fluidity. AKGs are important in the treatment of haematopoiesis and radiation sickness (Devaraj and Jialal 2000; Pedrono *et al.* 2004).

It has also been proved that long chain *n*-3 PUFAs (James *et al.* 2003) lower the incidence of inflammatory diseases such as asthma and arthritis (Calder 2006). These dietary fatty acids are known to reduce the levels of arachidonic acid metabolites and lower the formation of proinflammatory compounds, like prostaglandins and leukotrienes, by blocking their activity (Olivera *et al.* 2004). Early studies reviewed by Stamp *et al.* (2005) and Calder (2006) attributed the anti-inflammatory effects of fish oils to competition with arachidonic acid for production of inflammatory eicosanoids. EPA and DHA contained in fish oils also help to increase levels of digestive enzymes in the body thereby providing nutrients needed to build anti-inflammatory prostaglandin series 1 and 3, which helps in weight loss (Simopoulos 1991).

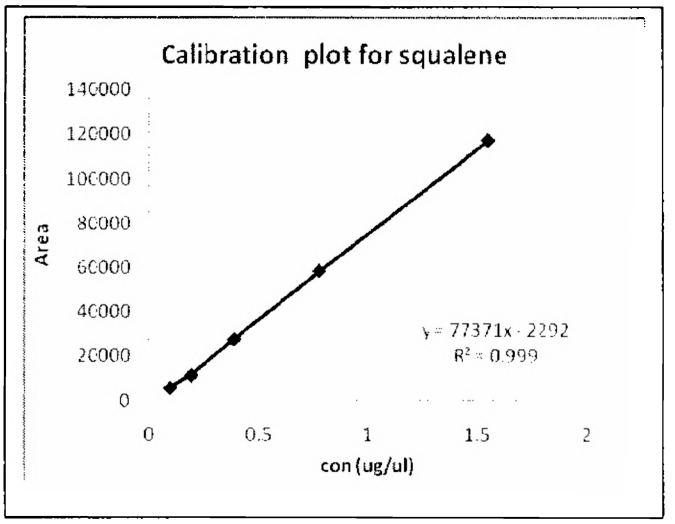
In addition, the high levels of squalene (Ko *et al.* 2002) and tocopherol (Devaraj and Jialal 2000) in the liver lipids of *N. pinnata* help reduce inflammation by decreasing C-reactive protein levels by blocking the activity of TNF-α (tumour necrosis factor-alpha) series 2-prostaglandins (PGE-2) and cyclooxygenases (James *et al.* 2003). The role of squalene as an antilipidemic agent (Qureshi *et al.* 1996) and membrane stabilizer has been well documented (Sabeena *et al.* 2004).

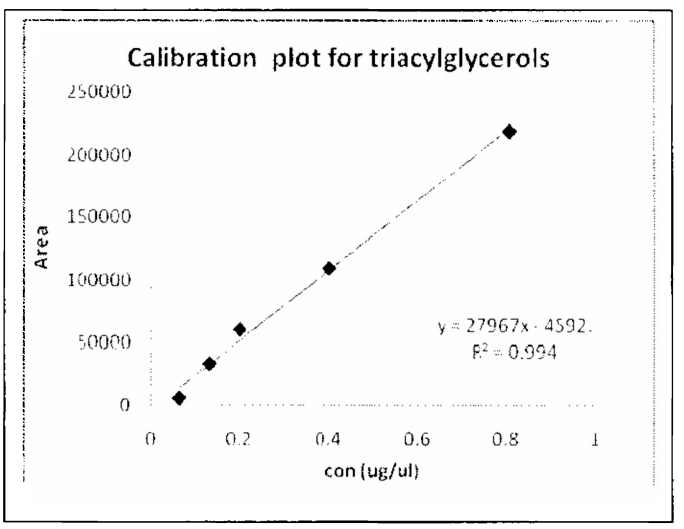
All the instruments used in the study had been calibrated for the purpose of quantification of the standards. Most of the calibration curves reported in the literature for the Iatroscan follow the same basic form and are fitted by a power law equation (FID response versus weight of lipid, $y=ax^b$) for loads ranging from 1 to 10

ug of standard lipid. Usually, FID response is linear, but the range of load used in TLC–FID is too large to obtain this linearity in one curve. Besides, the high sensitivity of the FID detector allows the quantification of low amounts of lipid (0.1 ug) with a low coefficient of variation (S.D.<6%, n=3) for each standard class. Thus, it was unnecessary to fit calibration curves in a larger range than 0.1 to 2 ug lipid. Thus, the power law equation was accurate enough for a satisfactory fitting (r^2=0.9905 to 0.9992, n=3) and easier to use in data processing.

Linear response curves have been reported for the detection of lipid classes by ELSD, although only for a narrow range of amount injected. Torres *et al.* (2005) prepared standard curves for non-polar lipids and observed that the relationship between light scattering and solute concentration was generally linear, but second order polynomial regression analysis gave the best fit. Figure 2.1 shows the calibration curves of the different lipid classes studied. The amount of sample injected was in the range between 1 and 50 ug injected. This broad range of concentrations studied permits one to simultaneously quantify minor constituents in fats and oil in concentrations as low as 1% (w/w) of the total and simultaneously with the rest of neutral lipid existing in the sample. Hence, this methodology not only separates the different lipid classes but also is able to estimate the relative proportion in which they are found in a broad range of concentrations.

Figure 2.1 Calibration curves of lipid components using TLC-FID





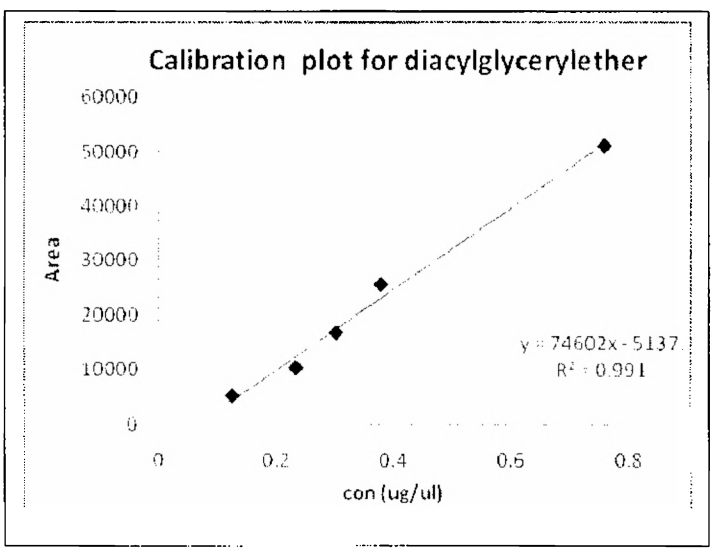
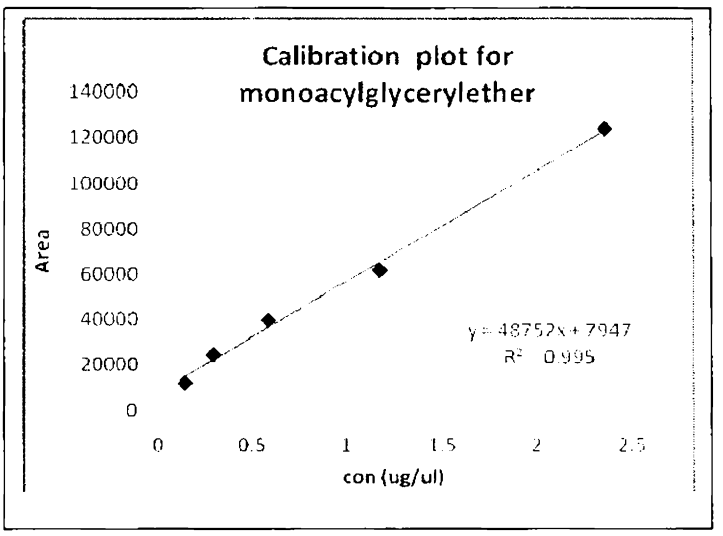
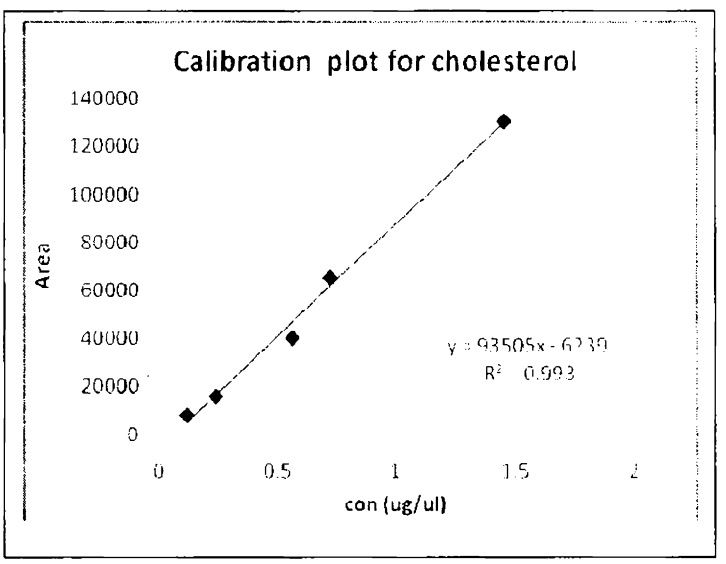


Figure 2.1 Calibration curves of lipid components using TLC-FID (contd.)





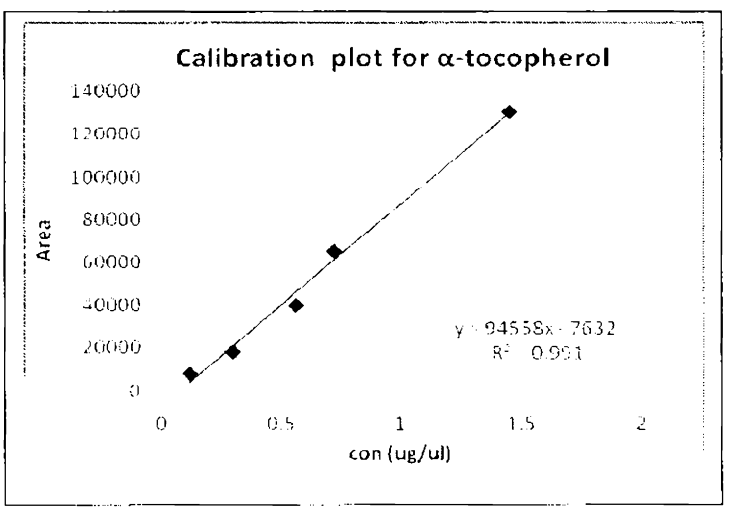
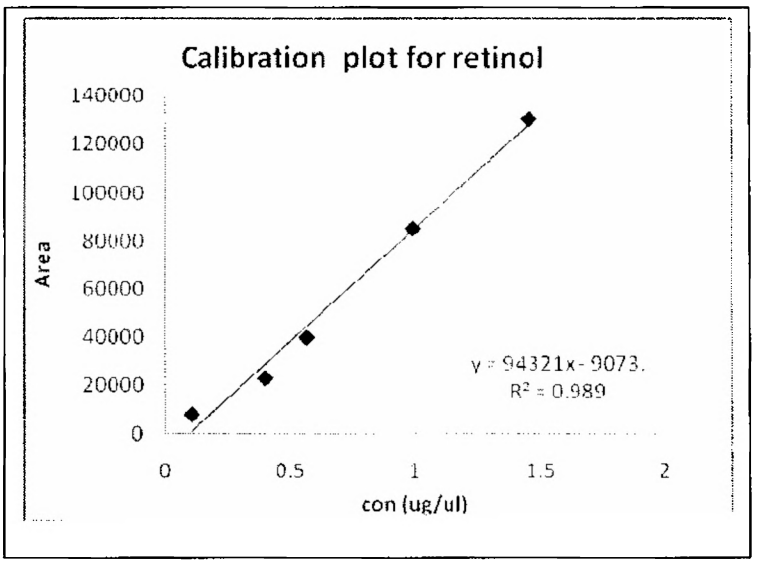
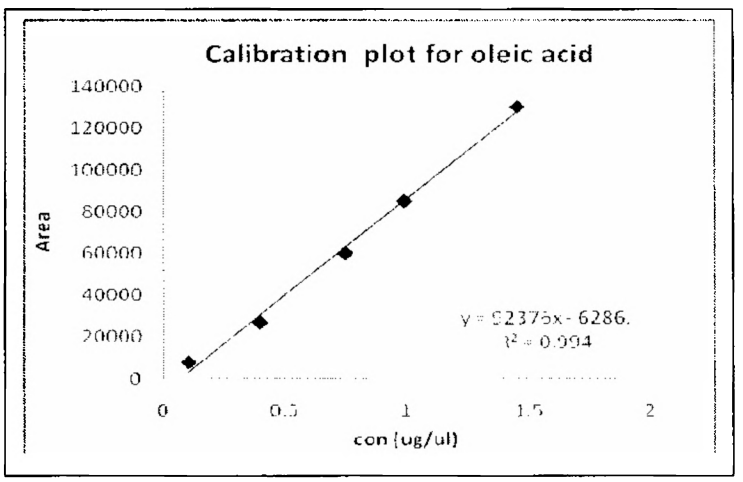
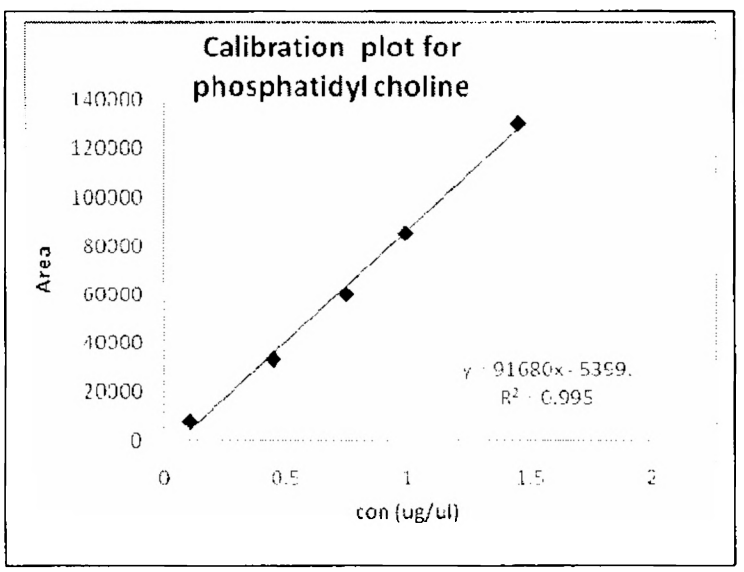


Figure 2.1 Calibration curves of lipid components using TLC-FID (contd.)







2.6 SUMMARY AND CONCLUSION

Studies on the potential health benefits of lipids from deep sea fishes is a rapidly emerging area. Our study is only a preliminary initiative to assess the bioactive potentials of marine lipids using species otherwise considered by people as wastes.

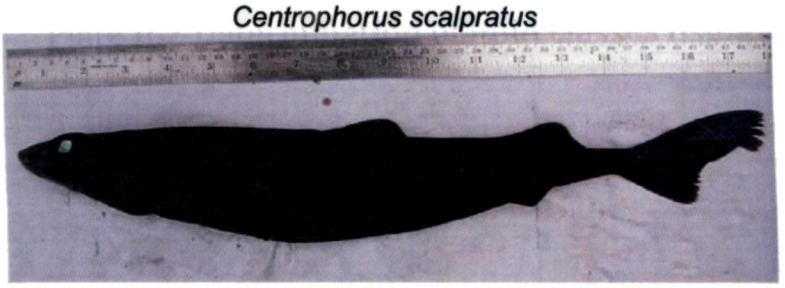
The major findings of the study were as follows

1) *Apristurus indicus*, *Centrophorus scalpratus*, *Centroselachus crepidater*, *Neoharriotta raleighana* and *Harriotta pinnata* were the major elasmobranchs that dominated our catches during the cruises along the south west coasts of the Indian EEZ.
2) There was an approximately 20% ratio of body to liver weights of almost all the species analysed.
3) High levels of non-saponifiable matter was observed in the liver oils of the CS, CC and NR species whereas those of AI recorded high levels of the saponifiable matter in their liver oils.

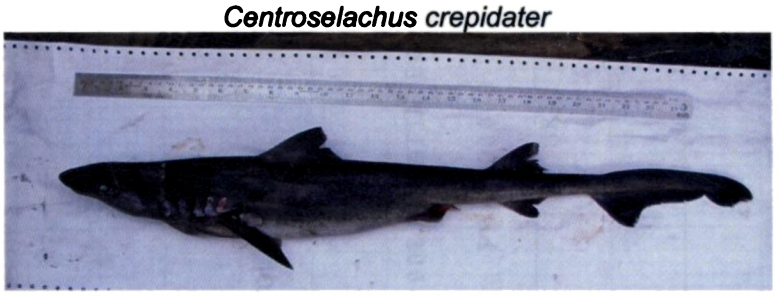
The use of these liver oils in biomedical applications needs to be looked upon. The liver oils of deep sea sharks and chimaeras possess multitudes of bioactives whose immense pharmacological applications deserve strong scrutiny.

PLATE II.1 Elasmobranch resources from the Indian EEZ

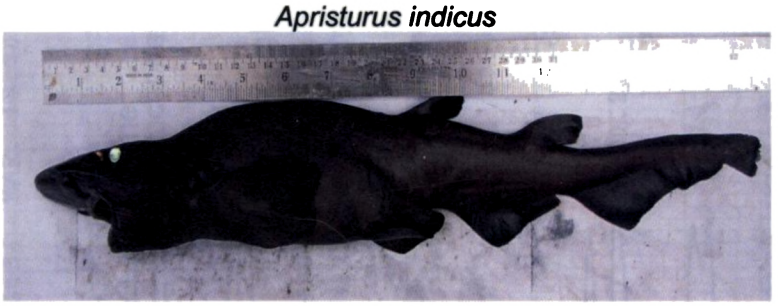
Centrophorus scalpratus



Centroselachus crepidater



Apristurus indicus



Neoharriotta raleighana

Neoharriotta pinnata

Plate II.2 Determination of lipid classes using TLC-FID

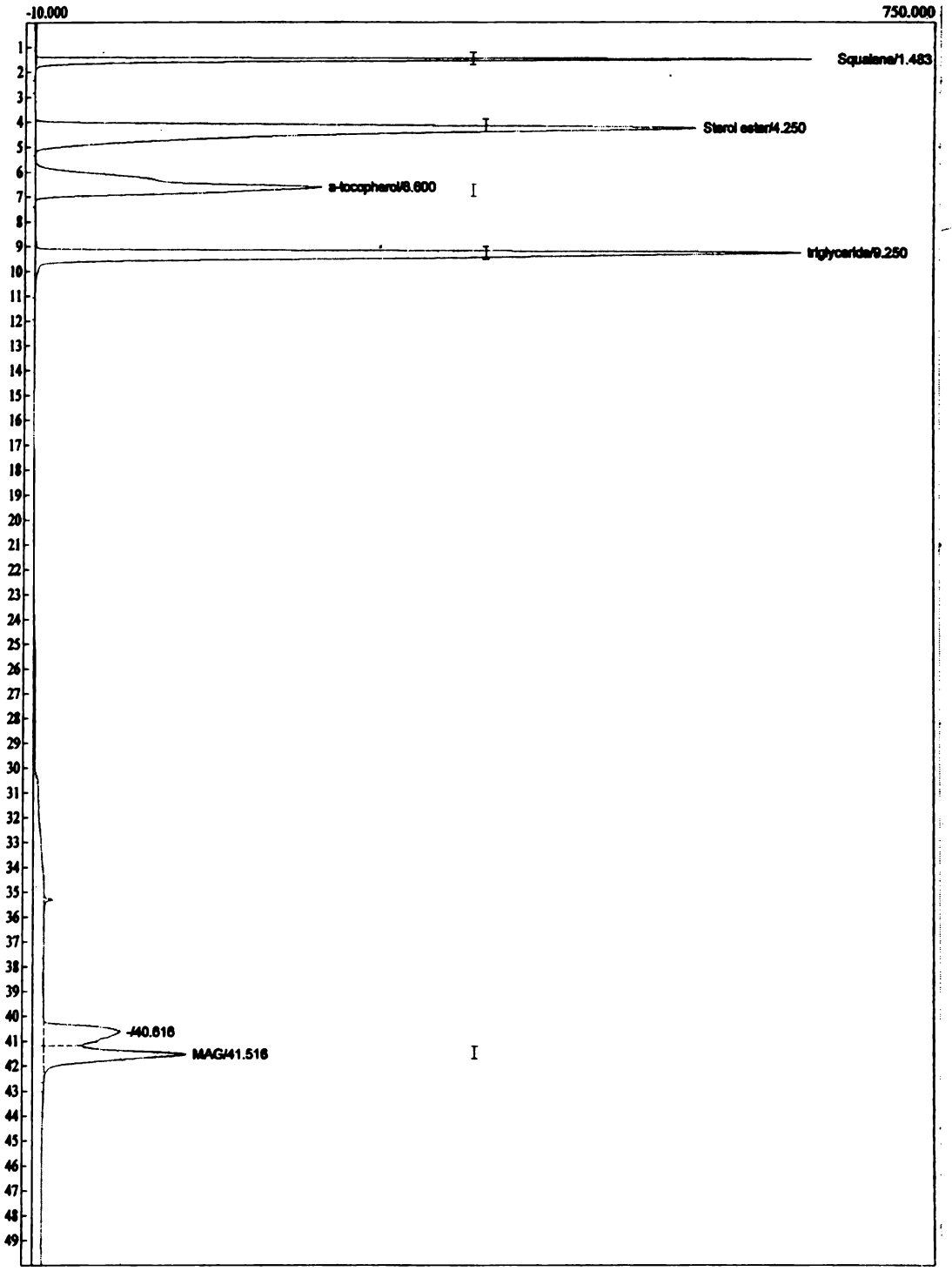


Plate II.3 Determination of lipid classes using HPLC-ELSD

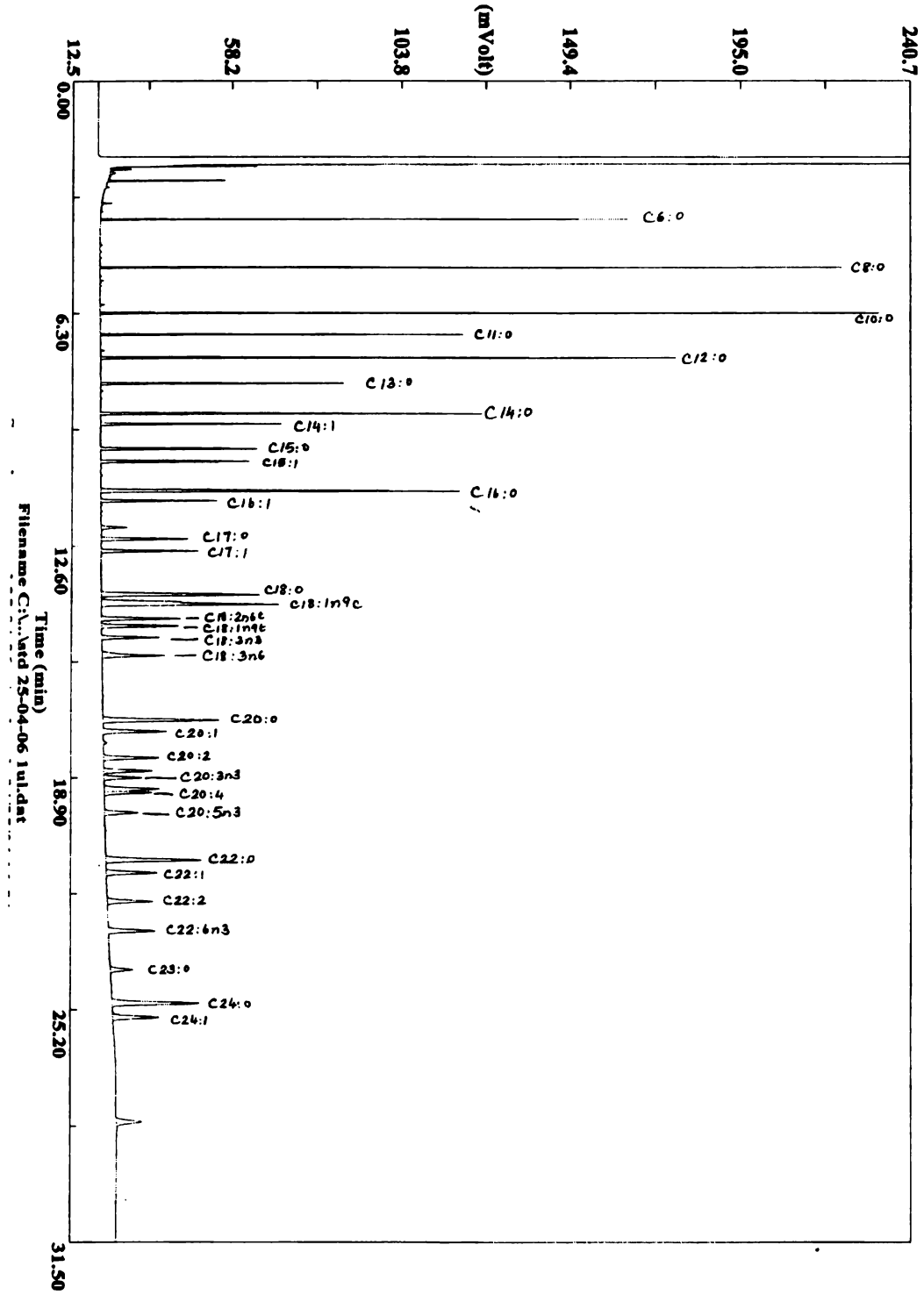


Plate II.4 Standard fatty acid profile

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Chapter 3.

Shark liver oils as analgesic, anti-inflammatory, antipyretic and anti-ulcer agents

Chapter 3. Shark liver oils as analgesic, anti-inflammatory, antipyretic and anti-ulcer agents

3.1 INTRODUCTION

Studies on the pharmacological properties of liver oils from sharks and chimaeras of Indian EEZ, inhabiting the waters beyond 600 m. depth are scanty. Shark liver oils contain high fractions of health boosting unsaponifiable matter and unsaturated fatty acids that could render beneficial effects. Therapeutic use of shark liver oil is evident from its use for centuries as a remedy to heal wounds and fight flu (Neil *et al.* 2006). Japanese seamen called it *samedawa*, or "cure all". Shark liver oil is being promoted worldwide as a dietary supplement to boost the immune system, fight infections, to treat cancer and to lessen the side effects of conventional cancer treatment. These days more emphasis is laid on the nutritive benefits of shark liver oils especially on the omega 3 polyunsaturated fatty acids (PUFAs) (Anandan *et al.* 2007) and alkylglycerols (AKGs) (Pugliese *et al.* 1998) contained in them due to the high rise of inflammatory disorders such as arthritis, asthma and neurodegenerative diseases like Alzheimer's, Parkinson's and Schizophrenia.

Higher concentrations of AKGs in shark liver oils are now considered to be responsible for their high immune boosting ability (Pugliese et al. 1998). These AKGS are essentially a class of lipids with an ether linkage and a glycerol backbone. In addition, shark liver oils also contain, antioxidant vitamins and squalamine (Brunel *et al.* 2005), a substance which has shown a promising behaviour towards fighting cancers of the breast, lung, brain, and skin (melanoma specifically) by choking off the tumor's blood supply. These lipid bioactives contained in deep sea fishes also render anti-ageing and hypocholesterolemic values. The pharmaceutical values associated with shark liver oils are abundant; they form the active ingredients of many different formulations ranging from vitamin supplements to skin based ointments and creams (Neil *et al.* 2006).

In the present study, the pharmacological behaviour of the liver oils of *Neoharriotta raleighana* (NR), *Centrosymnus crepidater* (CC), *Apristurus indicus* (AI) *and Centrophorus sculpratus* (CS) were evaluated and compared with a standard drug in each case, to understand its efficacy, when administered to laboratory animals. While the analgesic property was determined using the acetic acid-induced

mouse writhing and hot plate reaction time, the anti-inflammatory activity was evaluated using the formalin (0.1 ml 1% v/v) -induced rat paw oedema. The efficacy of fish oils against yeast-induced pyrexia and HCl induced gastric ulcers was also determined. The oils were administered at a dose of 1.0 g/kg bodyweight in the animals after performing acute toxicity studies.

Thus the biomedical applications of liver oils from *Neoharriotta raleighana* (NR), *Centrosymnus crepidater* (CC), *Apristurus indicus* (AI) *and Centrophorus sculpratus* (CS) caught from the Arabian Sea and the Indian Ocean were evaluated. As the information available with regard to these properties is relatively scanty, an attempt has been made to explore their ability as therapeutic agents. The major objectives of this study may be summarized as below.

1. To determine the analgesic activity of the liver oils from the four different deep sea elasmobranch resources using the hot plate reaction and writhing tests in Albino mice.
2. To evaluate the anti-inflammatory activity of the four oils with respect to a standard drug ibuprofen and thereby calculate the percentage of reductions in the edema, upon formalin-induced inflammations in Albino rats.
3. To analyse and compare the antipyretic effects of the fish oils of *Centrophorus scalpratus* and *Neoharriotta raleighana* with a standard drug paracetamol upon yeast-induced pyrexia experiments in Albino rats.
4. To understand the anti-ulcerative property of *Centrophorus scalpratus* liver oil in hydrochloric acid/ethanol – induced gastric ulcerations in Albino rats and compare its effect with normal placebo animals with and without induced ulcers.
5. To understand the components of the fish oil of *Centrophorus scalpratus* responsible for the observed pharmacological behaviour by carrying out Pearson's correlation tests with the level of significance set at $p<0.05$.

3.2 REVIEW OF LITERATURE

Products from nature have played an important role throughout the world in treating and preventing human diseases. The potential of natural products has been recognized since antiquity. They remain vital to modern life in such forms as antibiotics, anti-cancer drugs, cardiac drugs, and insecticides. Currently more than 50% of drugs in clinical use have a natural-product origin, and about half of the world's 25 best-selling pharmaceutical agents are natural-product derived. Of all available anti-cancer drugs developed from 1940 to 2002, 40% were natural products or natural-product-derived with another 8% being considered as natural-product mimics.

The sea is one of the most important frontiers for medical research and development. The marine environment is a rich source of both biological and chemical diversity. This diversity has been the source of unique chemical compounds with the potential for industrial development as pharmaceuticals, cosmetics, nutritional supplements, molecular probes, fine chemicals and agrochemicals. In recent years, a significant number of novel metabolites with potent pharmacological properties have been discovered from the marine organisms.

The marine environment, covering 70% of the earth's surface and 95% of its tropical biosphere represents 34 of the 36 phyla of life and provides a fascinating variety of biodiversity exceeding that of the terrestrial environment. Not surprising is that marine organisms produce an unprecedented molecular diversity by the incorporation of elements like bromine that are not readily available to terrestrial species. Partially responsible for the unique secondary metabolism of marine life are the ecological pressures in the marine ecosystem including significant competition for space, deterrence of predation and a high level of symbiosis between different species (Konig *et al.*, 1994). Due to the biogenetic origin, the secondary metabolites of marine organisms possess a number of structural differences as compared to terrestrial natural products. In addition, marine plants, mostly of the algal class (non vascular), are unrelated to the majority of the terrestrial flora; similarly, the marine invertebrates such as sponges (Porifera), coelenterates (Cnidaria) and molluscs. Mollusca are also not closely related to their terrestrial counterparts (Pelletier, 1986). Over 12,000 compounds from marine invertebrates (phyla: Annelida, Arthropoda,

Brachiopoda, Bryozoa, Chordata, Cnidaria, Echinodermata, Hemichordata, Mollusca, Nematoda, Platyhelminthes, Porifera), algae (phyla: Chlorophycota, Chromophycota, Chromophyta, Cyanophycota, Euglenophycota, Rhodophycota), and microorganisms (bacteria, fungi and protozoa), have been discovered by a relative few marine research groups (Blunt and Munro, 2001). Natural products from the oceans have been evaluated for infectious diseases such as for antiparasitic drugs (Kayser *et al.*, 2002), antituberculosis agents (El Sayed *et al.*, 2000), and antiHIV agents (Gochfeld *et al.*, 2003).

Oceans are unique resources that provide a diverse array of natural products (Marris 2006) primarily from invertebrates such as sponges, tunicates, bryozoans and molluscs as well as from marine bacteria and cyanobacteria. While bioactive compounds of varied origin have been explored from deep sea resources worldwide, the discovery of natural drugs from the fishery resources of the Indian Exclusive Economic Zone (EEZ) is still in its infancy. Over the past few years, the perception of marine nutraceuticals (Shahidi, 2007) to the health care professional and consumer has been popularized to fish oils from the greatest predators of the sea – the sharks.

Sharks are an important resource among the marine fisheries caught in India. While the marine waters up to 50 m depth have been studied extensively, the waters beyond this depth remain unexplored. Statistics (Fowler *et al.* 2005) have shown that over 30,000 tons of pelagic sharks and certain species like squalene sharks (inhabiting waters beyond 600 m) available in India's EEZ have hardly been exploited. While shark fishing gained momentum over the years much of their commercial value has been limited to the sale and supply of shark fins. The nutraceutical values associated with the liver oils of sharks from the Indian EEZ remain unexplored.

Despite the great progress in science, knowledge about the etiology of diseases is still limited, and for many life-threatening illnesses no effective treatments exist. Nature always has been a valuable source of drugs and, despite the unprecedented opportunities afforded by medicinal chemistry, continues to deliver lead compounds. Traditionally, research on natural sources was focused on terrestrial plants and microorganisms. More recently, however, organisms of marine origin are also being investigated.

3.2.1 Competition yields diversity.

The diversity of chemical compounds in the marine environment may be due in part to the extreme competition among organisms for space and resources. It is hypothesized that sessile marine organisms (for example, sponges, octocorals, tunicates and algae), have developed a diverse array of chemical compounds known as "secondary metabolites" or natural products for defense and competition. These compounds provide evolutionary advantages by preventing predation and fouling, or by helping an organism effectively compete for space (Reed *et al.*, 2005).

Both toxic and non-toxic compounds may be produced by marine organisms. Toxic compounds may poison predators or adjacent organisms, allowing continued growth. Non-toxic compounds may reduce predation through a reduction of palatability. Secondary metabolites can also act as chemical messengers (pheromones) between individual organisms (Reed *et al.*, 2005).

3.2.2 The biomedical potential offered by marine organisms

In order to evaluate the biomedical potential of any plant or animal, one must consider both the chemical ecology of the organism and its evolutionary history. It is probable that chemical defense mechanisms evolved with the most primitive microorganisms but have been replaced in many more advanced organisms by physical defenses and/or the ability to run or swim away and hide. Sessile, soft-bodied marine invertebrates that lack obvious physical defenses are therefore prime candidates to possess bioactive metabolites. If it is assumed that secondary metabolites evolved from primary metabolites in a random manner, any newly produced secondary metabolite that offered an evolutionary advantage to the producing organism would contribute to the survival of the new strain. The specific evolutionary pressures that led to chemically rich organisms need not be defined but the longer the period of evolution, the more time the surviving organism has had to perfect its chemical arsenal (Faulkner, 2000).

Sessile marine invertebrates have a very long evolutionary history and have had ample opportunity to perfect their chemical defenses. Chemical defense mechanisms cannot be directly equated with potential biomedical activity, but it is remarkable how well the two correlate in reality. This could be explained by the fact that targets of the chemical defenses, primary metabolites such as enzymes and

receptors are highly conserved compared with secondary metabolites. Among the many phyla found in the oceans, the best sources of pharmacologically active compounds are bacteria (including cyanobacteria), fungi, certain groups of algae, sponges, soft corals and gorgonians, sea hares and nudibranchs, bryozoans, and tunicates. Some marine organisms such as dinoflagellates, echinoderms and some fish are well-known for their ability to produce potent toxins, but these are usually too toxic for medicinal use (Faulkner, 2000).

3.2.3 Etiology and the pharmacological behaviour of the bioactives

3.2.3.a) Analgesic activity

Pain is a sensorial modality which in many cases represents the only symptom for the diagnosis of several diseases. It often has a protective function. Throughout history man has used many different forms of therapy for the relief of pain and medicinal herbs are highlighted due to their popular use. An example is *Papaver somniferum* from which morphine was isolated. It is regarded as the prototype of opiate analgesic drugs. In the relief of pain, opiates are generally considered to act on the central nervous system exercising their effects through three opioid receptors, such drugs are especially important for the treatment of chronic pain (Batista *et al.*, 2009).

Although morphine has reigned for centuries as the king of painkillers, its rule hasn't been totally benign. There are concerns about its addictive properties and side effects, which include respiratory depression, drowsiness, decreased gastrointestinal motility, nausea and several alterations of the endocrine and autonomous nervous systems (Batista *et al.*, 2009). Substances derived from natural products have been utilized since the beginning of time for various purposes including the treatment of pain. Opium, for example, has been used since the earliest records of time, some 7000 years ago. Not until the 19th century individual components of different natural product remedies were identified and purified. Today, drug discovery has become a complex field far beyond the use of only natural products. However, natural products have dominated the drug industry for many years and several marketed drugs are based on isolates from nature. There has been a recent resurgence in the study of natural products, especially from the dietary supplement industry. The pharmaceutical industry has begun to revitalize

programs on the screening of natural products. Academic research has continued to be a strong leader in the field of natural products, especially with respect to newly discovered chemical entities.

It is estimated that the analgesics are one of the highest therapeutic categories on which research efforts are concentrated (Elisabetsky and Castilhos, 1990). Analgesic compounds available in the market, still present a wide range of undesired effects (Katzung, 2001) leaving an open door for new and better compounds. Natural products are believed to be an important source of new chemical substances with potential therapeutic applicability. Several plant species are traditionally used as analgesics (Mills and Bone, 2000). There are reports about analgesic effects of medicinal plants (Cakci *et al.*, 1997, Garrido *et al.*, 2001, Hajhashemi *et al.*, 2002, Khanna and Bhatia, 2003, Mandegary *et al.*, 2004, Monsef *et al.*, 2004 and Vian *et al.*, 2003).

A large number of Indian medicinal plants are attributed with various pharmacological activities. Because it contains diversified class of phytochemicals, it is believed that current analgesia-inducing drugs such as opiates and non-steroidal anti-inflammatory drugs are not useful in all cases, because of their side effects and low potency (Ahmadiani *et al.*, 1998). In case of morphine there are chances of acute morphine poisoning, hypotension, drug dependence, etc. As a result, a search for other alternatives seems to be necessary and beneficial. Medicinal plants are having a wide variety of chemicals from which novel analgesic agents could therefore be discovered (Malairajan *et al.*, 2006).

Research in the area of pain management and drug addiction was originally focused on natural products exclusively. More recently, analogs have been made from natural substances and completely synthetic compounds based on natural pharmacophores have been introduced into the market. The research and medical fields still struggle with side-effect profiles from these analgesic substances that are undesirable.

Apart from rational drug design and completely novel synthetic efforts, natural products are still being investigated for novel chemical structures that may interact with known analgesic targets. The pharmacology of pain has become a complex field

and as more systems approaches are explored, more potential drug targets are being identified.

3.2.3.b) Pain mechanisms and control

Pain can be simply defined as undesirable physical or emotional experience. Pain is the most common reason that individuals seek medical attention. It can be divided into two types, acute pain and chronic pain. Acute pain serves as a warning system to remove oneself from particular pain stimuli.

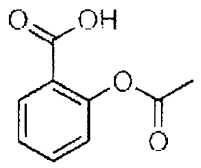
Chronic pain can exist for undefined times and undefined reasons and seems to serve no clear purpose. Treatment of chronic pain is a major problem due to the use of available medications and their undesirable side-effect profiles. The side effects of currently used pain medications vary based on the class of agents used. However, most medical personnel are concerned with addiction, tolerance, gastrointestinal effects, and abuse. Most recent clinical studies (Ballantyne, 2004) suggest that proper use of pain treatment has low risk of producing addicts and because of these prescribing efforts seem to be changing. Regardless, we can separate physical pain into at least four stimuli groups: mechanical, thermal, chemical, or electrical. The stimulation of nociceptive nerve endings of C fibers or activation of A-fibers carry the painful stimuli (Catheline, 1999).

It should be mentioned that endogenous inflammatory pain-producing substances can act in a synergistic way to increase pain levels. Nociceptors (or pain receptors) can be found in the skin but also in other areas. Signals transmitted through these receptors are interpreted as pain in the cognitive centers of the brain. Pain may also arise in the absence of stimuli and is known as phantom pain. The complex relationship of pain and injury indeed makes the perception of pain an important research issue. The brain and spinal cord play a major role in central pain mechanisms as previously mentioned. However, the knowledge of brain mechanisms is still relatively limited. The dorsal horn of the spinal cord is endowed with several neurotransmitters and receptors including: substance *P. somatostatin*, neuropeptide Y, excitatory amino acids, inhibitory amino acids, nitric oxide, endogenous opioids, adenosine, and the monoamines, among others. It is clear that pain transmission to the brain is under diverse physiological control. Undoubtedly,

this makes for a difficult challenge in the discovery of ways to inhibit pain sensations without causing side-effects.

3.2.3.c) Aspirin

Aspirin or acetylsalicylic acid (1, Fig. 1), derived from salicylic acid, extracted from the bark of the Willow tree (Salix alba), is one of the most widely used and available compounds for the management of mild pain. Aspirin served as the first non steroidal anti-inflammatory drug (NSAID) and inhibits the arachidonic acid pathway that eventually leads to the synthesis of eicosanoids, potent mediators of pain (Vane, 1971). The use of aspirin, that specifically inhibits the cyclooxygenase (COX) enzymes, led to the discovery of other synthetic nonsteroidal anti-inflammatory drugs (NSAIDs). In fact, the study of the biochemical cascade of the COX system led to the discovery of the COX-2 enzyme inhibitors once praised as having safer profiles than other NSAIDS (which inhibit the COX-1 enzyme).



Structure of aspirin.

3.2.3.d) Opioids

Opioid is the common name for all compounds that have the same mechanism of action as the constituents of opium. The use and abuse of opium juice from Papaver somniferum, has been known before history was recorded. All opioids interact with the endogenous opioid receptor system that presently includes four receptor subtypes (Dhawan *et al.*, 1996) designated as mu, delta, kappa, and ORL-1 (opioid receptor like receptor). These receptors are widely distributed in the mammalian system and have been found in all vertebrates. There is a relatively high density in the brain and spinal cord but they are also found in the gastrointestinal system and the cells of the immune system. Each subtype seems to play a slightly different role. The research on opioid systems has focused around three groups of modulators.

More recently, some novel chemical structures have appeared in the literature that interact either directly with opioid receptors, or through some other mechanism of controlling opioid receptor signaling. These compounds are of interest from a drug design perspective as most of them do not contain nitrogen. For many years, the extraction and fractionation methods used in natural products research either discarded lipid like molecules or were unable to partition them using acid/base extraction methods as they are neutral compounds.

The first reported (Roth *et al.*, 2002) non-nitrogenous selective kappa opioid receptor ligand, salvinorin A, has recently attracted much research attention. Salvinorin A, the active component of extracts from Salvia divinorium, is one of the most potent hallucinogens known to date (Capasso, 2006). For many years the molecular target for salvinorin A was unknown since researchers believed that it should interact with known targets for hallucinogens, namely the serotonin receptor system. Salvinorin A was initially screened against a battery of receptors that did not include opioid receptors (Siebert, 1994). Roth *et al.* (2002) followed up this screen & that showed salvinorin A to selectively interact with the kappa opioid receptor.

Mitragyna speciosa, have appeared in the literature as opioid receptor ligands (Matsumoto *et al.*, 2004). The herb has been used for many years in Thailand as a replacement for opium. More recently, Takayama (2004) has reviewed the individual components of the active extracts. They have identified at least two compounds that have opioid receptor activity. The first and one of the major alkaloidal components has been called mitragynine. Mitragynine is a corynanthe based compound that acts as a partial agonist at opioid receptors having about 26% the activity of morphine, but 7-hydroxymitragynine is structurally different from morphine. (Takayama and Ueda, 2005). The second, and possibly more interesting compound, 7-hydroxymitragynine, has activity of greater than 1000 times the potency of morphine (Takayama, 2004). It however, is a minor component of the plant but has been demonstrated to be orally active (Matsumoto *et al.*, 2004). These compounds have also been shown to interact with other receptor systems including the descending noradrenergic and serotonergic receptor systems (Matsumoto *et al.*, 2004). These systems are also known to play a role in centrally mediated nociceptive responses.

Many natural products have been found to interact with voltage-gated ion channels. Probably the most well known ligand that blocks sodium channels is cocaine, isolated from Erythroxylon coca. Cocaine is mostly known and studied for its ability to block the dopamine transporter due to its ability to create a euphoric state (Kuhar *et al.*, 1991). However, its utilization as a local anesthetic is known by its interactions through sodium channel blockage.

Tetrodotoxin, isolated from the puffer fish, blocks sodium channels and causes great harm to those that ingest it. It does produce numbness in the lip and tongue within 20 min of ingestion but quickly leads to paralysis and in some cases death. This is a major concern in Japan where the puffer fish liver dish, fugu, is a delicacy. Other species have been reported to contain tetrodotoxin, but they seem to not be desirable for food sources. The use of this as a lead compound for analgesic development has been limited by its toxic nature.

There is a large, unmet medical need in the treatment of pain. The dominant classes of analgesic drugs, the non steroidal anti-inflammatory drugs (NSAIDs) and the opiates, are limited by their efficacy and tolerability. Less than 30% of patients with chronic pain obtain adequate relief with current therapies, and there are many adverse effects, particularly with long-term administration (Kalso,. *et al.* 2004).

3.2.4 Experimental trials of analgesic activity using plant extracts

The methanol extract of *Caesalpinia bonducella* (MCEB) leaves were investigated for analgesic at the doses of 50, 100 and 200 mg/kg, body weight. and combination of above doses of extract with the standard drug morphine 5 mg/kg (Hot plate reaction time in mice) was administered to eight groups of six Swiss albino mice of both sex weighing between (18-22 g)in each paradigm. The hot plate method was used to assess analgesic activity. The extract produced significant ($P < 0.01$) analgesic activity. It potentiated the morphine induced analgesia. The study exhibits that the methanol extracts of leaves of *C. bonducella* possess analgesic activities (Gupta *et al.*, 1989).

In a study conducted by Malairajan *et al.,* (2006) on some of the Indian medicinal plants, *Sida acuta* whole plant (Malvaeae), *Stylosanthes fruticosa* (whole plant) (Papilionaceae), *Toona ciliata* (heart wood) (Meliaceao), *Bougainvilla spectabilis* (leaves) (Nyctaginaceae), *Ficus glomerata* (bark, leaves) (Moraceae) and *Polyalthia longifolia* (leaves) (Annonaceae), as plants were used in folklore medicine in the treatment of toothache and strengthening of gums, anthelmintic, kidney diseases, analgesic, anti-inflammatory, hepatoprotective, antihyperglycemic, antihyperglycemic and anticancer, ethanol extract of the powdered material was used . They were used at three dose level (100, 300 and 500 mg/kg) (Malairajan *et al.*, 2006).

The hot plate and tail immersion methods were employed, which were considered to be selective for opioid like compounds in several animal species (Janssen *et al.*, 1963). *Sida acuta* were reported to contain ecdysterone; ephedrine; hentriacontane; hypolaetin-8-glucoside, *Stylosanthes fruticosa,* sterols and amino acids, *Toona ciliata* bergapten, *Bougainvilla spectabilis* pinitol a antidiabetic compound, *Ficus glomerata* friedelin along with behanate; berganin; lupeol and its acetate, *Polyalthia longifolia* quercetin and bulbocapnin. These chemicals were thought to be responsible for narcotic analgesic, anti-inflammatory and antidiabetic activity. The steroids (β-sitosterol, stigmasterol and campesterol), which are common to these plants, were also thought to be contributing to the observed analgesic activity (Malairajan *et al.*, 2006).Studies have demonstrated that various flavonoids such as rutin, quercetin, luteolin, biflavonoids and triterpenoids produced significant antinociceptive activities (Mendes *et al.*, 2000).

In a successive extraction of the dried rhizomes of *Curcuma zedoaria Rosc* with hexane, chloroform and methanol, and their subsequent screening for the analgesic activity, the chloroform and methanol extracts showed significant activity on Swiss albino mice as compared to hexane fraction (Ali *et al.*, 2004).

In an investigation by Heidari *et al.*,2006 to evaluate the analgesic effect of the methanolic extract of the petals of the plant *Echium amoenum* Fisch & C.A. Mey. , which has been used in Iranian traditional medicine as demulcent and analgesic long ago, it was found that male albino mice injected intraperitoneally with different doses 5, 10, 20 and 30 mg/kg of the extract, when subjected to hot-plate test showed significant analgesic effect. The results showed that the dose of 10 mg/kg of extract

had the highest analgesia in hot-plate test ($P < 0.01$) compared to the control group (Heidari *et al.*, 2006).

In the discussion of the above study it was stated by Heidari *et al.*, (2006) that since hot-plate test is a central antinociceptive test (Paulino *et al.*, 2003), the extract may act via central mechanisms. The peak of analgesic effect of *Echium amoenum* extract was seen in 45 min after injection; it seemed that the concentration of extract reached to maximum in this time or due to some active analgesic metabolite (Heidari *et al.*, 2006). The phytochemical studies on *Echium amoenum* had revealed the presence of many chemicals such as anthocyanidine (13%), flavonoid aglycons (0.15%) and trace amount of alkaloid and yielded 0.05% of a clear lemon-yellow volatile oil with δ-cadinene (24.25%) as major component. Presence of rosmarinic acid as a phenolic compound in another species *Echium vulgare* and its pharmacological activity such as anti-inflammatory (Mills and Bone, 2000) and presence of flavonoids in *Echium amoenum*, have been demonstrated.. In conclusion, Heidari *et al.*,2006 have stated that the *Echium amoenum* extract had a suitable analgesic effect, which paralleled the traditional use of this plant as analgesic and anti-inflammatory medicine.

Koster *et al.* (1959) and Williamson *et al.* (1996) have postulated that acetic acid-induced writhing and hot-plate test methods are useful techniques for the evaluation of peripherally- and centrally-acting analgesic drugs, respectively. In consonance with the above statement it was hypothesized that the peripheral analgesic effect of the plant's extract may be mediated via inhibition of cyclo-oxygenases and/or lipoxygenases (and other inflammatory mediators), while the central analgesic action of the extract may be mediated through inhibition of central pain receptors. The central analgesic response of an extract can be evaluated by the tail flick (spinal analgesia) and hot plate (supra-spinal analgesia) methods.

The effectiveness of the hot-plate test is an indication that the analgesic agent acts primarily in the spinal medulla and/or higher central nervous system levels or by an indirect mechanism (Hunskaar and Hole, 1987; Yaksh and Rudy, 1978). In a study by Asongalem *et al.*, intended to evaluate the analgesic activity of an aqueous extract of *Erigeron floribundus* (H.B. & K) or (syn": *Conyza sumatrensis* (Retz) E.K. Walker) (Asteraceae), thermal stimuli by hotplate tests was used. Phytochemical screening had showed the presence of saponins, flavonoids, glycosides, alkaloids,

oils, phenols and tannins and it was proposed that they significantly increased the reaction time of hotplate, showing the central acting effect of the plant (Asongalem *et al.*, 2004).

3.2.5 Hot plate reaction time in mice.

Thermal painful stimuli are known to be selective to centrally but not peripherally acting analgesic drugs. The method was originally developed by MacDonald (1946). The paws of mice and rats are very sensitive to heat at temperature, which are not damaging the skin. The response is in the form of jumping, withdrawal of the paws or the licking of the paws (Eddy and Leimback, 1953). The animals were placed on Eddy's hot plate kept at a temperature of 55 ± 0.5 °C. A cut off period of 15 s, was observed to avoid damage to the paw. Reaction time and the type of response were noted using a stopwatch.

Mice were screened by placing them on a hot plate maintained at 55 ± 1°C and the reaction time in seconds for hind paw licking or jumping were recorded (Turner ',1965). Only mice which reacted within 15 sec and which did not show large variation when tested on four separated occasions, each 15 min apart, were used in this study. Morphine (5 mg/kg, i.p.) was used as standard. The time for hind paw licking or jumping on the heated plate of analgesiometer was taken as the reaction time.

The hot plate method was originally described by MacDonald *et al.*,(1946) (Woolfe and MacDonald, 1994). This test had been found to be suitable for the evaluation of centrally but not of peripherally acting analgesics. The validity of this test has been shown even in the presence of substantial impairment of motor performance (Plummer *et al.*, 1996). The above findings of the study indicated that the MECB may be centrally acting.

In a study, conducted to evaluate the analgesic activity of the plant *Momordica charantia* Linn. of the family of Cucurbitaceae (commonly called bitter gourd) which is well known in India for its medicinal value and its fruit has been used in the Ayurvedic system of medicine, for the treatment of diabetes mellitus and hepatic disorders and for pain relief in gout and rheumatism. Powdered seeds also showed

analgesic activity which were serially extracted with benzene, methanol and 50% ethanol in water. The extracts were dried using a flash rotary evaporator. Pilot studies with the various extracts revealed that only the methanol extract (yield 9.4 g/100 g of dry seeds) exhibited a significant analgesic response (Biswas *et al.*, 1991). The pain threshold was considered to have been reached when the animals lifted and licked their paws or attempted to jump out of the 2 l beaker placed on the hot plate at 55°C.

3.2.6 Analgesia testing

Analgesia was assessed by the writhing assay in mice (Koster *et al.*, 1959) and by the tail-clip assay in mice and rats. In the first assay, the number of writhings induced by 0.6% acetic acid (10 ml/kg i.p.) during 10 min after acetic acid injection was counted. In the tail-clip assay, a bull-dog clip with thin rubber sleeves was applied at the root of the tail of mouse or rat. The reaction time, i.e., the time taken by the animal to make an effort to dislodge the clip was noted. The extract was administered subcutaneously 30 min prior to the challenge. Any increase in the reaction time or the percentage inhibition of writhing was plotted against the dose. In each instance, the ED_{50} (the dose which produced 50% of maximum response) was estimated by graphical means. The time course of the analgesic response was also studied using the tail clip assay by recording the reaction times 15, 30, 45 and 60 min after subcutaneous injection of the extract. In addition, the possible role played by the opioid system was analyzed by administering naloxone (Endo Labs, 5 mg/kg i.p.) 15 min prior to the extract (8 mg/kg). For comparison, the prototype analgesic (morphine sulphate), opium and alkaloids were used. The dose-response curve was obtained using the writhing assay and the ED_{50} values recorded.

3.2.7 Anti-Inflammation

Inflammation normally occurs in response to a local disruption to the tissues after injury, however it is also seen in conditions such as Alzheimer's disease (Pasinetti, 1996), cancer,(Vane, 2000) and irritable bowel syndrome (Langmead *et al.*, 2002). It is believed that controlling inflammation may help alleviate these conditions or even prevent them. The inflammatory process is considered to be one of the

elements by which the body repairs injury (the other elements are fibroblasts, platelets, and epithelial cells) (Schwentker *et al.*, 2002).

Inflammation or phlogosis is a pathophysiological response of living tissue to injuries that leads to the local accumulation of plasmatic fluid and blood cells. Although it is a defense mechanism, the complex events and mediators involved in the inflammatory reaction can be induced, maintain or aggravate many diseases (Sosa *et al.*, 2002). However, studies have been continuing on inflammatory diseases and the side effects of the currently available anti-inflammatory drugs pose a major problem during their clinical use. Therefore, development of newer and more powerful anti-inflammatory drugs with lesser side effects is necessary.

During the different phases of inflammation, several mediators are released such as histamine, serotonine, chemotactic factors, bradykinins and prostaglandins. The first phase (acute phase) is characterized by local vasodilatation and increased capillary permeability resulting in an exudation of fluid from the blood into the interstitial space, the second one by infiltration of leukocytes into the inflamed tissues and the third one by granuloma formation. This granulomatous tissue consists of giant cells, undifferentiated connective tissue and fluid infiltrate. The granuloma can be experimentally developed by subcutaneous implantation of a pellet of compressed cotton in rats. Reports on granuloma development have been described by Levy *et al.* (2005).

The inflammatory response is a complex process with several characteristic features that include the activation of monocytes, granulocytes, and lymphocytes and the release and activation of inflammatory mediators, complement systems, and humoral mediators (Gannon *et al.*, 1998). The inflammatory process begins with a stimulus that causes the release of prostaglandins from cells. Stimuli such as lipopolysaccharides can induce an enzyme responsible for the production of nitric oxide (inducible nitric oxide synthase, or iNOS) and another enzyme known as cyclooxygenase-2 (COX-2). COX-2 acts by producing prostaglandins, particularly prostaglandin E2 (PGE2). In turn, PGE2 acts inside the cell to produce various types and quantities of cytokines, which are proinflammatory agents that complete the process by bringing active leukocytes to the injury sites (Pass *et al.*, 1998).

The most widely used primary test to screen new anti-inflammatory agents measures the ability of a compound to reduce local edema induced in the rat paw by injection

of an irritant agent (Winter *et al.* 1962). This edema depends on the participation of kinins and polymorphonuclear leukocytes with their pro-inflammatory factors including prostaglandins (Damas *et al.*1986). Many have demonstrated anti-inflammatory activity by different mechanisms such as inhibition of cyclo and lipoxygenase pathways. In formalin induced inflammation the subcutaneous injection of 0.25 to 5.0% formalin in the plantar surface of one hind paw of the rat produced a concentration-dependent increase in plasma extravasation. (John, *et al.*, 1998).

Taylor *et al.*, (1998) reported that injection of dilute formalin into the hind paw not only evokes inflammation and pain-related behavior, but it also increases ACTH and corticosterone to a greater extent than restraint and saline injection alone. This difference was particularly robust during the final periods of pain-related behavior in the formalin test, when the ACTH and corticosterone (B) levels in the restraint/saline control group had returned to normal. These results indicate that formalin-evoked increases in ACTH and B reflect nociceptive input, rather than the stress associated with handling,

Ahmadiani *et al*, (1998) had reported that in the formalin test, nociception occurs in two phases (Abbott *et al.*,1995). The first phase starts immediately after formalin injection and continues for 5 min, after which, nociception appears to diminish. The second phase is marked by a return to high levels of nociception beginning 15–20 min after formalin injection and continuing for ~60 min. The first phase is probably a direct result of stimulation of nociceptors in the paw, while the second phase may reflect the inflammation process, and at least to some degree, the sensitization of central nociceptive neurons (Coderre and Melzack, 1992).

It was also reported by Ahmadiani *et al*, (1998) that the i.p. administration of methanol rhizome extract of *Sambucus ebulus* from the family Caprifoliaceae using formalin induced paw edema method, produced an anti-inflammatory activity during inflammatory tests, it may be neurogenic inflammation, where some peripheral end of capsaicin sensitive sensory neurons releases substance P and other inflammatory peptide mediators. The peripheral end of these neurons also contains inhibitory opioid receptors. Opiates may be considered to be inhibitors of substance P containing neurons and inhibitors of developing inflammation.

According to Alcaraz and Jimenez (1988) some flavonoids can affect the inflammatory response in different models of experimental inflammation, associated

with a high margin of safety and weak analgesic effects and several studies have suggested that the anti-inflammatory effect of flavonoids is probably related to anti-histamine, anti-bradykinin, or anti-serotonin properties or to inhibition of enzymes such as 12-lipoxygenase and 5-lipoxygenase (Kimura *et al.*, 2003). The mechanism of action of sesquiterpene lactones has also been found to inhibit both cyclooxygenase (COX-2) and pro-inflammatory cytokines in macrophages (Hwang *et al.*, 1996).

There are also reports that the flavonoids and terpenoids have anti-inflammatory effects (Ahmadiani *et al.*, 2000), and a number of previous studies have suggested that flavonoids may interact directly with the prostaglandin system (Panthong, *et al.*, 1989). Also various flavonoids have been shown both to inhibit and to substitute as a cofactor for prostaglandin generation, to inhibit arachidonate lipoxygenation and to inhibit enzymes involved with inactivation or biotransformation of prostaglandins (Alcaraz and Hoult, 1985). Studies had indicated that certain flavonoids have anti-oxidant and vitamin-C sparing effect. In addition, some flavonoids have a catecholamine-sparing activity, due to inhibition of catechol-*O*-methyltransferase. The anti-oxidant and catecholamine-sparing activities were considered to be important determinants of the recognized anti-inflammatory activity of flavonoids (Middleton and Drzewiecki, 1987).

The anti-inflammatory role of fish oils has been documented in chapters 4 and 5 of this book.

3.2.8 Antipyretics

Although a significant progress for the understanding of mechanisms of thermoregulation has been achieved in past 30 years (Conn *et al.*, 1991), the number of safe and effective antipyretics available in the clinics remained practically unaltered during this period.

Fever is a manifestation often seen in case of infection by bacteria, viruses and spirochaetes, as well as in case of tumors and traumata. As a possible mechanism of fever, it is suggested that endotoxins released from invading microorganisms may possibly stimulate monocytes and macrophages to inducibly produce febrile

Cytokines (interleukin-1, interleukin -6, interferon, tumor necrosis factor, macrophage inflammatory protein-1,etc) (Wyler *et al.*,1984).These endogenous pyrogens could be transported by blood to the organum vasculosum laminae terminalis to promote prostaglandin release, which subsequently could react with the preoptic/ anterior hypothalamus to change the activity of temperature-sensitive neurons in the hypothalamus.

Blood-borne cytokines, such as interleukin-1b (Sauder *et al.*,1984), interleukin-6 and tumour necrosis factor-a (Roth *et al.*, 2002), convey pyrogenic signals from peripheral sites of inflammation to the brain, ultimately increasing prostaglandin synthesis within the hypothalamus and causing fever (Tull and Schilling, 2009). Interruption of this host-generated inflammatory response to exogenous pyrogens attenuates the development of fever. For example, fever is attenuated by neutralizing circulation of cytokines (Roth *et al.*, 2002) and by inhibiting prostaglandin synthesis.

Although the benefits of inhibiting fever have been questioned, antipyretic therapy still is widely employed by healthcare professionals in their treatment of febrile patients (Plaisance and Mackowiak, 2000). The antipyretics typically employed to alleviate fever include the non steroidal anti-inflammatory drugs and paracetamol, which work by inhibiting the synthesis of prostaglandins, the final chemical mediators of fever (Plaisance and Mackowiak, 2000).

Many investigators have already found that non steroidal anti inflammatory drugs, such as aspirin and indomethacin, produce antipyretic actions by inhibiting prostaglandin synthesis, in the hypothalamus, in addition to their anti-inflammatory and analgesic actions (Vane, 1971)

Fever may be a result of infection or one of the sequelae of tissue damage, inflammation, graft rejection, or other disease states. Antipyretics are drugs which reduce elevated body temperature. Regulation of body temperature requires a delicate balance between the production and loss of heat, and the hypothalamus regulates the set point at which body temperature is maintained (Parimaladevi *et al.*, 2004).

Fever may be a result of infection or one of the sequelae of tissue damage inflammation, graft rejection, or other disease states. Antipyretics are drugs, which reduce the elevated body temperature. Regulation of body temperature requires a delicate balance between the production and lose of heat. Hypothalamus regulates the set point at which body temperature is maintained. In fever this set point is elevated and drugs like paracetamol do not influence body temperature when it is elevated by factors such as exercise or increases in ambient temperature.

3.2.9 Mechanism of action

Physicians since antiquity have used various physical means to lower body temperature (Plaisance and Mackowiak, 2000). Applying Peruvian cinchona bark as an antipyretic dates to the early 1600s, but by the 18th century over harvesting of cinchona created scarcity (Aronson, 1994) and a search for substitutes. In 1763, Reverend Stone reported to the Royal Society of London on the antipyretic effects of "fever bark" from English willow (Aronson, 1994). Although his finding appeared novel, it simply confirmed what was known to Hippocrates, Galen, and ancient Egyptians centuries before. Salicylic acid was first prepared in 1838 from the glucoside salicin, the active component in willow bark. Another derivative, acetylsalicylic acid (aspirin) was later synthesized in 1853 and made commercially available as an antipyretic in 1899 (Plaisance and Mackowiak, 2000). Since then, numerous antipyretics have been introduced into clinical medicine.

The prescription of acetaminophen for fever is more recent. Although precursors such as acetanilid and phenacetine were developed in the second half of the 19th century, the popular use of acetaminophen as an antipyretic and analgesic did not occur until the 1950s (Spooner and Harvey , 1976). The antipyretics in common use today include acetaminophen, aspirin, and other non steroidal anti-inflammatory drugs (NSAIDs). The principal action of antipyretics rests in their ability to inhibit the enzyme cyclooxygenase (COX) and interrupt the synthesis of inflammatory prostaglandins (Weissmann, 1997). Recent studies on the mechanism of antipyretic action of these drugs, however, reveal effects independent of COX inhibition as well.

3.2.10 Normal thermoregulation

At the heart of thermoregulation is an integrated network of neural connections involving the hypothalamus, limbic system, lower brainstem, the reticular formation, spinal cord, and the sympathetic ganglia (Boulant, 1997). An area in and near the rostral hypothalamus is also important in orchestrating thermoregulation. This region, the "preoptic area," includes the preoptic nuclei of the anterior hypothalamus (POAH) and the septum.

In simple terms, the POAH maintains mean body temperature around a set point. This thermoneutral set point temperature is modulated by the balanced activities of temperature-sensitive neurons. These neurons integrate afferent messages regarding core body and peripheral (skin) temperatures and evoke various behavioral and physiologic responses controlling heat production or dissipation (Boulant, 1997).

Fever describes a regulated rise in body temperature after an increase in the hypothalamic set point (Saper and Breder, 1994). Under the influence of the hypothalamus, physiologic and behavioral functions favoring heat production and heat retention are stimulated until arriving at a newly elevated set point temperature (Saper and Breder, 1994). Typical early behavioral changes prior to fever include seeking a warmer environment or adding clothing. Physiologic alterations include cutaneous vasoconstriction, shivering, and non shivering thermogenesis through enhanced release of thyroid hormones, glucocorticoids, and catecholamines (Boulant, 1997). Upon reaching the elevated set point of fever, an increase or decrease in core temperature will stimulate thermoregulatory mechanisms similar to those evoked at normal body temperature (Cooper, 1994). In other words, normal thermoregulation modulates at this higher set point.

3.2.11 The pathogenesis of fever

The critical "endogenous pyrogens" involved in producing a highly regulated inflammatory response to tissue injury and infections are polypeptide cytokines. Pyrogenic cytokines, such as interleukin-1b (IL-1b), tumor necrosis factor (TNF), and interleukin-6 (IL-6), are those that act directly on the hypothalamus to affect a fever response (Luheshi, 1998). Exogenous pyrogens, such as microbial surface

components, evoke pyrexia most commonly through the stimulation of pyrogenic cytokines. The gram-negative bacterial outer membrane lipopolysaccharide (endotoxin), however, is capable of functioning at the level of the hypothalamus, in much the same way as IL-1b.

These signals trigger the release of other mediators, most notably prostaglandin E2 (PGE2), in the region of the POAH (Saper and Breder, 1994). PGE2 is believed to be the proximal mediator of the febrile response. Preoptic neurons bearing E-prostanoid receptors alter their intrinsic firing rate in response to PGE2, evoking an elevation in the thermoregulatory set point. There are four known cellular receptors for PGE2: EP1 through EP4. The particular receptor subtype involved in pyrogenesis is unknown.

Fever is tightly regulated by the immune response. Inflammatory stimuli triggering the generation of propyretic messages provoke the release of endogenous antipyretic substances. Substances such as arginine vasopressin (AVP), a-melanocyte stimulating hormone, and glucocorticoids act both centrally and peripherally to limit pyrexia. The cytokine interleukin-10 (IL-10) has numerous anti-inflammatory properties, including fever suppression. In addition, a class of lipid compounds known as epoxyeicosanoids, generated by certain cytochrome P-450 enzymes play an important role in limiting the fever and inflammation.

3.2.12 The Role Of Prostaglandin E2

PGE2 is synthesized from arachidonic acid, which is released from cell membrane lipid by phospholipase. Arachidonic acid is metabolized by two isoforms of the COX enzyme, COX-1 and COX-2. COX-1 usually is expressed constitutively and generates prostanoids important to housekeeping functions supporting homeostasis. COX-2, on the other hand, is inducible by inflammatory signals such as the pyrogenic cytokines, IL-1b, TNF, and IL-6, and bacterial lipopolysaccharide. Genetically engineered mice that lack either the COX-1 or COX-2 gene demonstrate that the inducible isoform is responsible for hypothalamic PGE2 production during a febrile response. As COX-2 is the key provider of PGE2 during pyrexia, it is not

surprising that the selective COX-2 antagonist, rofecoxib, is an effective antipyretic in humans.

3.2.13 The Cyclooxygenase Hypothesis

The antipyretic drug aspirin was in wide clinical use for more than 70 years before. Vane demonstrated in 1971 that it exerted its physiologic action by inhibiting the production of prostaglandins. Further work suggests a current model of how aspirin and similar NSAIDs act as antipyretics.

Aspirin interferes with the biosynthesis of cyclic prostanoids derived from arachidonic acid, such as thromboxane A2 and prostaglandins. As a nonselective COX inhibitor, aspirin has been widely studied for its anti-inflammatory, antipyretic, and antithrombotic traits. The major mechanism of action of aspirin and other antipyretics involves lowering PGE2 by directly inhibiting COX enzyme activity. Worth noting, however, is that sodium salicylate, aspirin's major metabolite, exhibits similar antipyretic and anti-inflammatory properties as aspirin but shows only weak inhibition of COX-1 and COX-2 in vitro. NSAIDs are also capable of reducing PGE2 production by down-regulating the expression of COX enzymes, as opposed to directly inhibiting their enzymatic action. The antipyretic effects of acetaminophen, aspirin, and other NSAIDs are complex and repress inflammatory signals at many levels. Although COX enzyme inhibition plays a central role in the antipyretic actions of these drugs, other immunomodulatory actions appear to contribute (Aronoff and Neilson, 2001).

3.2.14 Experimental trials of antipyretic activity using plant extracts

Subcutaneous injection of yeast induces pyrexia by increasing the synthesis of prostaglandins and is a useful model for screening antipyretic effect of substances (Al-Ghamdi, 2001). The potent activity of a plant extract against yeast induced pyrexia therefore, shows that the extract has some antipyretic effect at that dosage (Adzu *et al.*, 2002).

The root extract of *Clitoria ternatea* L. (Family: Fabaceae), a well-known perennial, twining herb, found abundantly in Indochina, the Phillipines and Madagascar causes a significant antipyretic effect in yeast-provoked elevation of body temperature and

the effect extended up to 5 hours after the drug administration. The anti-pyretic effect of the extract was comparable to that of paracetamol (150 mg/kg body wt., p.o.), a standard anti-pyretic agent (Parimaladevi *et al.*, 2004).

The powdered roots of *Clitoria ternatea* L were extracted using methanol in a Soxhlet extraction apparatus.As methanol is the solvent which brings out most of the components present in any material, the present investigation was carried out using a methanol extract of whole plant (Parimaladevi *et al.*, 2003). The solvent was removed *in* vacuo to provide a dry extract (9.8%w/w, as compared to the powdered material). The chemical constituents of the methanol extract were identified by qualitative analysis and confirmed by thin layer chromatography for the presence of flavonoids, tannins, steroids and saponins (Parimaladevi, 2004).

Cleome viscosa Linn. (Capparidaceae) is a widely distributed sticky herb with yellow flowers having strong penetrating odour, used in Indian traditional medicine, found throughout the greater part of India, often in waste places (Gupta and Dixit, 2009). In Ayurvedic system of medicine, this plant is used in fever, inflammations, liver diseases, bronchitis, and diarrhea and infantile convulsions. The seeds are used as anthelmintic and the leaves are useful in healing the wounds and ulcers externally. The juice of the plant was diluted with water and given internally in small quantities to fight against fever (Anonymous, 1966).

The antipyretic activity of a methanol extract of *Cleome viscosa* Linn. (CVME) was investigated for its, potential on normal body temperature and yeast-induced pyrexia in albino rats. The CVME, at doses of 200, 300, and 400 mg/kg BW, showed significant reduction in normal body temperature and yeast-provoked elevated temperature in a dose-dependent manner. The effect also extended upto 5 h after the drug administration. The anti-pyretic effect of CVME was comparable to that of paracetamol (150 mg/kg,), a standard anti-pyretic agent (Parimaladevi *et al.*, 2004).

3.2.15 Fish oils and antipyretic effects

The antipyretic effects of fish oils have been studied by several workers on humans as well as animal models (Petursdottir and Hardardottir, 2009; Schwerbrock *et al.*,2009). The effect of dietary fish oil supplementation on fever and cytokine

production in human volunteers had been studied by Cooper *et al.*,1993. Half of the subjects were supplemented their normal diet with 4.5 g/day of fish oil for 6-8 weeks. Injection of typhoid vaccine in unsupplemented subjects caused an increase in white cell count, resting heart rate, metabolic rate, oxygen consumption, and oral temperature. Fish oil supplementation inhibited the tachycardia and attenuated the maximal increases in oral temperature and metabolic rate following typhoid vaccine. However, interpretation of these latter results were complicated by similarly attenuated responses in saline-injected subjects. The in vitro production of interleukin-1 and interleukin-6 from whole blood was suppressed by fish oil supplementation, but production of tumor necrosis factor alpha was not significantly altered. Fish oil supplementation may therefore provide a non-pharmacological approach of attenuating several of the responses associated with injury and infection and this may be related to reduced cytokine (IL-1 and IL-6) production (Cooper *et al.*,1993).

The effects of dietary supplementation with fish oil on in vivo production of inflammatory mediators in clinically normal dogs were investigated by LeBlanc *et al.*,2008. Serum activity of IL-1, IL-6, and PGE2 significantly increased after LPS (an endotoxin) injection in all groups but to a lesser extent in dogs receiving the fish oil diet, compared with results for dogs receiving the sunflower oil diet. Serum activity of TNF-alpha and PAF concentration also increased significantly after LPS injection in all groups but did not differ significantly among groups. The waorkers concluded that a fish oil-enriched diet consisting of 1.75 g of EPA/kg of diet and 2.2 g of DHA/kg of diet (dry-matter basis) with an n-6:n-3 fatty acid ratio of 3.4:1 was associated with significant reductions in serum PGE2 concentrations and IL-1 and IL-6 activities. Results also supported the use of EPA- and DHA-enriched diets as part of antiinflammatory treatments for dogs with chronic inflammatory diseases. Micheali *et al.*, (2007) observed that fish oil supplementation during fever significantly blunted fever and cortisol plasma levels (with no effect on cytokine release). Fish oils blunted the peak norepinephrine after fever induction and even helped reduce body temperatures.

3.2.16 Antiulcer property

Gastric ulcer therapy faces a major drawback in modern days due to the unpredictable side effects of the long term uses of commercially available drugs. As it affects 5% of the global population (Datta *et al.*, 2002), the treatment of this painful disease and its prevention has become one of the challenging problems today. Hence the search is still on to find out a drug possessing anti ulcer properties which will serve as a powerful therapeutic agent to cure gastric ulceration, and the search has been extended to the systematic development of natural products, from ancient times.

It is now generally agreed that almost all gastroduodenal ulcer disease results from an abnormality in the mucosal barrier. In recent years, attention has focused on *Helicobacter pylori* and NSAIDs as important causes of this failure of barrier function. Uncommonly, ulcer disease of the upper GI tract is attributable to excessive secretion of hydrochloric acid rather than to a primary failure of the barrier itself (Filaretova, 2005).

An early hypothesis on the inherent resistance of the stomach to autodigestion was that of John Hunter in 1772. On observing the rapidity of post-mortem gastric autolysis, he ascribed the ability of the stomach not to digest itself during life to the presence of a "living principle." This "living principle" depended, in Hunter's view, on the continuing circulation of blood through the gastric tissue. In 1853, Virchow refined this hypothesis, proposing that the acid in the gastric juice diffused back into the mucosa, where it was neutralized by circulating alkaline blood. Gastric ulcers were considered to be secondary to a restriction in local blood supply, with resultant ineffective neutralization of absorbed acid, leading to localized areas of autodigestion.

Role of prostaglandin is also emphasized in ulcer formation. Prostaglandin induces maintenance of the stomach's non wettable surface properties protecting the underlying epithelium from aqueous acidic/proteolytic damaging agents in the lumen (Giraud *et al.*, 1997). This hydrophobic acid-resistant property of the gastric surface active phospholipid layer (SAPL) is rapidly attenuated by NSAIDs. As in humans, *Helicobacter* infection in mice is associated with a significant reduction in both gastric surface hydrophobicity and the phospholipid concentration of the oxyntic mucosa (Lichtenberger *et al.*, 2007).

The importance of cytoprotection by prostaglandin-E2 now receives considerable attention because of increasing concerns regarding NSAID-induced ulcers. However, these prostaglandins reduce gastric mucosal blood flow, which theoretically should be deleterious rather than helpful. It has been agreed that enhanced mucus production, with thickening of the mucus layer, is the key to prostaglandin-induced cytoprotection (Filaretova, 2005).

Peptic ulcers are defects in the gastrointestinal mucosa which extend through the muscularis mucosae. They persist as a function of the acid or peptic activity in gastric juice. Peptic ulcer disease is an important cause of morbidity with expenditures in the United States attributed to recent ulcers estimated at $5.65 billion per year (Sonnenberg and Everhart, 1997).

Global expansion of consumption of alcohol and non-steroidal anti-inflammatory drugs (NSAID) and inappropriate diets have contributed to growing ulcer etiopathology (Peskar and Maricic, 1998). In this way, the peptic ulcer is considered a disease of modern times, related to the addictions that are increasingly frequent in the society and to its stressful lifestyle. Treatment with natural products presents promise of a cure. Plants have been raw material for the synthesis of many drugs and they remain an important source of new therapeutic agents. Borrelli and Izzo (2000) presented a review that demonstrated the enormous variety of chemical substances isolated from plants that present antiulcerogenic activity, indicating their great potential in the discovery of new therapies for ulcers.

Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and piroxicam remain among the most commonly used pharmacological agents (Garner *et al.*, 2005). However, these classes of substances may cause gastrointestinal ulceration, due to the ability of these agents to suppress prostaglandin synthesis. Piroxicam is a preferential COX-1 inhibitor. Cyclooxygenase was constitutively expressed in the gastrointestinal tract in large amounts and has been reported to maintain mucosal integrity through continuous generation of prostaglandins (Halter *et al.*, 2001).

3.2.17 HCl/ethanol-induced ulcer

To produce gastric ulcer in animals, necrotizing agents of gastric mucosa such as HCl and absolute ethanol are used. Compounds containing sulfhydryl (SH)

groups such as glutathione and agents that modify SH groups protect the gastric mucosa against aggressive factors. The protective role of these endogenous SH groups have been demonstrated in ethanol-induced gastric injury (Avila *et al.*, 2009).

It is known that ethanol is among many factors that increases risk of gastric ulcer formation due to stress, use of steroids and non-steroidal anti-inflammatory drugs (NSAIDs). Ethanol is widely used to induce experimental gastric ulcer in animals (Yam *et al.*, 2004). Oxygen derived free radicals, primarily super oxide anions, hydroxyl radicals and lipid peroxides play an important role in the pathogenesis of acute experimental gastric lesions.

The ability of the gastric mucosa to resist injury produced by endogenous secretions (acid, pepsin and bile) and by ingested irritants (e.g., alcohol), can be attributed to a number of factors that have been collectively identified as mucosal defense (Wallace, 2001). The formation of gastric mucosal lesions by necrotizing agents such as HCl and EtOH has been reported to involve the depression of these gastric defensive mechanisms (Kinoshita *et al.*, 1995). HCl/EtOH-induced gastric ulcers also promote stasis in gastric blood flow that contributes to the development of the hemorrhagic and necrotic aspects of tissue injury.

In the HCl/EtOH-induced gastric ulcers, the lesions were characterized by multiple-hemorrhage red bands of different sizes along the long axis of the glandular stomach. Ulcerations were found deep within the muscular layer. EtOH-induced ulcers are inhibited by agents that enhance mucosal defensive factors such as prostaglandins (Morimoto *et al.*, 1991).

Absolute ethanol is the main factor that leads to intense damage of the gastric mucosa and it induces multiple hemorrhagic red bands (patches) of different sizes along the long axis of the glandular stomach (Mincis *et al.*, 1995). The pathogenesis of ethanol-induced gastric mucosal damage is still unknown, but the solubility of mucus constituents, a concomitant fall in the transmucosal potential difference, increases the flows of Na^+ and K^+ into the lumen, pepsin secretion, and the histamine content in the lumen, but depresses tissue levels of DNA, RNA and proteins leading to flow stasis in damaged areas and formation of oxygen-derived

free radicals, which are considered the main reasons for mucosa injury (Watanabe *et al.*,1990; Satoh *et al.*,1984).

3.2.18 Anatomy and function of the Stomach

The stomach produces a very strong acid which helps to digest and break down food before it enters the small intestine (duodenum). The lining of the stomach is covered by a thick protective mucous layer, which prevents the acid from injuring the wall of the stomach.

3.2.19 Causes of Ulcer

An ulcer is an open wound in the lining of the stomach or intestine, much like mouth or skin ulcers. Peptic ulcers are eventually caused by acid and pepsin, a digestive stomach enzyme. In the end, it is the production of large amounts of acid in the stomach, passing under the term 'hyperchlorhydria' that causes the injury to the stomach or bowel lining. In a few cases, cancerous tumors in the stomach or pancreas can cause ulcers. Peptic ulcers are not caused by stress or eating spicy food, but these can make ulcers worse. However, a revolutionary and startling recent discovery is that most peptic ulcers result from a stomach infection caused by the bacteria, *Helicobacter pylori*. Use of non-steroidal anti-inflammatory drugs (NSAIDs) will also cause ulcers.

3.2.20 *Helicobacter pylori* (*H. pylori*)

Barry Marshall and Robin Warren's in 1982 (D'Elios 2007) discovered that bacteria are the primary cause of stomach and duodenal ulcers excluding those caused by aspirin or arthritis drugs. This bacterium has a twisted spiral shape and infects the mucous layer lining of the stomach. This infection produces an inflammation in the stomach wall called gastritis. The body even develops a protein antibody in the blood against it. The bacterium is probably acquired from contaminated food or from a drinking glass. It is only after *H. pylori* bacteria injure the protective mucous layer of the stomach, allowing damage by stomach acid, that an ulcer develops. It takes

advantage of the stomach's own mucus for protection. Any acid that does reach the bacteria is converted by *H. pylori*'s urease enzyme in the following reaction

urea + stomach acid + water --> bicarbonate + ammonia

$C{=}O{\cdot}2NH_2 + H^+ + 2H_2O \rightarrow HCO_3^- + 2NH_4^+$

The products of this reaction, bicarbonate and ammonia, are strong bases that further protect the bacteria because of their acid-neutralizing capability. The body's immune system responds to the presence of *H. pylori* and sends infection-fighting cells to the area. However, the neutrophils cannot reach the *Helicobacter pylori* infection because they cannot easily get through the stomach lining. Inflammation in the stomach tissue occurs as the neutrophils die and release superoxide radicals on the stomach wall, damaging tissue. The immune system sends in more nutrients to help the neutrophils, and the *H. pylori* can feed on these nutrients. It may not be the *H. pylori* itself that causes a stomach ulcer, but inflammation in the stomach lining as part of the immune response. Age is also a factor in *H. pylori* infection. In Western countries, children are unlikely to be infected. *H. pylori* infections occur in about 20% of persons below the age of 40 years, and 50% of persons above the age of 60 years.

3.2.21 Non Steroidal Anti-inflammatory Drugs (NSAIDs) and other ulcer inducing agents

NSAIDs include ibuprofen, fenoprofen, aspirin, diclofenac, sulindac, diflusinal, naproxen, tolmetin and many others. They can damage the mucous layer of the stomach, after which the gastric acid causes the final injury.

There are two components to NSAID-induced ulceration. First, there is a local acid effect of the dissolved drug. Most NSAIDs are weakly acidic, lipid-soluble compounds. Since the cell membranes on the stomach wall contain lipids for protection against strong acids, they offer little resistance to the lipid-soluble NSAID. The NSAID acts against the cell membrane, increasing its permeability. This results in cell swelling and death. The local acid effect of NSAIDs has been reduced by

enteric-coating the drug, delaying dissolution until later in the digestive process. However, not all NSAIDs are enteric-coated as it increases cost. In addition, enteric-coating does little more than improving the symptoms of upset stomach. Patients must be informed that enteric-coated NSAIDs are still just as likely to cause stomach ulcers as regular NSAIDs. The second and much more significant component to NSAID-induced ulceration is the systemic effect after being absorbed into the bloodstream. NSAIDs inhibit COX-1, reducing prostaglandin production. Normal COX-1 present in stomach tissue produces prostaglandins which:

- increase mucous and bicarbonate production,
- inhibit stomach acid secretion,
- increase blood flow within the stomach wall.

By acting on COX-1, NSAIDs restrict these self-protection mechanisms, allowing stomach ulcers to develop. It is primarily through this mechanism, not a local acid effect, that NSAIDs cause stomach ulcers.

So, *H. pylori* and certain drugs are the two major factors that cause ulcers. In rare cases, a patient will produce very large amounts of acid and develop ulcers. This condition is called Zollinger -Ellison syndrome. Finally, some people get ulcers for unknown reasons. Besides NSAIDS, certain chemicals can also cause gastrointestinal damage. In a study conducted by Petrovic *et al.* (2003) ulcer was induced with indomethacin. Tan *et al.* (2002) induced ulcer by the administration of HCl-ethanol. Reserpine, an alkaloid can also be used for the induction of ulcer. Kobayashi *et al.* (2010) induced ulcer in rat models by using acetic acid.

3.2.22 Symptoms of gastric ulcer

Ulcers cause gnawing, burning pain in the upper abdomen. These symptoms frequently occur several hours following a meal, after the food leaves the stomach but while acid production is still high. The burning sensation can occur during the night and be so extreme as to wake the patient. Instead of pain, some patients experience intense hunger or bloating. Antacids and milk usually give temporary relief. Other patients have no pain but have black stools, indicating that the ulcer is bleeding. Bleeding is a very serious complication of ulcers.

3.2.23 Diagnosis

A diagnosis of peptic ulcers can be suspected from the patient's medical history. However, the diagnosis should always be confirmed either by an upper intestinal endoscopy, which allows direct examination of the ulcer or by a barium x-ray of the stomach. Rarely an ulcer can be malignant. With endoscopy, a biopsy specimen can be obtained to determine if this is so.

3.2.24 Treatment and medication

Therapy of peptic ulcer disease has undergone profound changes. There are now available very effective medications to supress and almost eliminate the outpouring of stomach acid. These acid-suppresssing drugs have been dramatically effective in relieving symptoms and allowing ulcers to heal. If an ulcer has been caused by aspirin or an arthritis drug, then no subsequent treatment is usually needed. Avoiding these latter drugs, should prevent ulcer recurrence.

The second major change in peptic ulcer disease treatment has been the discovery of the *H. pylori* infection. When this infection is treated with antibiotics, the infection, and the ulcer, do not come back. Increasingly, physicians are not just suppressing the ulcer with acid-reducing drugs, but they are also curing the underlying ulcer problem by getting rid of the bacterial infection. If this infection is not treated, the ulcers invariably recur.

There are a number of antibiotic programs available to treat *H. pylori* and cure ulcers. Antacid, H_2 receptor antagonists (ranitidine, cimetidine, nizatidine, famotidine), gastric acid pump inhibitor (omeprazole), barrier agent (sucralfate) etc. were developed to heal ulcers, but they failed to prevent the occurrence of NSAID-induced ulceration (Agrawal, 1995; Blower, 1996). These treatments are often effective in alleviating ulceration symptoms. They are also used in combination with antibiotic treatment for *H. pylori*-induced ulcers. However, the treatments listed earlier do not prevent NSAID-induced ulcers. Only misoprostol, a synthetic prostaglandin, has been shown to prevent NSAID-induced ulcers.

Some plant extracts were also found effective in the treatment of ulcer. Tan *et al.* (2002) proved the gastric cytoprotective anti-ulcer effects of the leaf methanol extract of *Ocimum suave* (Lamiaceae) in rats. Xing *et al.* (2002) discovered that sea buckthorn (*Hippophae rhamnoides*) seed and pulp oils have both preventive and

curative effects against experimental gastric ulcers in rats. Sea buckthorn (*Hippophae rhamnoides*) is a Euro–Asian wild, newly cultivated, edible berry with exceptionally high contents of nutrients and phytochemicals such as lipids, water and fat soluble vitamins, and flavonoids. Petrovic *et al.* (2003) have reported the gastroprotective effects of *Tanacetum larvatum*.

3.2.25 Ibuprofen

Ibuprofen comes under a class of drugs called Non steroidal Antiinflammatory Drugs (NSAIDs) (Tyagi *et al.*, 2005). Ibuprofen reported to have analgesic properties (Polat and Karaman, 2005), works by reducing hormones that cause inflammation and pain in the body. Ibuprofen inhibits cyclooxygenase (COX); thus inhibits prostaglandin synthesis. Ibuprofen appears to have gastrointestinal adverse drug reactions of all NSAIDs.

3.2.26 Structure

Ibuprofen is chemically 2-(p-isobutylphenyl) propionic acid (Fig. 5). It has an empirical formula of $C_{13}H_{18}O_2$. Ibuprofen like other 2-arylpropionate derivatives (including ketoprofen, flurbiprofen, naproxen etc.) contains a chiral carbon in the β-position of the propionate moiety. As such there are two possible enantiomers of ibuprofen with the potential for different biological effects and metabolism for each enantiomer.

Indeed, it was found that S-ibuprofen (sinisteribuprofen) was the active form both invitro and invivo. Logically then, there was the potential for improving the selectivity and potency of ibuprofen formulations by marketing ibuprofen as a single-enantiomer product (as occurs with naproxen, another NSAID). Further, invivo testing, however, revealed the existence of an isomerase, which converted R-ibuprofen to the active S-enantiomer. Thus, due to the expense and futility that might be involved in marketing the single enantiomer, all ibuprofen formulations currently marketed are a racemic mixture of both enantiomers.

3.2.27 Properties

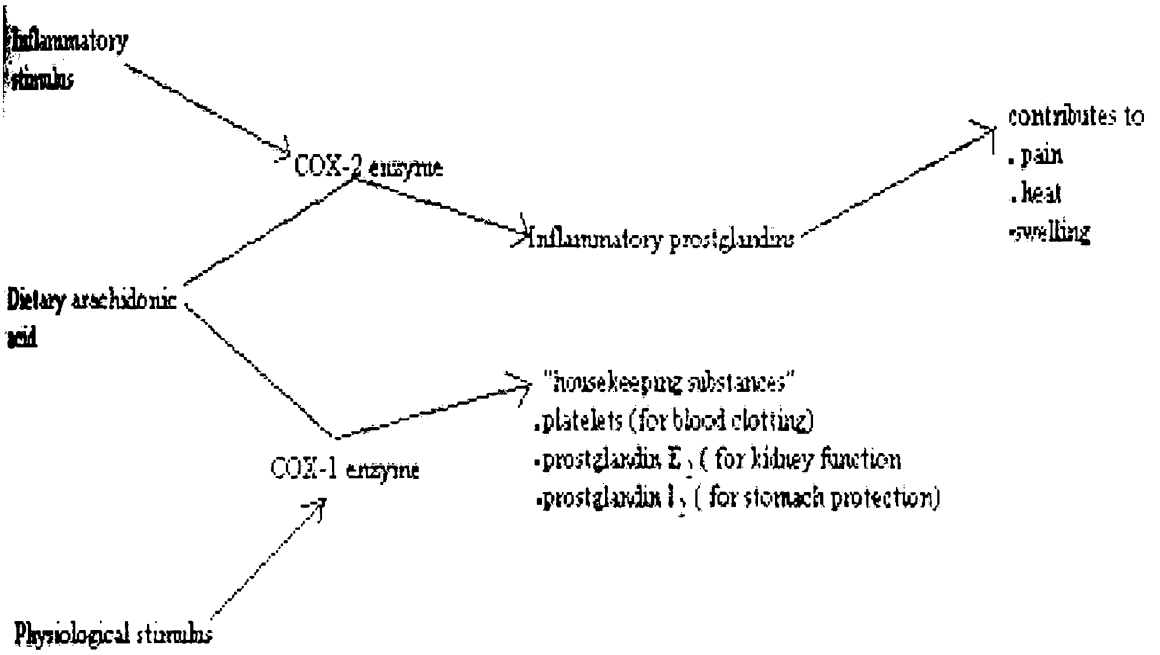
Ibuprofen has a molecular weight of 206.3 g. It has hepatic metabolism and has renal excretion. Common adverse effects include nausea, dyspepsia, gastrointestinal ulceration/bleeding, raised liver enzymes, diarrhoea, headache, dizziness,

salt and fluid retention, hypertension. Infrequent adverse effects include oesophageal ulceration, heart failure, hyperkalaemia, renal impairment, confusion, bronchospasm, rash etc. As with other NSAIDs, ibuprofen has been reported to be a photosensitizing agent (Castell *et al.*, 1987). Ibuprofen however has a very weak absorption spectrum which does not reach into the solar spectrum. The molecule contains only a single phenyl moiety and no bond conjugation, resulting in a very weak chromophore system. Ibuprofen, therefore, is only a very weak photosensitizing agent when compared with other members of the 2-arylpropionic acids.

3.2.28 Mechanism of action

Nonsteroidal anti-inflammatory drugs work by interfering with the cyclooxygenase pathway (Gisbert *et al.*,2001). The normal process begins with arachidonic acid a dietary unsaturated fatty acid obtained from animal fats. This acid is converted by the enzyme cyclooxygenase to synthesize different prostaglandin. The prostaglandins go on to stimulate many other regulatory functions and reactionary responses in the body. Earlier research (Tannenbaum, 1996; Vane 1971) have shown that there are two types of cyclooxygenase, denoted COX-1 and COX-2. Each type of cyclooxygenase lends itself to producing different types of prostaglandins.

Different mechanisms stimulate the two types of cyclooxygenase. COX-1 is stimulated continuously by normal body physiology. The COX-1 enzyme is constitutive meaning that its concentration in the body remains stable. It is present in most tissues and converts arachidonic acid into prostaglandins. These prostaglandins in turn stimulate normal body functions, such as stomach mucus production and kidney water excretion, as well as as platelet formation formation. The location of the COX-1 enzyme dictates the function of the prostaglandins it releases (Vane, 1996). For example, COX-1 in the stomach wall produces prostaglandins that stimulate mucous production. In contrast, the COX-2 enzyme is induced. It is not normally present in cells but its expression can be increased dramatically by the action of macrophages the scavenger cells of the immune system (Tannenbaum, 1996)



The Cyclooxygenase Pathway

COX-2's most important role is in inflammation. COX-2 is involved in producing prostaglandins for an inflammatory response. COX-1 is stimulated continually, and COX-2 is stimulated only as a part of an immune response.

3.2.29 Biological applications

Ibuprofen is used to reduce the fever, pain, inflammation, and stiffness caused by many conditions, such as osteoarthritis, rheumatoid arthritis, and abdominal cramps associated with menstruation. Ibuprofen is used widely in the community for the relief of headache including migraine. It is also widely marketed as an analgesic agent rather than as an anti-inflammatory and is often used for general pain conditions including those arise from various injuries such as sporting injuries, illness such as influenza, shingles, gout and post operative pain. As with other NSAIDs, ibuprofen inhibits platelet aggregation, but is not used therapeutically for this action since it is a minor and reversible effect. Litkowski *et al.* (2005) studied the analgesic effect of ibuprofen with oxycodone in combination therapy. According to Rostom *et al.* (2005), ibuprofen can cause hepatotoxicity. An earlier study (Hollenz and Labenz, 2004)

reported that administration of ibuprofen could lead to gastrointestinal injury like ulcer. Long-term use of ibuprofen will lead to Nephrogenic adenoma, which is an infrequent benign lesion of the urinary system (Scelzi *et al.*, 2004). Dokmeci (2004) proved that Ibuprofen might be a promising new therapeutic avenue for the treatment of neurodegenerative diseases such as Alzheimer's disease (AD).

3.2.30 Anti–ulcerogenic activities in plants

Gastric ulcer is now believed to be due to an imbalance between acid and pepsin and weakness of the mucosal barrier. Several mechanisms have been suggested for the effect of anti-ulcerogenic principles, including increasing the gastric hexosamine level and enhancing the strength of the gastric barrier either physically or by blocking the H^+,K^+-ATPase pump (Sumbul 2010), stimulation of membrane stabilization by interference with Ca^{2+} influx, scavenging oxygen generated free radicals and inhibiting peroxidation of biological membranes (Koch and Loffler, 1985). Each of the tested plant extracts might exert its activity by one or more of these proposed mechanisms. However, it should be pointed out that all of the effective plant extracts contain tannins and/or flavonoids to which the anti-ulcerogenic effects could be attributed.

Andreo *et al.*, (2006) reported significant antiulcerogenic activity for *Mouriri pusa* Gardn. (Melastomataceae) at various doses i.e 250, 500 or 1000 mg/kg evaluated by HCl/ EtOH solution and it was suggested that the methanolic extract probably had an antiulcerogenic effect related to cytoprotection. It was also stated by Andreo *et al.*, (Andreo *et al.*, 2006.) that the large amount of condensed tannins (~70%) in the crude MeOH extract of *Mouriri pusa* may promote precipitation of proteins that could prevent the development of ulcers. Tannins have the capacity to precipitate micro proteins on the site of the ulcer, forming a protective pellicle that prevents the absorption of toxic substances and resists the attack of proteolytic enzymes (John and Onabanjo, 1990). Condensed tannins were also detected as major compounds. These compounds are formed by several catechin moieties. Plants rich in tannins are also used in folk medicine to treat several health problems, including gastrointestinal diseases and inflammation. Andreo *et al.*,(2006) correlated the effects of the *Mouriri pusa* extracts with its chemical composition, flavonoids and catechins which are secondary metabolites present in plants that have attracted the

attention of many researchers due to the wide range of their biological activities (Harborne, 1996) . There are many studies related to antiulcerogenic properties of flavonoids (Gracioso *et al.*, 2002). In low doses, flavonoids have anti-inflammatory effects because they increase the capillary resistance; however, in high doses (1000 mg/kg) there is evidence that flavonoids increased pro-inflammatory factors (Gracioso *et al.*, 2002). Especially interesting are those compounds with an *ortho*-dihydroxy nucleus, which were reported as possessing antioxidant activity.

In most cases, the antioxidant activity of flavonoids seems to depend on the substitution pattern of the hydroxyl groups and the critical condition for efficient radical scavenging is the 3',4'-dihydroxy arrangement in the B ring as well as 4-keto group in the C-ring. The presence of 3- and/or 5-OH groups also provides the flavonoid with a catechol-like structure, via intramolecular rearrangement, and it is a supportive feature for the antioxidant activity of flavonoids. The $\Delta^{2,3}$ double bond in the C-ring allows phenoxyl electron delocalization from the B ring (and thus radical stabilization), increasing the radical scavenging activity (Cao *et al.*, 1997) . The flavonoids with these structural features, play a role in the gastroprotective action (Andreo *et al.*, 2006). Literature reports that *ortho*-dihydroxy grouping at ring A of flavonoids can also lead to potent antioxidants (Rice-Evans *et al.*, 1996) property contributing to the anti-ulcerogenic activity.

Fejes *et al.* (1998) demonstrated that flavonoids and phenolic compounds have a synergistic antioxidant activity. Repetto and Llesuy (2002) reported that low concentrations of phenolic compounds stimulated the prostaglandin H-synthase (PGHS), whereas high concentration inhibited the production of PGHS. Since increasing amounts of flavonoids may change their properties from antioxidant to pro-oxidant (Gracioso *et al.*, 2002), the absence of the gastroprotective activity observed , may possibly be due to the flavonoid content of this extract, which may induce modulation of endogenous PGs (Andreo *et al.*, 2006). In addition, saponins and terpenoids (Repetto and Boveris 2001), triterpenes (Gonzalez *et al*, 2001) are also known to possess antiulcer activity.

3.2.31 Fish oil and ulcer

Several workers (Manjari and Das, 2000; Güzel *et al.*,1995) have studied the protective effects of fish oil against induced gastric ulcers in animal models.

Bhattacharya *et al.*,(2006) studied the effect of fish oil derived from Scomberoides commersonianus containing omega-3 polyunsaturated fatty acids on gastric ulcers and on offensive and defensive factors in gastric mucosal damage, following experimental gastric ulceration. Fish oil significantly reduced the severity of ulceration in gastric ulcers induced by aspirin, cold-restraint stress (CRS), alcohol and pylorus ligation. Fish oil decreased the offensive acid-pepsin secretion and augmented the defensive factors like mucin secretion, cellular mucus and life span of mucosal cells following pylorus ligation. It significantly increased activity of anti-oxidant enzymes (catalase and glutathione peroxidase) and decreased lipid peroxidation in gastric mucosa of CRS rats.

Ulak *et al.*,(1995) demonstrated the effect of fish oil and olive oil on the gastric mucosal damage induced by cold-restraint stress in rats. They observed that a diet containing fish oil, when ingested for 3 weeks before exposure to stress, protected from gastric ulceration significantly ($p < 0.01$) and led to a statistically significant increase both in mucus and phospholipid content of the gastric mucosal barrier ($p < 0.02$ and $p < 0.001$, resp.) in cold-restraint stress-induced gastric injury in rats. Drago *et al.*,(1999) hypothesized that the EPA and DHA components of fish oil are responsible in vitro bactericidal activity of fish oils on Helicobacter pylori infection in ulcers. al-Harbi *et al.*,(1995) proved that fish oil rich in eicosapentaenoic acid possessed both antisecretory and antiulcerogenic effects.

3.3 MATERIALS AND METHODS

Liver oils extracted from the elasmobranchs (source of the elasmobranchs and lipid extraction procedure have been discussed in the previous section) *Neoharriotta raleighana*, *Centrosymnus crepidater*, *Apristurus indicus and Centrophorus sculpratus* were used for the pharmacological analyses. These were fed to the animals (either wistar strain male Albino mice or rats, as the case may be) orally after evaluating their lethal dosages. All chemical reagents used in the experiments were purchased from Merck (Darmstadt, Germany). Paracetamol and Ibuprofen, the standard reference drugs used for the animal experiments were obtained from Sigma-Aldrich Chemical Inc. (St. Louis, MO).

3.3.1 Experimental animals.

Wistar strain male albino rats (120-200 g) and mice (35-40 g) were used in the experiments. They were housed individually in polypropylene cages under hygienic conditions and were provided standard food and water *ad libitum*. The animals were maintained on a 12:12 h light:dark photoperiod under standard conditions of temperature and ventilation. The experiments were performed as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and with the approval of the Institutional Animal Ethics Committee (IAEC).

3.3.2 Experimental protocol.

The analgesic, anti-inflammatory anti-pyretic and anti-ulcer activities of the shark liver oils were assessed after checking their lethal dosages in rats and mice as animal models. Analgesic activity was determined using the acetic acid-induced writhing and hot-plate tests while the anti-inflammatory activity was determined using the formalin-induced rat paw oedema test.

Acute toxicity of the four shark liver oils was carried out on male albino rats and mice using Karber's arithmetical method for the determination of LD_{50} (lethal dose that causes mortality by 50%) (Turner[a] 1965). In this assay increasing doses of the test substance, oil along with the vehicle dimethylsulfoxide (DMSO) (oil:DMSO, 4:1), were administered orally to groups of four animals at different doses (1.0-7.0 g/kg).

The animals were observed for one week, the number of survivors was counted and the optimum average dosage was determined.

$$LD_{50} = \textit{Maximal Dose} - \underline{\Sigma xy} / n$$

Where *Maximal Dose* is the dose at which 100% mortality occurred, *x* is the dose administered (mg/kg body weight) and *y* is the mean mortality rate.

3.3.3 Analgesic activites

a) Acetic acid-induced writhing assay. In vivo determination of antinociceptive activity was carried out using the abdominal constriction test (Koster *et al.* 1959). Mice were divided into six groups of 5 animals each after initial screening. All shark liver oils were administered orally (50 mg/ 0.2 mL/ animal) as a suspension in DMSO (oil:DMSO, 4:1) one hour prior to the intraperitoneal administration of acetic acid (0.6 % v/v). Ten minutes after the administration, the number of constrictions per animal was recorded for 20 min. Control animals received an equal volume of vehicle. The standard reference drug paracetamol was administered at 100 mg/kg body weight. Antinociceptive activity was reported as percent inhibition of constrictions compared to the placebo control group.

b) Hot-plate activity. The analgesic activity was investigated in male albino mice, using the hot-plate test (Turner[b] 1965). Mice were divided into six groups of 5 animals each after initial screening. Each of the shark liver oils was administered orally at a dose of 50 mg/ 0.2 mL/ animal as a suspension in DMSO (oil:DMSO, 4:1). Control animals received an equal volume of the vehicle. One group received the reference drug paracetamol at 100 mg/kg bodyweight. The animals were dropped gently on the hot-plate maintained at 53 ± 1 C; this was done 5 min prior to the administration of the vehicle, oils and paracetamol and at 30, 60, 90, 120, 150 min following administration. The time between placement and the first sign of paw licking or jumping was recorded as latency. The basal latencies were 6-10 s. A cut-off time of 30 min was established to prevent injury to the paws. The mean values were recorded.

3.3.4 Anti-inflammatory activity.

The anti-inflammatory activity was determined, by the method of Hunskaar and Hole (1987), using the formalin-induced rat paw oedema test. Male albino rats weighing between 160-180 g were divided into five groups of 6 animals each. The oil along with the vehicle DMSO (oil:DMSO, 4:1) was administered orally at a dose of 1.5 g/kg bodyweight prior to the induction of inflammation by the subcutaneous injection of 0.1 ml sterile saline solution of 3.5% formalin in the right hind paw. The control group received sterile saline solution (1 ml 0.9% NaCl solution) while the reference drug ibuprofen at dose of 100 mg/kg bodyweight was administered to the standard group intraperitoneally at least 30 min before the induction of oedema. Paw sizes were measured with a calibrated screw guage before the administration of formalin, then thereafter at 1, 2, 3 and 4 h after the injection of the inflammatory agent. The average size of the paw measured in millimeters was calculated from 3 measurements which did not differ from more than 1%. These individual measurements allowed us to determine the average paw size for each group (s_m) and then the percentage of oedema by comparison with the average size obtained for each group before any treatment (s_o).

Percentages of inflammation-inhibition were obtained for each group using the following calculation:

$$\frac{[(s_m - s_o)\text{control} - (s_m - s_o)\text{treated}] \times 100}{(s_m - s_o)\text{control}}$$

Where s_m is the mean paw size for each group after formalin treatment and s_o is the mean paw size obtained for each group before the treatment (Owolabi and Omogbai 2007).

3.3.5 Antipyretic activity

Antipyretic activity of drug was measured by slightly modifying the method described by Yogna *et. al.*(2005). Rats were fasted overnight with water *ad libitum* before the experiments. Pyrexia was induced by subcutaneously injecting 20% (W/V) brewer's yeast suspension in saline solution (10 ml/kg) into the animal's dorsum region. The rectal temperature of each rat was measured at 19th hour after injection using a thermometer. Only rats that showed an increase in temperature of at least 0.7 °C

were employed for the experiments. The fish oils CS, NR and AI (solvent free and diluted in DMSO, 1:4 ratio of DMSO: oil) were administered orally (1.5 g/kg) and the temperature was monitored at 0, 1, 2 and 3 h after drug administration. Paracetamol IP (200 mg/kg) was used as standard for comparison of antipyretic activity, and all control animals received dimethyl sulfoxide as the vehicle. A significant drop in the body temperature of the animals indicated better anti-pyretic effects of the administered sample.

3.3.6 Anti-ulcer studies

Male and female Wistar rats (180–200 g) kept in standard laboratory conditions, were fasted overnight in single wire-net floor cages with free access to tap water and were randomly assigned to four groups of 5–6 animals. Group I and III served as the normal control groups. While group I received oral administrations of the vehicle DMSO (diluted 1:3,v/v with double distilled water) group III received CS oil along with the vehicle (oil:DMSO=4:1) at 1.5g/kg bodyweight. Groups II and IV were induced with ulcer. However group IV had been pre-treated with CS oil (along with the vehicle) prior to induction of ulcer. All animals were fasted overnight before the induction of ulcer. Hydrochloric acid and ethanol (0.6% v/v) was used as ulcerogenic agent which was administered intraperitoneally at a dose of 2.0 ml/kg bodyweight. After 4 hours, all animal groups underwent surgery as per the procedure of Takeuchi *et al.* (1976) and gastric juice was collected. Rats were then sacrificed with over dose of chloroform and the stomach was removed after the esophagus had been clamped. The gastric juice was centrifuged and the volume was noted. The stomach was inflated with normal saline and then incised through the greater curvature and examined for the number of lesions. The total acidity was determined by titration with 0.02 N NaOH with phenolphthalein used as indicator. The mucosal tissue was scraped from the stomach and the gastric mucosa was homogenized in ice-cold 0.1 M Tris-HCl, pH 7.2 and centrifuged. The supernatant was used for further biochemical analyses.

3.3.6.a) Estimation of pepsin

The activity of pepsin was estimated by the method of Anson (1938).

Reagents

1. Pepsin substrate: haemoglobin in 0.06 N HCl
2. Sodium Carbonate: 0.55 M
3. Trichloroacetic acid: 7%
4. Standard tyrosine: 10 mg of tyrosine dissolved in 100ml distilled water
5. Folin's reagent

Procedure

1.0 ml of the substrate was added to 0.2 ml of the enzyme and incubated at 37^0C for 10 minutes. The reaction was terminated by adding 1.0 ml of 7% TCA and the mixture was centrifuged at 1000x g for 10 minutes. 1.0 ml of the supernatant was made alkaline by 5.0 ml of 0.55 M sodium carbonate. Then 0.5 ml of 1 N Folin's reagent was added. The optical density was measured at 660 nm after 30 minutes at room temperature in Shimadzu spectrophotometer. The results were expressed as umoles of tyrosine liberated/mg protein/h.

3.3.7 Statistical analyses

All data were expressed as mean ± SD and analysed statistically by one-way ANOVA using Duncan's test with a level of significance set at $P<0.05$. The statistical software, SPSS for Windows version 16, was employed for the analyses. Pearson's correlation test was performed between the various lipid components analyzed and the percentage of inhibition in inflammation to determine the factor(s) responsible for the observed pharmaceutical effects. A positive correlation was established when the level of significance was set at $P<0.05$ or $P<0.01$ as the case may be.

3.4 RESULTS

3.4.1 Acute toxicity of the oils

In the acute toxicity trial no mortality was observed for doses (oral administrations) of up to 3.8 g/kg bodyweight for either mice or rats. No significant changes in the body weight were observed at this dose. However, 50 % mortality was observed for both rats and mice at a dose of 6.2 g/kg body weight after one week. Based on these observations the oils were administered at an optimum average dose of 1.5 g/kg bodyweight.

3.4.2 Analgesic activity of oils

All the four shark liver oils (AI, CC, CS, NR) exhibited a highly significant ($P<0.05$) analgesic activity when compared to the control group. Table 3a illustrates the antinociceptive activity of the four shark liver oils, in the acetic acid-induced constrictions in mice, when administered at 1.5 g/kg bodyweight. The analgesic behavior of AI, CC oils and that of the standard drug paracetamol were significantly different ($P<0.05$) from each other and from CS and NR oils. Even though there was no significant difference in activity between CS and NR oils they differed significantly ($P<0.05$) from AI, CC oils and paracetamol. While mice treated with AI oil showed only a 29.5% inhibition, those treated with CC, CS and NR oils showed 52.7, 59.0 and 57.8% inhibitions, respectively ($P<0.05$). Paracetamol at 100 mg/kg bodyweight showed 47.2% inhibition.

The analgesic activity for all the four oils in the hot plate test i.e. in the latency vs time test was similar. However, only NR and CS oils exhibited an analgesic behavior (Fig. 3.1) similar to that of the standard drug paracetamol (latency time of 8.3 ± 0.5 s). NR, CS and CC oils showed a significant ($P<0.05$) analgesic activity from the 60^{th} to 90^{th} min. While mice treated with AI oil showed a significant ($P<0.05$) analgesic behavior up to 90 min of its administration, its effect started to decline afterwards. The latency time for control mice was 2.6 ± 0.5 s while NR, CS, CC and AI rats showed 9.3 ± 0.0, 8.6 ± 0.5, 8.0 ± 0.0 and 6.3 ± 0.5 s, respectively, from the 60 to 90^{th} min of the treatment. Of the four oils analysed only the animals treated with AI oil showed the least antinociceptive activity.

Table 3a. The Effect Of Shark Liver Oils And Paracetamol (Standard Reference Drug) On Acetic Acid-Induced Writhing Test In Mice. Oils Of *A. Indicus*(AI), *C. Crepidater*(CC), *C. Scalpratus* (CS) And *N. Raleighana* (NR) Were Administered Orally At 1.5 G/Kg And Paracetamol At 100 Mg/Kg Animal Weight. Values Are Mean Number Of Writhes ± Sd (N = 5 Per Group). ^{a,b,c,d,e}number Of Writhes With Different Superscripts Are Significantly Different (P<0.05). Inhibition Is Reported As Percent W.R.T. Control.

Treatment	Number of writhes	Inhibition (%)
Control	84.6 ± 1.52^{a}	-
Paracetamol	44.6 ± 3.05^{b}	47.27
AI	59.6 ± 2.08^{c}	29.51
CC	40.0 ± 2.00^{d}	52.75
CS	34.6 ± 2.08^{e}	59.05
NR	35.6 ± 3.05^{e}	57.83

Table 3b. Inhibitory Effects Of Shark Liver Oils On The Formalin-Induced Rat-Paw Oedema. Oils Of *A. Indicus*(AI), *C. Crepidater*(CC), *C. Scalpratus* (CS) And *N. Raleighana* (NR) Were Administered Orally At 1.5 g/Kg And Ibuprofen At 100 mg/Kg Animal Weight. Values Are Mean Percentage Inhibition ± Sd (N = 6 Per Group). For Each Hour ^{a,b,c,d,e}percentage Inhibition Values With Different Superscripts Are Significantly Different (P<0.05).

	Percentage Inhibition			
Treatment	1h	2h	3h	4h
Ibuprofen	62.94 ± 0.75^{a}	64.58 ± 1.09^{a}	51.29 ± 3.18^{a}	55.63 ± 1.86^{a}
AI	20.97 ± 2.11^{b}	27.95 ± 0.65^{b}	54.23 ± 3.55^{a}	39.42 ± 0.94^{b}
CC	50.20 ± 1.49^{c}	58.30 ± 1.15^{c}	63.18 ± 1.40^{b}	61.17 ± 0.75^{c}
CS	56.18 ± 1.62^{d}	60.31 ± 0.60^{d}	65.72 ± 0.56^{b}	69.51 ± 0.78^{d}
NR	46.12 ± 0.49^{e}	58.11 ± 0.43^{c}	65.02 ± 5.81^{b}	48.68 ± 3.89^{e}

3.4.3 Anti-inflammatory activity of oils

The percentage inhibition in the formalin-induced rat paw oedema is shown in Table 3b. In the formalin-induced inflammation, the oils showed a peak inhibition of inflammation at the 3rd hour. Significant ($P<0.05$) inhibition of inflammation was shown by the standard drug ibuprofen and the four shark liver oils within one hour from the onset of inflammation. Ibuprofen showed a maximum inhibitory activity at the 2nd hour (64.5 %). CS and NR oils showed 65.0 % reduction in oedema at the 3rd hour while AI oil was able to reduce it by only 54.2 %. At the 4th hour, while all the oils as well as the standard paracetamol started to show a decline in the inhibitory activity, only CS oil showed a peak inhibitory activity of 69.5 % ($P<0.05$).

3.4.4 Antipyretic effect

Table 3c illustrates the highly significant ($P < 0.05$) antipyretic effect of the liver oils of *Centrophorus sclapratus* (CS), *Neoharriotta raleighana* (NR) *and Apristurus indicus* (AI) compared with the standard reference drug Paracetamol and placebo control, in yeast – induced pyrexia in rats. Fig. 2 shows the reduction in body temperature in rats treated with root extracts of CS, NR and AI at doses of 1.5g / kg bodyweights. NR and AI oils showed an antipyretic effect (lowering in body temperature observed) similar to the standard reference drug paracetamol, at a dose of 100mg / kg bodyweight, upto two hours of its administration. Maximum antipyretic effect was observed in rats treated with the CS oils, wherein a 1.5 degree decline in temperature was observed within one hour of its administration; CS oil was found to be 65% better than the standard drug used.

3.4.5 Anti-ulcer effects of CS oil

Figure 3.2 represents the highly significant anti-pyretic effects of *Centrophorus scalpratus* liver oil against acid induced ulcerations in Albino rats. Ulcer induced and untreated group II animals recorded maximum number of gastric erosions when compared to the control animals group I, III and the ulcer induced and treated group IV, as shown in fig 3.2 and plate III.2 . there was a significant decrease ($p<0.05$) in protein content for Group II animals w.r.t. groups I, III and IV. However the ulcer induced and CS oil treated group IV recorded significantly higher ($p<0.05$) protein content w.r.t. group III animals. The ulcer induced and untreated group II animals

Fig. 3.1. Effect Of Shark Liver Oils On The Hot Plate Reaction Time Vs. The Basal Latency(s). Oils Of *A. Indicus*(AI), *C. Crepidater*(CC), *C. Scalpratus* (CS) And *N. Raleighana* (NR) Were Administered Orally At 1.5 G/Kg Animal; Paracetamol At 100 Mg/Kg Animal. Data Are Expressed As Mean Latency ± SD Values. ^{a,b,c,d,e}latency Values With Different Superscripts Are Significantly Different (P<0.05).

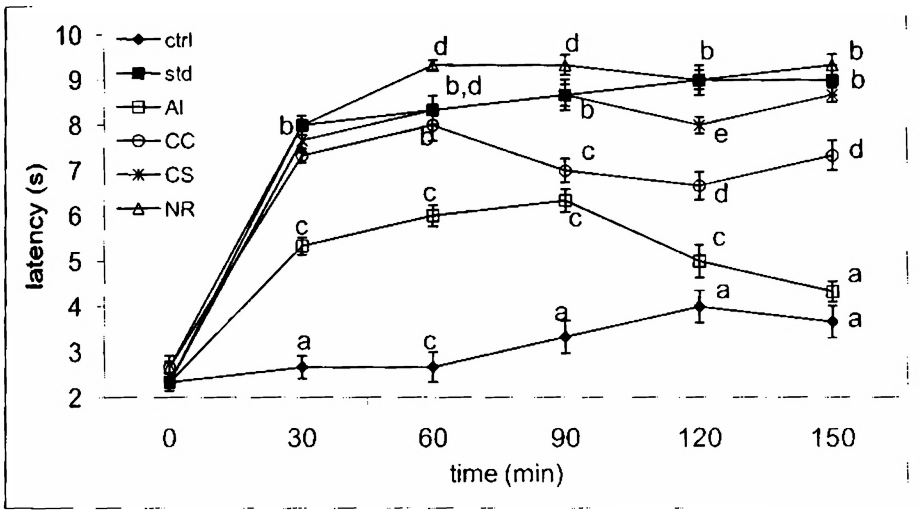


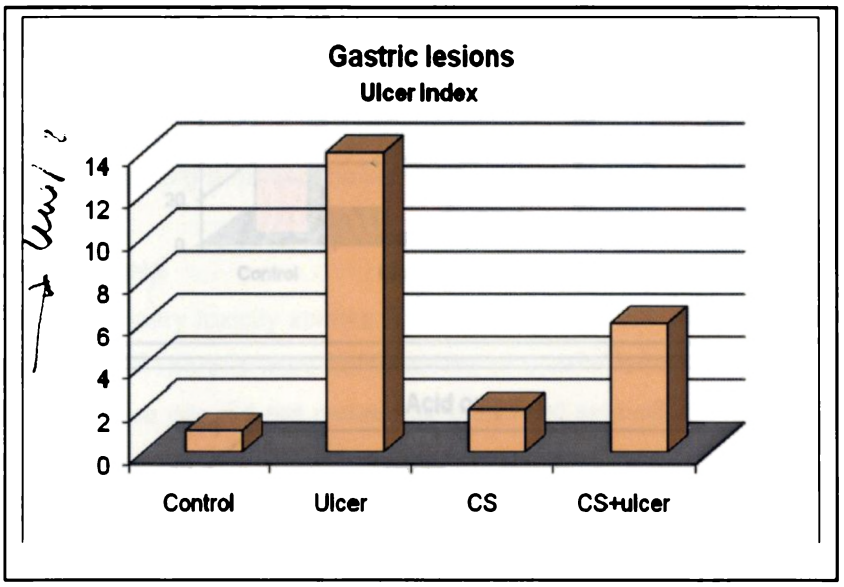
Table 3c. Antipyretic effect of liver oils of *Centrophorus sclapratus* (CS), *Neoharriotta raleighana* (NR) *and Apristurus indicus* (AI)on yeast – induced pyrexia in rats. The drug Paracetamol is used as the reference standard at 100 mg/kg body weight.

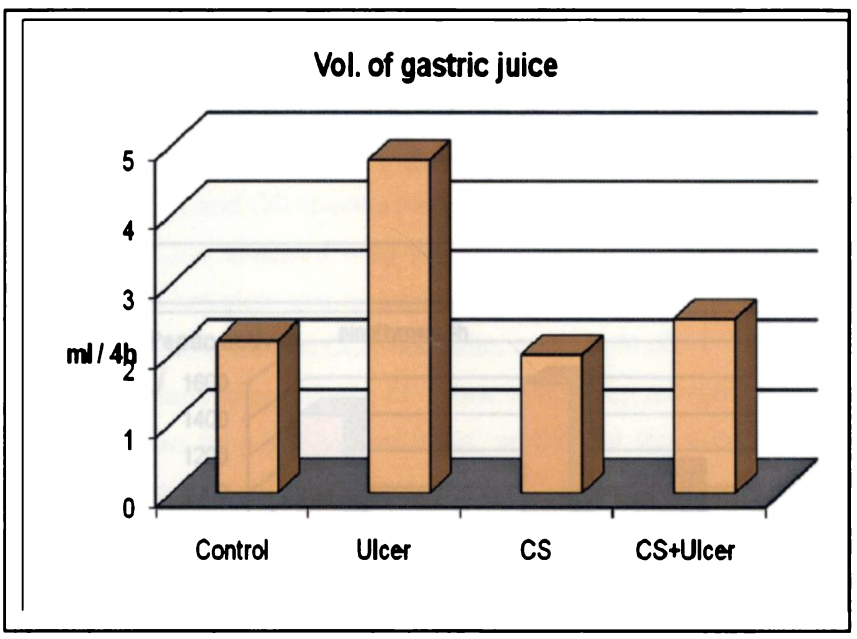
Time (h)	Control	Paracetamol	CS	NR	AI
0	39.70 ± 0.10	39.56 ± 0.05	39.50 ± 0.10	39.60 ± 0.10	39.66 ± 0.05
1	39.53 ± 0.15	38.96 ± 0.05	37.96 ± 0.06*	38.70 ± 0.17	38.93 ± 0.06
2	38.93 ± 0.11	38.10 ± 0.15	37.70 ± 0.11*	38.10 ± 0.16	38.56 ± 0.05
3	38.70 ± 0.10	37.40 ± 0.10	37.56 ± 0.15	37.76 ± 0.12	37.90 ± 0.10
4	38.66 ± 0.20	36.83 ± 0.21*	37.26 ± 0.20	37.50 ± 0.10	37.80 ±0.11

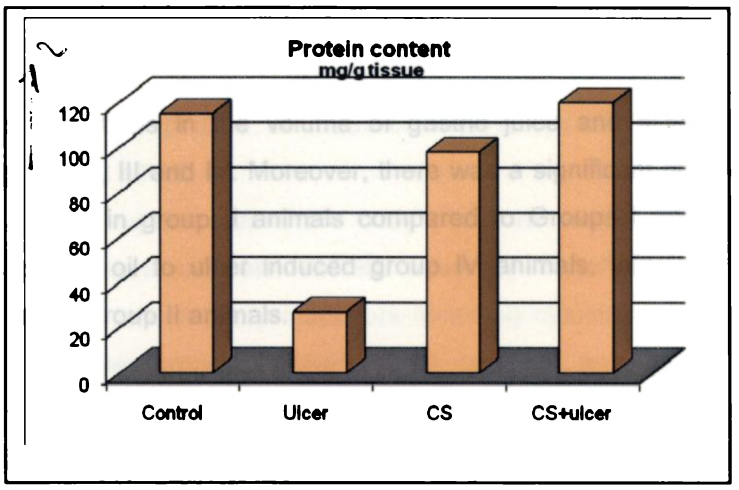
Values are expressed as means (in degree Celsius) ± SD (n = 6).

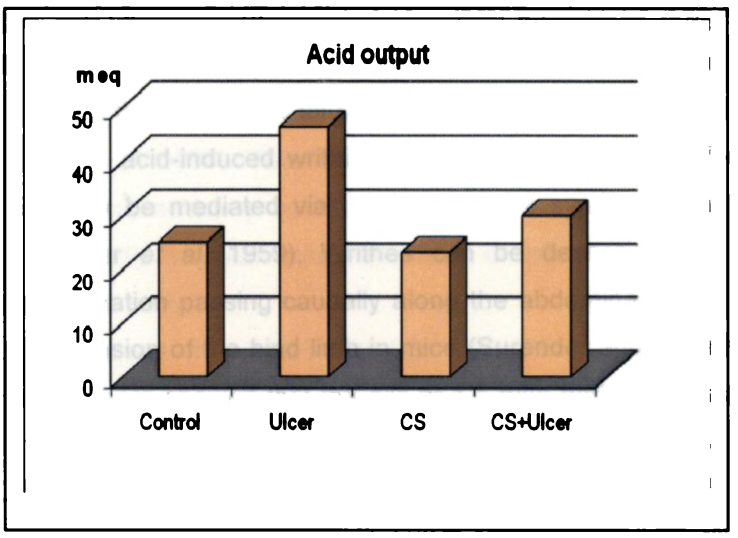
* indicates values are significantly different compared to the control group (P < 0.05).

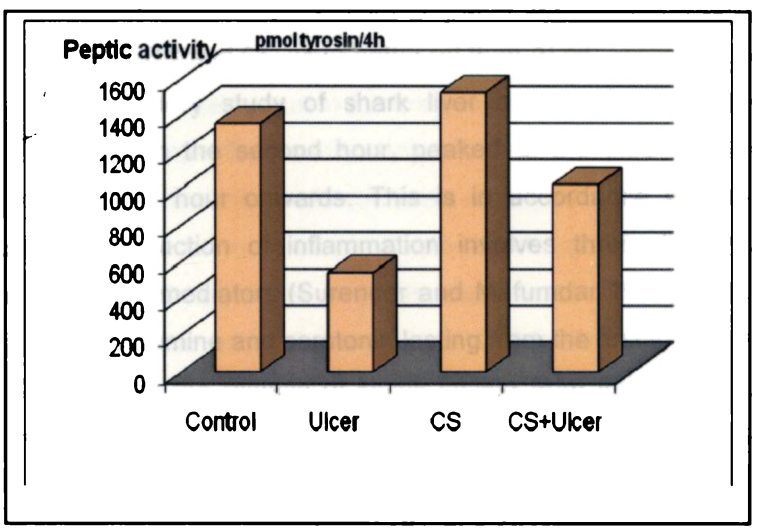
Figure 3.2 Anti-ulcer effect of CS oil against HCl-induced gastric ulcer in rats











Effect of CS oil on HCl-induced gastric ulcers in rats on protein content, acid output and peptic activity.

had significant elevations in the volume of gastric juice and acid output when compared to groups I, III and IV. Moreover, there was a significant decline ($p<0.05$) in the peptic activity in group II animals compared to Groups I and III. However administration of CS oil to ulcer induced group IV animals, improved the peptic activity compared to group II animals.

3.5 DISCUSSIONS

In the preliminary toxicity studies 50% mortality was observed at a dosage of 6.2 g/kg bodyweight. The oils were administered at a safer level of 1.5 g/kg bodyweight per animal. These oils did not cause any unwanted side-effects in the experimental animals indicating that they were safe for consumption.

Inhibition of acetic acid-induced writhing in mice suggested that the analgesic effect of the oils may be mediated via inhibition of the synthesis and release of prostaglandins (Koster *et al.* 1959). Writhes can be described as a wave of constriction and elongation passing caudally along the abdominal wall with twisting of the trunk and extension of the hind limb in mice (Surender and Mafumdar 1995). The results obtained here showed that the oils at 1.5 g/kg bodyweight had a higher rate of inhibition than the standard drug paracetamol. Of the four oils analysed those belonging to CS, NR and CC species had a better analgesic effect (59, 57 and 52 %, respectively) than the standard drug paracetamol (47 %) within 90 min from the onset of pain. The hot-plate test also confirmed our findings that the antinociceptive ability of the oils of NR, CS and CC were better off than AI oil.

In the anti-inflammatory study of shark liver oils, a significant inhibition of inflammation began from the second hour, peaked at the third hour and started declining from the forth hour onwards. This is in accordance with a previously reported study that induction of inflammation involves three distinct phases of release of inflammatory mediators (Surender and Mafumdar 1995). The first phase being the release of histamine and serotonin lasting from the first to the second hour; the second phase being the release of kinins lasting from the second to the third hour while the third phase being the release of prostaglandins and lasting from the third to the fifth hour (Surender and Mafumdar 1995). Thus it can be inferred that the mechanism through which the oils of CS, NR and CC elicited its effects might be through the inhibition of the synthesis of kinins and prostaglandins, since the oils had

been effective at these phases of mediator release. AI oil showed the least inflammation inhibitory effect.

Inflammation is a normal part of the body's immune response to infection or injury. Activated white blood cells secrete a variety of inflammation-promoting compounds or rather inflammatory mediators including cytokines like interleukin-6 (IL-6) and C-reactive proteins (CRP), free radicals and eicosanoids like prostaglandins and leucotrienes, to fight germs and to dispose off damaged cells (Ridker *et al.* 2000). It can be assumed that the mechanism of action of liver oils in the present study is via the inhibition of the synthesis of kinins and prostaglandins and this might be through the action on cyclooxygenase (COX) enzyme. COX-2 is responsible for the biosynthesis of prostaglandins under acute inflammatory conditions (Nantel *et al.* 1999). This inducible COX is believed to be the target enzyme for the anti-inflammatory activity of nonsteroidal anti-inflammatory drugs (NSAIDs) (Lau *et al.* 1993) which reduce the level of inflammatory mediators and alleviate the pain in the body.

The behavior of shark liver oils, from CS, NR, CC and AI species, in the descending order of anti-inflammatory activity was 69, 65, 63 and 54 %, respectively, as was similar to the standard drug ibuprofen (64 %) in the formalin-induced rat paw oedema test, thus confirming their antinociceptive profile and their action might be similar to that of NSAIDs. Earlier reports have shown that NSAIDs inhibit the activity of cyclooxygenases (Lau *et al.* 1993) and lower the levels of myeloperoxidase (Faurschou and Borregaard 2003) in the tissues and that they attenuate the pain response in the second phase but not in the first phase of the formalin test in rats.

Antinociceptive and anti-inflammatory components present in the shark liver oils are yet to be explored. Shark liver oils comprise mainly of 1-O-alkylglycerols which constitute about 10-30% of the unsaponifiable matter of the oils (Hallgren and Larsson 1962). These alkylglycerols or AKGs are indeed responsible for reducing pain or inflammation in the body (Pedrono *et al.* 2004). The exact mechanism by which they function has not been fully understood but it has been proposed that they work by either inhibiting the synthesis, release or action of inflammatory mediators, namely histamine, serotonin and prostaglandins that might be involved in inflammation. It has been reported that naturally occurring AKGs have potent biological activities on various cells or systems (Devaraj and Jialal 2000). Shark liver

oils also contain high proportions of γ-linolenic acid (Zurier *et al.* 1996), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (James *et al.* 2003). It has been shown that these long chain n-3 PUFAs (James *et al.* 2003) lower the incidence of inflammatory diseases such as asthma and arthritis (Shahidi and Senanayake 2006). These dietary fatty acids are known to reduce the levels of arachidonic acid metabolites and lower the formation of proinflammatory compounds, like prostaglandins and leukotrienes, by blocking their activity (Olivera 2004). Early studies reviewed by Stamp *et al.* (2005) and Calder (2006) attributed the anti-inflammatory effects of fish oils to competition with arachidonic acid for production of inflammatory eicosanoids. Anti-inflammatory effects of EPA and DHA have been studied by several workers. (Arita *et al.* 2005; Lukiw *et al.* 2005; Hudert *et al.* 2006). EPA and DHA contained in fish oils provide nutrients needed to build anti-inflammatory prostaglandin series 1 and 3 (Simopoulos 1991). In addition, shark liver oils are rich in antioxidants like vitamin E (Devaraj and Jialal 2000) which reduce inflammation by decreasing C-reactive protein levels and by blocking the activity of TNF-α (tumour necrosis factor-alpha) series 2-prostaglandins (PGE-2) and cyclooxygenases (James *et al.* 2003). Antioxidants are well known to alleviate the inflammatory processes mediated by allergic substances. They also curb inflammation by quenching hazardous molecules called free radicals, which stimulate inflammation (Vittala and Newhouse 2004). Sharks inhabiting waters beyond 600m depth are believed to possess reasonably high content of the hydrocarbon squalene (Ko *et al.* 2002), yet another antioxidant with potent pharmaceutical values. Its role as an antilipidemic agent (Qureshi *et al.* 1996) and membrane stabilizer has been reported (Sabeena *et al.* 2004).

Pyrexia or fever is a frequent medical symptom that describes an increase in internal body temperature to levels that are above the normal body temperature i.e. above 36.8±0.7 °C or 98.2±1.3 °F. Fever is most accurately characterized as a temporary elevation in the body's thermoregulatory set-point, usually by about 1-2°C. In the yeast-induced pyrexia maximum antipyretic effect was observed in rats treated with the liver oils of CS, wherein a 1.5 degree decline in temperature was observed within one hour of its administration; CS oil was found to be 65% better than the standard drug used. NR and AI oils showed an antipyretic effect similar to the standard reference drug paracetamol, at a dose of 100 mg / kg bodyweight, up to

two hours of its administration. This result seems to support the view that the fish oils of CS, NR and Al have some influence on prostaglandin biosynthesis because prostaglandins are believed to be a regulator of body temperature.

Temperature is regulated in the hypothalamus, in response to PGE2. PGE2 release, in turn, comes from a trigger, a pyrogen (substance that induces fever). The hypothalamus generates a response back to the body, making it increase the temperature set-point (Aronoff and Neilson, 2001). The endogenous pyrogens such as interleukin 1, interleukin 6 (IL-6), and the tumor necrosis factor-alpha (TNFα) are a part of the innate immune system, produced by phagocytic cells and cause the increase in the thermoregulatory set-point in the hypothalamus (Luheshi, 1998). The endogenous pyrogens such as interleukin 1, interleukin 6 (IL-6), and the tumor necrosis factor-alpha (TNFα) are a part of the innate immune system, produced by phagocytic cells and cause the increase in the thermoregulatory set-point in the hypothalamus (Cooper *et al.*,1994). These cytokine factors are released into general circulation where they migrate to the circumventricular organs of the brain, where the blood-brain barrier is reduced. The cytokine factors bind with endothelial receptors on vessel walls, or interact with local microglial cells (Boulant *et al.*,1997). When these cytokine factors bind, they activate the arachidonic acid pathway, PGE2 release comes from the arachidonic acid pathway. This pathway (as it relates to fever), is mediated by the enzymes phospholipase A2 (PLA2), cyclooxygenase-2 (COX-2), and prostaglandin E2 synthase (Cooper *et al.*,1994). These enzymes ultimately mediate the synthesis and release of PGE2. The role of fish oils in reducing fever might be due to their action on PGE2 or on endogenous pyrogens or on the arachidonic pathway and their mode of action have to be explored further.

Gastric ulcer is now believed to be due to an imbalance between acid and pepsin and weakness of the mucosal barrier (Dutta *et al.*, 2002 Satoh *et al.*,, 1984). The gastroprotective activity of the liver oils of *Centrophorus scalpratus* on the acid induced ulcer model was investigated using Albino rats administration (i.p.) of hydrochloric acid to the control group I clearly produced characteristic haemorrhagic lesions with large linear patches of mucosal necrosis. The oral administration of CS oil significantly decreased the gastric lesions. The anti- ulcerogenic effect may be probably related to cytoprotection of fish oil components. (Andreo *et al.*, 2006.)

The preliminary biochemical screening of the fish oil (CS) had revealed the presence of alkoxyglycerols, tocopherol, squalene, n3 fatty acids. Several mechanisms have been proposed to explain the biological role of fish oils against acid induced ulcers including increase of mucosal prostaglandin content, decrease of histamine secretion from mast cells, inhibition of acid secretion and inhibition of *Helicobacter pylori* growth. In addition, these fish oil components have been found to be free radical scavengers; free radicals play an important role in ulcerative and erosive lesions of the gastrointestinal tract (Borrelli and Izzo, 2000).

Diets enriched in fish oils protect duodenal and gastric mucosae against ethanol injury. The polyunsaturated fatty acids present in fish oils are reportedly converted to a family of trienoic eicosanoids with reduced inflammatory potential (Manjari and Das, 2000; Bhattacharya *etal.*,2006). The n-3 fatty acids are readily incorporated into gastric phospholipids, displacing arachidonic acid and potentially affecting both the cyclooxygenase and lipoxygenase pathway. The result is a likely decrease of prostaglandin PGE2 and thromboxane A2 synthesis and an increase in that of PGE3 and leukotriene B5 which may be less biologically active in inflammation (Ulak *et al.*,1995). Furthermore, in some *in vitro* studies, dietary fish oil reduces leukotriene B4, free radicals, and platelet-activating factor, all of which are considered significant mediators of mucosa damage.

Prostaglandin and nitric oxide influence the various components of mucosal defenses: they inhibit acid secretion, stimulate mucus and bicarbonate secretion, elevate mucosal blood flow, and accelerate the healing of ulcers (Andreo *et al.*,2006; Manjari and Das, 2000; al-Harbi *et al.*,1995; Halter *et al.*,2001). Among the eicosanoids are also lipid mediators of inflammation, such as leukotrienes and thromboxane, which may contribute to the pathogenesis of gastric ulcers. The n-3 fatty acid EPA present in fish oils has been demonstrated to be metabolized to trienoic eicosanoids, and in some models these have exhibited less inflammatory potential than the arachidonate-derived analogs. The n-3 fatty acids are readily incorporated into gastric phospholipids, displacing arachidonic acid as described above and potentially affecting both the cyclooxygenase and lipoxygenase pathways. It has been shown that gastric prostaglandin synthesis can be adversely affected by ingestion of fish oil (Halter *et al.*,2001; Bhattacharya *et al.*,2006). In the present study, the antioxidant effects of squalene and tocopherol, the membrane

stabilizing and anti-inflammatory potentials of alkoxyglycerols and n3 PUFAs (Ko *et al.* 2002; Pedrono *et al.* 2004; Arita *et al.* 2005) would have decreased the offensive acid-pepsin secretion and augmented the defensive factors like mucin secretion, celllular mucus and life span of mucosal cells following pylorus ligation in ulcer induced and CS oil treated animals.

Pearson's correlation test was used to determine the influence of the HC squalene and AKGs upon anti-inflammation (Table 3d). Significant correlations were observed between NSM and anti-inflammation for all the four oils used in the study ($P<0.01$ for CS, CC and AI, $P<0.05$ for NR). HC and AKG contributed significantly towards anti-inflammation in CS, NR and AI oils whereas it was the AKGs which were responsible for the observed anti-inflammatory activity in CC oils ($P<0.01$). Significant correlations were also observed between HC squalene and antinociception (Table 3d); $P<0.01$ for oils of CC, NR and AI species, $P<0.05$ for CS species. AKG component of NSM significantly influenced analgesic responses in oils of CC, NR and CS species ($P<0.05$). Pearson's correlation test proved a significant correlation on the influence of fatty acids upon anti-inflammation ($P<0.05$). Saturated and MUFAs showed a negative correlation co-efficient with anti-inflammation/antinociception which meant that as their levels increased the anti-inflammatory responses decreased. Positive correlations were observed between levels of PUFA and anti-inflammation thus confirming their roles in lowering an inflammation (Table 6). The cumulative effects of the various lipid components (squalene, alkylglycerols and poly-unsaturated fatty acids) studied are indeed responsible for the observed anti-inflammatory and antinociceptive effects of the extracted shark liver oils (Ko *et al.* 2002; Pedrono *et al.* 2004; Arita *et al.* 2005).

Table 3d. Pearson's correlation coefficient test to determine the influence of various lipid components upon anti-inflammation and antinociception.

Anti-inflammation/ Antinociception	Oils		Lipid Components						
			NSM	HC	AKG	FA	SatFA	MUFA	PUFA
	AI	P.corr[a]	1.000**	1.000**	1.000**	1.000*	-0.777	-0.756	0.982
		P.corr[b]	1.000**	0.499	1.000**	1.000*	-0.997	-0.372	0.541
	CC	P.corr[a]	1.000**	0.492	1.000**	1.000*	-0.996	-0.365	0.548
		P.corr[b]	1.000*	1.000**	1.000**	1.000**	-0.783	0.849	0.408
	CS	P.corr[a]	1.000**	1.000**	1.000**	1.000*	-0.777	-0.756	0.982
		P.corr[b]	0.999*	1.000*	0.497	1.000**	0.406	-0.282	0.596
	NR	P.corr[a]	1.000*	1.000**	1.000**	1.000*	-0.780	0.851	0.404
		P.corr[b]	1.000**	0.499	1.000**	1.000*	-0.997	-0.372	0.541

* Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

P.corr[a] = Pearson's correlation coefficient w.r.t. anti-inflammation

P.corr[b] = Pearson's correlation coefficient w.r.t. antinociception

3.6 SUMMARY AND CONCLUSION

The biomedical applications of the liver oils of deep sea sharks and chimaeras of the Indian EEZ are immense. Marine lipids possess a wide range of bioactive potentials which has to thoroughly assessed before it can be prescribed for medical purposes.

The present investigation indicated that shark liver oils belonging to CS, NR and CC species showed a better analgesic and anti-inflammatory profile than that of AI oil. We propose the high NSM content and the presence of n3 PUFAs to be responsible for the observed findings. The liver oils of NR, CS and CC contained a high fraction of NSM (average 70 %); the AKGs, HCs and vitamin E could play a major role in lowering the incidence of inflammatory diseases by blocking the activity of prostaglandins and leukotrienes. AI oil recorded the lowest fraction of NSM (25%) and hence would have exhibited lower pharmacological effects.

In the yeast-induced pyrexia experiments, CS oil exhibited a comparatively better anti-pyretic effect compared to NR and AI oils. Similarly CS oil was able to reduce the gastric erosions and reduce acid-pepsin secretion in ulcer induced rats. CS oil components would have significantly inhibited the production of inflammatory prostaglandins and cytokines during the inflammation and hence contributed to better activity.

In conclusion, liver oils extracted from the sharks namely *Centrophorus sculpratus*, *Neoharriotta raleighana*, *Centrosymnus crepidater* and *Apristurus indicus* possess pharmacological activities, which could contribute to their use in treatment of pain, pyrexia, ulcer, arthritis or other inflammatory disorders. Alkylglycerols, long chain polyunsaturated fatty acids, vitamin E and squalene present in shark liver oils play a major role in reducing the level of inflammatory mediators during an inflammation. However, the bioactive potentials of marine lipids from creatures inhabiting the Indian EEZ and the ability of these oils to interfere with the inflammatory mediators deserve further investigation.

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Chapter 4.

The hypocholesterolemic fish oil

Chapter 4. The hypocholesterolemic fish oil

4.1 INTRODUCTION

The Indian subcontinent is home to 20% of the world's population and one of the regions with the highest burden of cardiovascular diseases (CVD) in the world. Most CVD cases arise due to lifestyle, unhealthy eating habits, smoking etc. and are more common in men. In 1990, there were an estimated 1.17 million deaths from CVD in India, and the number is expected to almost double by the end of 2010. Studies have indicated that South Asians have elevated levels of low density lipoprotein cholesterol and triglycerides, while also suffering from a deficiency in high density lipoprotein cholesterol (good cholesterol, which helps to clear fatty buildups from blood vessels). In addition, South Asians are more vulnerable and are at greater risk of developing heart diseases than others.

Contrary to popular belief, fats and oils are an important part of nutrition in a healthy diet. Healthful sources of fat contribute to our energy stores and act as excellent lubricants, lubing up our digestive tract and providing protective insulation to our organs as well as allowing for the absorption of fat-soluble vitamin A, vitamin D, vitamin E, and vitamin K.(He, 2009) Fish oils are rich in the fat soluble vitamins and they contain antioxidant hydrocarbons like squalene and immune boosting compounds like 1-O-alkyl glycerol ethers and are even blessed with the long chain polyunsaturated fatty acids especially the n-3 series eg. EPA, DHA.(Enas, 1996) These long chain n3 PUFAs have been shown to reduce cardiovascular risk factors and reduce symptoms in rheumatoid arthritis. Fish oil helps in reducing the risk of heart related disorders as it is a powerful anti-inflammatory and can help reduce the risk from the C-reactive proteins.(Ciubotaru *et al.*,2003) Secondly, the omega 3 fatty acids help to prevent clumping and stickiness of the blood. Thus, blood can flow more easily in the arteries reducing the probability of heart attacks or strokes. Fish oil also helps to prevent plaque build-up inside the arteries. The association between fish consumption and risk of cardiovascular disease (CVD) has been extensively studied.(Virtanen *et al.*,2008; Mozaffarian *et al.*,2008; Leaf *et al.*,2008) Although the results are inconsistent, the majority of studies are in favour of the cardio protective effects of fish consumption. Dietary n3 PUFA of marine origin rich in EPA and DHA are reported to be more effective than vegetable oils in decreasing plasma

triglyceride (TG) and cholesterol concentrations. The low incidence of coronary heart disease and inflammatory disorders among Greenland Eskimos has been attributed in part to the production of eicosanoids from n-3 PUFA by platelets and blood vessel walls.(Vrablík *et al.*,2009) The beneficial effects of fish consumption on the risk of CVD are the synergistic effects of many nutrients in fish, and the integrative effects of fish consumption may reflect the interactions of nutrients in fish (He, 2009). But despite its multiple benefits to the living system most people hesitate to use fish oil for cooking, owing to its pungent taste and unpleasant aroma and hence limit their use in food preparations.

Most vegetable cooking oils are low in saturated fats and are "heart healthy" with the important exception of tropical oils, such as coconut and palm oil, which are very rich in saturated fats. Coconut oil forms a major part of the diet among the South Asians. And most of the food, and fish especially, are prepared in coconut oil to add flavour and increase over-all acceptability. But coconut oil alone has its limitations. Coconut oil is rich in saturated fatty acids (SFA) namely lauric (C12:0) and myristic (C14:0) acids (Cox *et al.*,1995; He and Fernandez, 1998). The hypercholesterolemic action of dietary saturated fatty acids is well established (Reiser *et al.*,1985; Bonanome *et al.*,1992). Various authors have proposed that the C12-C18 saturated fatty acids are capable of raising serum cholesterol and triglyceride levels in the body. Saturated fats lead to deposition of fats in the blood vessels leading to atherosclerosis. Elevated blood cholesterol is the strongest risk factor for coronary artery disease, and dietary excess of saturated fats is its largest contributor. Though coconut oil contains no cholesterol, its cholesterol-raising potential is similar to or higher than most animal fats.

In view of the partial negative role played by coconut oil, the present study was taken up with an aim to replace coconut oil partially with a better substitute such as shark liver oils with more health boosting lipid components, thereby causing less health havoc to the consumer. The fish oil could be given as a supplement in the form of an encapsulation and this could reduce the ill effects of coconut oil. The main objectives of the study were

1. To compare the impact of four isolipidemic diets, namely, diets with 10% coconut oil (CO) alone, diets with 5% *Neoharriotta raleighana* (NR) liver oil

and 5% *Centrophorus scalpratus* (CS) liver oil, and 1% polyunsaturated fatty acid concentrate (PUFA), the latter three diets made isolipidemic upto 10% with coconut oil, on the levels of serum diagnostic marker enzymes during high fat diet induced hypercholesterolemia in male Albino rats.

2. To determine the pattern of food intake and thereby calculate the feed conversion ratio of the rats fed on different isolipidemic diets.
3. To determine the impact of these diets on the body weights, liver and heart weights of the respective animals.
4. To determine the effect of high fat diets on the level of the diagnostic marker enzymes AST, ALT and LDH in the blood sera, liver and heart tissues of Albino rats.
5. To determine the cholesterol lowering effects, and thereby analyse the impact on the lipoprotein levels in blood, of liver oils of *Neoharriotta raleighana* (NR) and *Centrophorus scalpratus* (CS) on the high fat diet induced hypercholesterolemia in male Albino rats
6. To analyse the levels of triglycerides, phospholipids, free fatty acids and lipid peroxides in the blood, liver and heart tissues of the rats fed on different isolipidemic diets.
7. To evaluate the hepatic fatty acid profile of rats when fed different high fat diets and hence derive the effective n6/n3 fatty acid ratio for the respective groups.
8. To assess the levels of enzymatic and non-enzymatic antioxidant defences in the heart of the high-fat diet fed animals.
9. To conduct Pearson's correlation tests to determine the components of the fish oil responsible for the hypocholesterolemic behaviour, in male Albino rats, when fed on high energy diets.

1.2 REVIEW OF LITERATURE

1.2.1 Cardiovascular diseases

Cardiovascular diseases (CVD) refer to the class of diseases that involve the heart or the blood vessels and are usually used to refer to those that are related to atherosclerosis (arterial disease). The risk of CVD increases with age, smoking, hypercholesterolemia, diabetes, hypertension and is more common in men and those who have close relatives with ischemic heart disease- a disease characterized by reduced blood supply to the heart muscle. Ischemic heart disease and stroke are the two most common causes of death worldwide (Murray and Lopez, 1996). According to the WHO, cardiovascular disease is now the leading cause of death, accounting for 29% of all deaths in 2005 (WHO, 2005). Cardiovascular disease (CVD) causes over 4.3 million deaths in Europe and over 2.0 million deaths in the European Union (EU) (Allender *et al.*, 2008). Over 80 per cent of deaths and 85 per cent of disability from cardiovascular disease (CVD) occur in low- and middle-income countries (Reddy, 2004; Leeder *et al.*, 2004; Yusuf *et al.*, 2001). The Indian subcontinent (including India, Pakistan, Bangladesh, Sri Lanka, and Nepal) is home to 20 per cent of the world's population and may be one of the regions with the highest burden of CVD in the world. Although studies have documented that immigrants from the Indian subcontinent (South Asians) living in Western countries have a higher burden of cardiovascular disease than other ethnicities (Anand *et al.*,2000; McKeigue *et al.*,1989; Enas *et al.*, 1992), less attention has been paid to the enormous burden of CVD in the Indian subcontinent itself. The huge burden of CVD in the Indian subcontinent is the consequence of the large population and the high prevalence of CVD risk factors. Moreover, the projected increase in deaths and disability from CVD is expected to follow closely leading to an explosion in the prevalence of traditional risk factors. Driving this steep rise in CVD risk factor burden is the rapid increase in the proportion of urban inhabitants (currently at 30% with a projected rise to 43% in 2021)8. Urbanization is characterized by a marked increase in the intake of energy-dense foods, a decrease in physical activity, and a heightened level of psychosocial stress, all of which promote the development of dysglycaemia, hypertension, and dyslipidaemia (Yusuf *et al.*, 2001).

1.2.2 Diabetes mellitus:

Diabetes mellitus type 2 or type 2 diabetes (formerly called non-insulin-dependent diabetes mellitus (NIDDM), or adult-onset diabetes) is a disorder that is characterized by high blood glucose in the context of insulin resistance and relative insulin deficiency (Rother 2007). The Indian subcontinent has a higher prevalence of diabetes mellitus than any other region in the world, and 2-3 times the reported prevalence in Western countries13. In India alone, an estimated 19.3 million people had diabetes in 1995, and this is expected to almost triple to 57.2 million in 2025 (King *et al.*, 1998). The Indian Council of Medical Research (ICMR) estimates that the prevalence of diabetes is 3.8 per cent in rural areas, compared with 11.8 per cent in urban areas8. This might be a substantial underestimate, according to some preliminary cross-sectional survey data from Bangalore as part of the ongoing Prospective Urban Rural Epidemiologic (PURE) study (Vaz M, Kurpad A and Yusuf S, unpublished data).

4.2.3 Hypertension:

Hypertension is a chronic medical condition in which the blood pressure is elevated. Persistent hypertension is one of the leading risk factors for strokes, heart failure and chronic renal failure. Hypertension had been even more prevalent (20-40% among urban and 12-17% among rural adults) (Gupta, 2004), and affected an estimated 118 million inhabitants, in India in 2000; this number is projected to almost double to 214 million in 2025 (Kearney *et al.*, 2005). Similarly, the 1990-1994 National Health Survey of Pakistan revealed that one third of the Pakistani population over the age of 45 yr had hypertension (Jafar, 2006).

4.2.4 Chronic kidney disease:

Concomitant with the rise in the prevalence of diabetes and hypertension is an increase in the prevalence of chronic kidney disease, also recognized as an independent risk factor for CHD (Go *et al.*,2004). A study from Pakistan reported a prevalence of 15-20 per cent of reduced estimated glomerular filtration rate among people of 40 yr of age or older (Jafar, 2006); data from India suggest a prevalence of chronic kidney disease of 0.8-1.4 per cent in urban areas (Mani, 2005; Agarwal *et al.*, 2005), although this is likely a significant underestimate given the absence of a

kidney disease registry in India and low rates of screening for chronic kidney disease.

4.2.5 Dyslipidaemia:

Dyslipidemia is a disruption in the amount of lipids in the blood. In the ICMR surveillance project reported a prevalence of dyslipidaemia (defined as a ratio of total to HDL cholesterol >4.5) of 37.5 per cent among adults aged 15-64 yr, with an even higher prevalence of dyslipidaemia (62%) among young male industrial workers (Reddy, 2005). The INTERHEART (Yusuf *et al.*,2004) investigators reported that the prevalence of dyslipidaemia (abnormal apolipoprotein ApoB/ ApoA1 ratio) among controls without acute myocardial infarction was higher among study participants living in the five South Asian countries (45%) compared with participants from the other 47 countries represented in the study (35%)(unpublished data). As in the overall INTERHEART population, abnormal ApoB/ApoA1 ratio was the single largest contributor to the population attributable risk for acute myocardial infarction in South Asian countries. The impact of dyslipidaemia on the burden of CHD has been otherwise understudied at a population level in native South Asians, despite its large contribution to CHD in other world populations.

4.2.6 Hypercholesterolemia (literally: high blood cholesterol) is the presence of high levels of cholesterol in the blood [1]. It is not a disease but a metabolic derangement that can be secondary to many diseases and can contribute to many forms of disease, most notably cardiovascular disease. It is closely related to "hyperlipidemia" (elevated levels of lipids) and "hyperlipoproteinemia" (elevated levels of lipoproteins) (Durrington, 2003). Elevated cholesterol in the blood arises due to abnormalities in the levels of lipoproteins, the particles that carry cholesterol in the bloodstream. This may be related to diet, genetic factors (such as LDL receptor mutations in familial hypercholesterolemia) and the presence of other diseases such as diabetes and an underactive thyroid. The type of hypercholesterolemia depends on which type of particle (such as low density lipoprotein) is present in excess.[1]

High cholesterol levels are treated with diets low in cholesterol, medications, and rarely with other treatments including surgery (for particular severe subtypes). This

has also increased emphasis on other risk factors for cardiovascular disease, such as high blood pressure (Durrington, 2003).

4.2.7 Diet Induced Obesity (DIO) and CVD

Obesity (defined as a body mass index [BMI] of 30 kg/m^2)is a medical condition in which excess body fat is accumulated to an extent that it may have an adverse effect on health, leading to reduced life expectancy (WHO, 2000). The World Health Organization (WHO) predicts that overweight and obesity may soon replace more traditional public health concerns such as under nutrition and infectious diseases as the most significant cause of poor health (Loscalzo *et al.*,2008). Obesity is a risk factor for CVD, especially coronary heart disease and heart failure. Cardiovascular disease (CVD) is still the leading cause of death in developed countries, despite a recent decline in coronary artery disease-related deaths. In the United States approximately 35% of all deaths are due to CVD (WHO, 2000).It is estimated that the relative risk of coronary heart disease in obesity is approximately 1.5 even after adjusting for all other traditional coronary heart disease risk factors that often co-migrate with obesity (e.g. hyperlipidemia, hypertension) (Loscalzo *et al.*,2008). Obesity is also associated with an increased risk of heart failure, with 11–14% of all heart failure thought to be attributable to obesity. Given that excess body weight now affects more than 300 million persons worldwide, prevention and treatment of obesity should be considered one of the cornerstones for the prevention of CVD.

4.2.8 CVD and the impact of lifestyle

Cardiovascular disease (CVD) is the world's leading killer, which accounted for 16.7 million or 29.2 per cent of total global deaths in 2003. With modernization, a large proportion of Asians are trading healthy traditional diets for fatty foods, physical jobs for deskbound sloth, the relative calm of the countryside for the stressful city. Heart-attack victims are just the first wave of a swelling population of Asians with heart problems. While deaths from heart attacks have declined more than 50 per cent since the 1960s in many industrialized countries, 80 per cent of global cardiovascular diseases related deaths now occur in low and middle-income nations, which covers most countries in Asia.

4.2.9 The estimated burden of CVD in the Indian subcontinent

The absence of reliable mortality data in the Indian subcontinent has necessitated estimates of the CVD burden based on cross-sectional studies that have been well described previously (Reddy *et al.*,2005; Gupta, 2005). In 2003, the prevalence of CHD in India was estimated to be 3-4 per cent in rural areas (two-fold higher compared with 40 yr ago), and 8-10 per cent in urban areas (six-fold higher compared with that 40 years ago), with a total of 29.8 million affected (14.1 million in urban areas, and 15.7 million in rural areas) according to population-based cross-sectional surveys (Gupta, 2005; Gupta, 2004). This estimate is comparable to the figure of 31.8 million affected, derived from extrapolations of the Global Burden of Diseases study1,9,10. However, these numbers are still likely underestimates as they do not account for those with silent myocardial infarction or otherwise asymptomatic CHD. In 1990, there were an estimated 1.17 million deaths from CHD in India, and the number is expected to almost double to 2.03 million by 2010 (Ghaffar, 2004). In addition to the high rate of CHD mortality in the Indian subcontinent, CHD manifests almost 10 yr earlier on an average in this region, compared with the rest of the world (Gupta 2005, Yusuf *et al.*,2004), resulting in a substantial number of CHD deaths occurring in the working age group. In Western countries where CVD is considered a disease of the aged, 23 per cent of CVD deaths occur below the age of 70; this compared with 52 per cent of CVD deaths occurring among people under 70 yr age group in India (Gupta, 2005; Ghaffar, 2004) . As a result, the Indian subcontinent suffers from a tremendous loss of productive working years due to CVD deaths: an estimated 9.2 million productive years of life were lost in India in 2000, with an expected increase to 17.9 million years in 2030 (almost ten times the projected loss of productive life in the United States) (Leeder, 2004). The health and economic implications of this staggering rise in early CVD deaths in South Asian countries are profound and warrant prompt attention from governing bodies and policy makers of these countries.

In India in the past five decades, rates of coronary disease among urban populations have risen from 4 per cent to 11 per cent. In urban China, the death rate from coronary disease rose by 53.4 per cent from 1988 to 1996. In a report released in 2009, the Earth Institute at Columbia University warned that without sustained effort on individual and national levels, the coming heart-disease epidemic will exact

a devastating price on the region's physical and economic health. In Professor Philip Poole-Wilson's, president of the World Heart Federation words. "We're trying to warn people sufficiently early so that they can do something about it, but this isn't a disease you can cure by turning on an electric switch."

The World Health Organization (who) estimates that 60 per cent of the world's cardiac patients will be Indian by the end of 2010. Dr Timothy Gill, an Asia-Pacific specialist with the International Obesity Task Force, a medical NGO that coordinates with the WHO on obesity issues feels that of all Asians, South Asians have by far the worst problems when it comes to heart disease. Nearly 50 per cent of CVD-related deaths in India occur below the age of 70, compared with just 22 per cent in the West. This trend is particularly alarming because of its potential impact on one of Asia's fastest-growing economies. In 2000, for example, India lost more than five times as many years of economically productive life to cardiovascular disease than did the U.S., where most of those killed by heart disease were above retirement age. Studies have indicated that South Asians have elevated levels of LDL cholesterol and triglycerides, while also suffering from a deficiency in HDL cholesterol (good cholesterol, which helps clear fatty buildups from blood vessels). In addition, South Asians tend to gain weight in the abdominal region (Waist: hip ratio >1.0 in men, >0.9 in women) and are at greater risk contracting of heart diseases. Environmental factors like low birth weight, malnutrition also possibly predisposes Indians to increased risk of diabetes and heart attacks in adulthood.

Statistics suggest that South Asians seem more naturally vulnerable to heart disease than other ethnic groups. Yusuf *et al.*,(2004) study showed that, even after adjusting for all known risk factors; South Asians in Canada appeared to have a higher rate of heart disease than Europeans or Chinese living there. Some doctors think that this vulnerability can be explained by the "thrifty-gene" theory, which holds that South Asians adapted over many generations to the region's frequent famines. Now with a very recent overabundance of food, their bodies are having difficulty making a metabolic U-turn and the result is high insulin intolerance, with accompanying raised levels of diabetes and obesity.

4.2.10 Risk factors of CVD

Statistics suggest that South Asians seem more naturally vulnerable to heart disease than other ethnic groups. Lancet 2000 study showed that, even after adjusting for all known risk factors; South Asians in Canada appeared to have a higher rate of heart disease than Europeans or Chinese living there. Some doctors think that this vulnerability can be explained by the "thrifty-gene" theory, which holds that South Asians adapted over many generations to the region's frequent famines. Now with a very recent overabundance of food, their bodies are having difficulty making a metabolic U-turn and the result is high insulin intolerance, with accompanying raised levels of diabetes and obesity.

***Cigarette smoking*:**

In 2002, a national survey of tobacco use reported that the Indian subcontinent, second only to China in both the production and consumption of tobacco products, had an alarming rate of current tobacco use of 56 per cent among Indian men aged 12-60 years (Reddy *et al.*,2005). Reddy and colleagues recently observed in a survey of sixth and eighth graders attending school in an urban setting that the prevalence of tobacco use (any history of use or current use) was 2-3 times higher among sixth graders compared with eighth graders (Reddy *et al.*,2006), suggesting a concerning new wave of smoking among India's youth that forebodes serious future public health consequences for the Indian subcontinent.

***Other forms of tobacco use*:**

In addition to the high prevalence of cigarette smoking, other forms of tobacco use are common in South Asia, including reported prevalences of smoking beedies (a small amount of tobacco wrapped in a temburini leaf) of 5.9 per cent among males, and of smokeless tobacco (chewing tobacco or chewing paan) of 7.3 per cent (5.5% in women and 7.6% in men) (Teo *et al.*,2006). Little data have existed regarding the association between the use of other forms of tobacco and the risk of CVD; however, a recent analysis of data from the INTERHEART case-control study *(INTERHEART was a global study led by McMaster University's Dr. Salim Yusuf that focuses on cardiovascular disease (CVD) and co-funded by the Canadian Institutes of Health Research (CIHR), the Heart and Stroke Foundation of Ontario and 37 other funding sources.)* of risk factors for acute myocardial infarction (MI) (Yusuf *et Al.*,2004) has documented that there is an increased risk of myocardial infarction associated with all forms of smoked and smokeless tobacco (Teo *et al.*,2006). In addition, there was a graded

increase in risk associated with the number of beedies smoked per day, a finding also demonstrated for cigarette smoking. The risk of having an acute myocardial infarction associated with the use of chewing tobacco was also increased. Furthermore, the INTERHEART investigators found that 40 per cent of people who used smokeless tobacco also smoked cigarettes, and in these individuals there was a compounded risk of acute myocardial infarction associated with the use of both chewing tobacco and smoking cigarettes. Although all of these reported odds ratios are for the entire INTERHEART cohort, analysis in only the South Asian countries revealed the same degree of risk associated with all forms of tobacco use. Therefore, use of tobacco in forms other than cigarette smoking (*e.g.*, beedies) is both common and important contributors to the CHD burden in South Asia.

The 10 leading selected risk factors for death and disability, in the order of priority, by type of country as given by WHO:

High-mortality developing countries	Low-mortality Developed countries	Developed countries
1) Underweight	Alcohol consumption	Tobacco consumption
2) Unsafe sexual practices	High blood pressure	High blood pressure
3) Unsafe water, poor sanitation and poor hygiene	Tobacco consumption	Alcohol consumption
4) Indoor smoke from solid fuels	Underweight	High cholesterol level
5) Zinc deficiency	Overweight	Overweight
6) Iron deficiency	High cholesterol level	Low fruit and vegetable intake
7) Vitamin A deficiency	Low fruit and vegetable intake	Physical inactivity
8) High blood pressure	Indoor smoke from solid fuels	Illicit drug use
9) Tobacco consumption	Iron deficiency	Unsafe sexual practices

10) High cholesterol level	Unsafe water, poor sanitation and poor hygiene	Iron deficiency

Alcohol consumption:

Mild to moderate alcohol consumption has been associated with lower rates of CVD events in multiple Western-based observational studies (Fuchs *et al.*,1995; Mukamal *et al.*,2003; Stampfer *et al.*,1988). However, Joshi and colleagues in the INTERHEART South Asia study reported that consumption of alcohol was not associated with myocardial infarction in any of the South Asian countries (OR for acute myocardial infarction associated with alcohol intake 1.06, 95 per cent CI 0.85-1.30 for South Asia, compared with OR 0.79, 95 per cent CI 0.74-0.85 for the rest of the world) (unpublished data). The authors postulated that this may be due to differences in the typical pattern of alcohol consumption among inhabitants of the Indian subcontinent (consumption of relatively large quantities of alcohol on an irregular basis, *i.e.*, binge drinking) compared with other world populations (consumption of relatively small quantities of alcohol on a regular basis). However, this observation requires further corroboration and more careful study of the association between patterns of alcohol intake and CVD risk in the Indian subcontinent.

4.2.11 Markers of CVD

Nearly 95 percent of people who developed a fatal cardiovascular disease had at least one of these major risk factors: high blood cholesterol, high blood pressure, smoking, diabetes besides a poor diet and overweight. But it can also develop in the absence of any traditional risk factors and evidence is accumulating that several other risk factors may help to predict or contribute to cardiovascular disease.

Among the leading new potential culprits: C-reactive protein (CRP), Homocysteine, Fibrinogen, Lipoprotein (a). Information about how these four substances are connected to cardiovascular disease is still emerging, and researchers continue to debate their importance. Routine screening of the general public for these markers is not recommended but there may be a role for screening in people who have a strong family history of cardiovascular disease, have early onset disease with no apparent traditional risk factors, or whose disease isn't well controlled despite optimal

management of traditional risk factors. It's not clear yet what role these four substances play in predicting or causing disease and testing for these substances isn't fully standardized. There is hope that they may help lead to additional prevention and treatment strategies for cardiovascular disease.

C-reactive protein :

(CRP) is a protein produced by the liver as part of the normal immune system response to injury or infection. CRP is an inflammatory marker and inflammation has a central role in atherosclerosis the accumulation of plaques of fats, cholesterol and other material in the arteries. High levels of CRP in the blood have been associated with an increased risk of cardiovascular disease, including heart attack and stroke. But it's not clear if CRP actually causes heart disease or is just a sign of inflammation, which may cause heart disease. The AHA and the Centers for Disease Control and Prevention recommend CRP screening for an intermediate risk a 10 percent to 20 percent chance of developing coronary heart disease in the next 10 years. Low risk: Less than 1 mg/L, Average risk: 1 to 3 mg/L, High risk: Over 3 mg/L. If the CRP is greater than 10 mg/L, it's likely the result of an infection or other condition and isn't useful in assessing the cardiovascular risk and the test should be repeated in about two weeks, or after the infection is gone, to assess cardiovascular risk.

Homocysteine:

It is an amino acid normally present in the blood and is utilized by the body to make protein and to build and maintain tissue. Studies indicate a link between high plasma levels of homocysteine and an increased risk of stroke, certain types of heart disease, and peripheral vascular disease. Raised levels may be associated with four times higher risk than normal homocysteine levels. The exact mechanism of its action isn't clear and as with CRP, it's not known if homocysteine is a cause of cardiovascular disease or a marker of its presence. Recent work suggests that increased homocysteine levels may eventually cause the tissues lining arteries to thicken and scar. Cholesterol can build up in those scarred areas, providing a surface for blood clots to form. There's no consensus on what homocysteine levels are optimal, but in general, less than 12 micromoles is desirable. Readings in healthy

people can range between 5 and 15 micromoles. Elevated homocysteine levels can be decreased by dietary supplementation of folate, vitamin B.

Fibrinogen:

Although fibrinogen is needed for normal blood clotting, its excess may promote excessive clumping of platelets and can result in thrombosis in an artery, leading to a heart attack or stroke. Besides inactivity, excessive alcohol consumption and estrogens, whether from birth control pills or hormone therapy, which elevate fibrinogen, smoking is the most significant lifestyle factor that raises fibrinogen levels. The normal range for blood (serum) fibrinogen is 200 to 400 mg/dL, and levels around 400 mg/dL is associated with a twofold increase in risk of heart attack or stroke.

Lipoprotein:

It's formed when a low-density lipoprotein (LDL) cholesterol particle attaches to a specific protein. Studies show that an increased level of LDL is associated with an increased risk of cardiovascular complications, including early coronary heart disease, heart attack and stroke. Elevated LDL level, generally do not respond to most lipid lowering agents but niacin, omega-3 fatty acids or estrogen may help in some cases.

Deaths from cardiovascular diseases, principally acute myocardial infarction and cerebrovascular accidents, have decreased substantially over the past two decades, largely as a result of advances in acute care and cardiac surgery, aggressive antihypertensive therapy, the recognition of the hazards of tobacco abuse, improved nutritional patterns coupled with a decrease in cholesterol values in the general population, and an increased emphasis on physical activity.

However, these developments have produced a growing population of patients who have survived a myocardial infarction or who have a stable, if not controlled, pattern of angina pectoris due to atherosclerotic coronary artery disease. These patients, and those with peripheral vascular disease, hypertension, hyperlipidemia, diabetes mellitus, and chronic obstructive pulmonary disease, are potential participants and likely benefactors of heart smart strategies that include change in dietary habits and cardiac-rehabilitation programs. These techniques are particularly useful in the

Indian context where the semi urban and rural population is largely unaware about the importance of lifestyle techniques in prevention of cardiovascular disease.

4.2.12 Steps to lower the CVD burden in the Indian subcontinent

***Steps in policy*:**

Recognition that the vast majority of the CVD burden in the Indian subcontinent is due to environmental factors has led to the understanding that the greatest impact on the CVD burden in the Indian subcontinent will come from implementing societal changes through policy interventions (Reddy *et al.*,2005; Yusuf *et al.*,2001). However, the Indian subcontinent is home to a heterogeneous population for which a "one size fits all" policy approach to addressing the CVD burden is likely to be insufficient. Different manifestations of CVD (reflecting different stages of the epidemiologic transition of CVD4 can be observed not only between urban and rural populations, but also in between poor and wealthy households that live side by side in the same neighbourhood, making even regional CVD prevention programmes (let alone national programmes) a challenge to implement effectively. Currently, public health programmes and health systems in South Asia have been designed to address primarily communicable diseases and conditions affecting maternal and child health8; prevention and control of chronic diseases (which have been incorrectly perceived as affecting only the wealthy population that seeks health care from the private sector) have not been integrated adequately by these public programmes.

***Tobacco cessation*:**

For the prevention of chronic diseases (in particular CVD), the highest priority for the Indian subcontinent is tobacco cessation, for which notable strides have already been achieved. The Indian government's enactment of the Tobacco Control Act in 2003 has mandated bans on smoking in public places, on tobacco advertising and tobacco use in the film industry, and on tobacco sales to minors or near educational institutions. India has also adopted the World Health Organization's Framework Convention on Tobacco Control. However, lack of a national regulatory authority for tobacco control hinders enforcement of these policies8. Greater concentration of tobacco cessation efforts must be applied to those groups in whom tobacco use rate trends are particularly disturbing, including the youth (Reddy *et al.*,2006), and rural

populations in whom the use of smokeless tobacco and use of tobacco among women are highly prevalent (Teo *et al.*,2006). Moreover, innovative means are required to address the even greater challenge of controlling the use of other forms of tobacco (beedies, chewing tobacco, and paan). Finally, in addition to the challenges in India, similar tobacco cessation policies and efforts in Pakistan, Nepal, Sri Lanka, and Bangladesh are lacking.

Lifestyle modification:

Structured programmes to promote healthy dietary patterns and physical activity must be developed to combat the deleterious effects of urbanization. Policy efforts are needed to (i) reduce carbohydrate intake on a population basis to lower rates of abdominal obesity and atherogenic dyslipidaemia (high triglycerides and low HDL cholesterol), (ii) reduce the sodium content of packaged foods (e.g., jarred pickle or snacks) to lower the prevalence of hypertension, and (iii) increase regular consumption of fresh fruits and vegetables to lower CHD risk. In addition, cultural norms that hinder the adoption of healthy lifestyles should be appreciated so that novel approaches to encourage healthy lifestyles can be developed and implemented in a culturally sensitive manner (*e.g.*, engaging and educating the leaders of the community to encourage leisure-time physical activity among urban men and women).

Cardiac rehabilitation:

Cardiac rehabilitation is a medically supervised exercise and counseling program designed to help overcome some of the physical complications of heart disease, limit the risk of developing additional heart trouble, help a person return to an active social or work schedule, and improve the psychological well-being. It has four main components: Medical evaluation, supervised exercise, lifestyle education and psychosocial support. Cardiac rehabilitation takes time at least six months and it's not always easy. It's also not suited for everyone with a heart problem, and the results may vary for reasons beyond the participant's control.

But for most people in cardiac rehab, the hard work put into it offers many rewards. Participation maximizes their ability to regain independence and provides the knowledge to ensure that healthy living will become a permanent part of their future.

Five heart-smart strategies directed towards healthy dietary habits.

1. Limiting intake of unhealthy fats and cholesterol

The best way to cut saturated and trans fat intake is to limit the amount of solid fat like butter, margarine that is added to food when cooking and serving. If fat is to be used, choose oils high in monounsaturated fat, such as olive oil or canola oil. Avoid butter, lard, bacon, gravy, cream sauce, nondairy creamers, hydrogenated margarine, cocoa butter found in chocolate, coconut, palm and palm kernel oils.

Use of monounsaturated fats lower the total cholesterol and low-density lipoprotein (LDL) cholesterol (the "bad" cholesterol).

2. Choosing low-fat protein sources

Although meat, poultry and fish along with dairy products and eggs are some of the best sources of protein, they are high in total fat, saturated fat and cholesterol. Skim milk rather than whole milk or skinless chicken breast rather than fried chicken patties are lower fat versions and may be substituted for the above. Fish is another good alternative to high-fat meats. Some types of fish such as cod, tuna have less total fat, saturated fat and cholesterol than do meat and poultry whereas certain types of fish like salmon, mackerel and herring are heart healthy because they're rich in omega-3 fatty acids. These fats may help lower triglyceride levels and may reduce the risk of sudden cardiac death.

Legumes like beans, peas and lentils are good sources of protein and contain less fat and no cholesterol, making them good substitutes for meat. Soybeans may be especially beneficial to the heart and may be regularly substituted for animal protein.

3. Eating more fruits and vegetables

Fruits and vegetables are low in calories, good sources of vitamins and minerals, and rich in dietary fiber. They also contain phytochemicals, substances found in plants that may help prevent cardiovascular disease. Eating more fruits and vegetables helps us indirectly also by satisfying hunger and thereby reducing intake of high-fat foods. Don't smother vegetables in butter, dressings, creamy sauces or other high-fat garnishes. Avoid fruits in cream or heavy sauces.

4. Selecting whole grains compared to polished ones

Whole grains do not have their bran and germ removed by milling, making them good sources of fiber, which the body can't digest besides other nutrients. A diet high in fiber can help lower blood cholesterol levels and reduce the risk of heart disease. Whole grains are also important sources of vitamins and minerals, such as thiamin, riboflavin, niacin, folate, selenium, zinc and iron. Doughnuts, biscuits, cakes, Buttered popcorn and high-fat snack crackers should be avoided.

5. Practice moderation and balance

Knowing which foods to eat is the first step in creating a heart-healthy diet. The next step is to know how much food to consume. Overloading can lead to excess calorie, fat and cholesterol intake. Keep track of the number of servings you eat - and use proper serving sizes - to help control how much food you eat.

A serving size is a specific amount of food, defined by common measurements such as cups, ounces or pieces. For example, the Food Guide Pyramid developed by the Department of Agriculture and Department of Health and Human Services suggests that one serving of pasta is 1/2 cup, or about the size of an ice cream scoop. A serving of meat, fish or chicken is 2 to 3 ounces or about the size and thickness of a deck of cards. Judging serving size is a learned skill. It is recommended to use measuring cups and spoons and a scale until you are comfortable with your judgement.

4.2.13 Fish Oil and Cholesterol

Fish oil supplements containing omega 3 fatty acids have been promoted on a commercial scale as being effective at lowering cholesterol levels (Padma and Devi, 2000). Fish oil is not only a good hypocholesterolemic agent but also a powerful anti-inflammatory, so it can help reduce the risk from the C-reactive protein. Second, the omega 3 fatty acids help prevent clumping and stickiness of the blood. Thus, blood can flow more easily in the arteries reducing the probability of heart attacks or strokes. Another important property of fish oil is that it helps prevent plaque build-up inside the arteries. Again, this is important, considering that what actually causes heart attacks, strokes and heart disease in general is this formation of plaques.

Fats of deep sea fishes are rich in ether lipids (Mathen *et al.*,2008), hydrocarbons like squalene and antioxidant vitamins besides containing the n3 polyunsaturated

fatty acids. All of these lipid bioactives serve as lipid lowering agents owing to their ability to retard the synthesis and limit the intake of the low density lipoprotein cholesterol during lipid metabolism in the body.

4.2.14 Fish oil and Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disorder that is characterized by progressive memory loss, intellectual decline, and eventually global cognitive impairment. Major symptoms include short- and long-term memory loss, impairments of language and speech, decline of abstract reasoning, and visual-spatial perceptual changes. The incidence of dementia in Western countries, of which AD is the leading cause, is estimated to be approximately 10% of the population over the age of 65 y and 47% of the population over 80 years of age (Julie *et al.*,2000).

Researchers reported that the omega-3 fatty acid docosahexaenoic acid (DHA) found in fish oil increases the production of LR11, a protein that is found at reduced levels in Alzheimer's patients and which is known to destroy the protein that forms the 'plaques' linked to the disease. The plaques are deposits of a protein called beta amyloid that is thought to be toxic to neurons in the brain, leading to Alzheimer's. Since having high levels of LR11 prevents the toxic plaques from being made, low levels in patients are believed to be a factor in causing the disease.

4.2.15 Fish oils and epilepsy & strokes

It has been reported by a team of scientists from France's National Research Centre that by acting on a protein called Trek-1, a known fatty acid target, the fats in deep sea fishes open a potassium channel, allowing potassium ions to cross cell membranes. The findings help to explain and support a number of studies that have demonstrated the positive impact of omega-3 fatty acids on brain health. Polyunsaturated fatty acids have a protective effect against certain heart diseases and are beginning to be recommended by healthcare professionals for heart health. But the new findings mean that fish oil manufacturers could have additional benefits to promote or even a future health claim.

In 2000, Michel Lazdunski and his team demonstrated that PUFAs like alpha-linoleic acid or docosahexanoic acid, found mainly in deep sea fish oils, reduced the damage to neurones caused by an ischaemic stroke or during an epilepsy attack.

More recently it has been discovered that this effect may be attributed to the action of PUFAs on the Trek-1 protein. The workers reported that mice in which the gene coded for Trek-1 had been removed were much more likely to have strokes and epilepsy attacks than normal mice. They also failed to respond to the protective effect of fatty acids on the brain, and died from minor strokes that normal mice would survive.

Strokes affect more than 1 million people each year in the European Union and are the second biggest cause of death in the world after heart disease. They are also a source of major disability for patients that survive them, causing paralysis of one side, depression or secondary epilepsy. The findings could also help researchers to develop new treatments for these illnesses. At least 20 per cent of epilepsy patients (around 2 per cent of the population) are resistant to current medication.

4.2.16 Fish oil and Atherosclerosis

Evidence from several lines of investigation supports the hypothesis that fish consumption may protect against atherosclerotic vascular disease. These studies support the early observations that Greenland Eskimos, who have a high fish and arctic mammal consumption, exhibit a very low incidence of death from ischemic heart disease (Bang *et al.*,1976; Dyeberg *et al.*,1975). The mechanism by which n-3 long-chain polyunsaturated fatty acids (PUFA), mainly eicosapentaenoic (EPA, 20:5) and docosahexaenoic (DHA, 22:6) acids, may exert their effects is not fully understood. It has been proposed that some of these effects occur through plasma lipids (Parks and Rudel, 1990). It has been well established that n-3 fatty acids reduce the level of plasma triacylglycerol and very low density lipoprotein (VLDL).

The effect of fish oil on plasma cholesterol is more uncertain (Harris, 1989), although this effect varies remarkably among different animal species (Spady *et al.*,1995). The n-3 fatty acids which are present in fish oil also have several biological effects that could be antiatherogenic, including a reduction of arachidonic acid (AA) in leukocytes and platelets with subsequent decreases in thromboxane A2 and leukotriene production (Lee *et al.*,1985; Lorenz *et al* ,1983) , an inhibition of platelet aggregation (Leaf, 1988), and a decrease of blood pressure (Morris *et al.*,1993). EPA is believed to be one of the major active fatty acids having hypolipidemic action (Mizuguchi[a] *et*

al.,1993). Ethyl-ester derivatives of EPA have been demonstrated effective in lowering serum or plasma lipids in rats (Mizuguchi[b] *et al.*,1993). Castillo *et al.*, (1999) reported a drastic inhibition of 3-hydroxy-3- methylglutaryl-CoA (HMG-CoA) reductase activity from neonatal chick liver after 1 week of diet supplementation with 10% menhaden oil.

4.2.17 Anti-arrhythmic properties of n-3 fatty acids are another area of interest associated with CVD. These effect may be direct consequences of the incorporation of n-3 fatty acids, especially DHA and EPA, into cell membranes (Morrand *et al.*,1988). Both EPA and DHA are readily incorporated into cell membranes following supplementation (Wilkinson *et al.*,2005). The membrane enrichment with EPA/DHA may result in increasing membrane fluidity in cardiac cells, thereby preventing atrial fibrillation (Schwalfenberg, 2006; Rosenberg, 2002) and reducing the binding of inflammatory cytokines to their receptors (Ergas *et al.*,2002). This may explain benefits of EPA/DHA in preventing cardiac events. Consuming 3 g/day encapsulated fish oil for 6 weeks reduced inducible ventricular tachycardia and risk of sudden cardiac death among patients with coronary artery disease (Metcalf *et al.*,2008). This could be the result of an attenuation in intracellular calcium and in the response to noradrenalin (Den Ruijter, 2008). Moreover, habitual consumption of fish and marine n-3 fatty acids was linked with particular heart rate variability constituents, including indexes of vagal activity, baroreceptor responses, and sinoatrial node function among American adults (Mozaffarian, 2008). Such enhancement of vagal control by tuna and other fish consumption could explain, in part, improved endothelial function and reduced resting heart rate after fish oil supplements in a randomized study in healthy men and women (Shah *et al.*, 2007).

4.2.18 Hypocholesterolemic effects of fish oils

Over the last eight decades, the efficacy of polyunsaturated marine oils in lowering serum cholesterol level in experimental animals and man has been established and has been reviewed by Peifer (1960). Hypocholesterolemic effect of fish in the diet of rats and human beings has been reported by Kinsell *et al.*.(1958); Bronte-Stewart *et al.*,(1956); Sen (1977), Hsu *et al.*. (2001). The hypocholesterolemic effect of oil-

sardine (*Sardinella longiceps*) fish and its oil in cholesterol bile salt stressed rats had been reported. It has been suggested that the cholesterol-depressant activities of marine oils and their fatty acid fractions are closely related to their high total unsaturation which results from their contents of longer chain linolenate homologues (Sen, 1977). Kahn (1964) have reported that a concentrate of cod liver oil with an iodine value (IV) of 375 was more effective than a concentrate with IV 315 in alleviating the hypocholesterolemia in the chicken.

4.2.19 The paradox behind the cholesterol-lowering effects of n-3 fatty acids

Several cases on decline in cholesterol levels upon feeding fish oil on high fat diet induced hypercholesterolemia have been studied, but the results are controversial. Contacos and colleagues (1993) observed a 1 nm increase in the diameter of low-density lipoprotein (LDL) particles after consumption of 3 g/day fish oil for 6 weeks. Kelley and associates (2007) also observed a significant 21% reduction in the number of small, dense LDL particles and a 0.6 nm increase in LDL particle size in men with hypertriglyceridemia receiving 3 g/day DHA. This may explain, in part, the increased LDL cholesterol levels observed in some clinical trials (Sanders *et al.*,2006; Normen *et al.*,2004). An increase in apolipoprotein B concentrations following n-3 fatty acid supplementation has been observed in healthy elderly subjects (Goyens and Mensink, 2006). n-3 fatty acid intake was also associated with small increases in high-density lipoprotein cholesterol concentrations in healthy volunteers and patients with familial hyperlipidemia (Breslow, 2006; Calabresi *et al.*,2004; Engler *et al.*,2004; Ferrier *et al.*,1995). Overall, benefits of fish oil on LDL and/or high-density lipoprotein cholesterol metabolism appear inconsistent, whereas influences of particle size seem to be key. Ikeda et al. (1998) reported a decrease in triglyceride and cholesterol concentrations in plasma, on arachidonic acid content in various tissue phospholipids and on thromboxane (TX) A2 production in platelets, when hypercholesterolemic rats were fed seal oil-rich diet for 160 days. n-3 fatty acids, EPA and DHA inhibit Δ6 desaturation of linoleic acid and hence reduce arachidonic acid content in tissue phospholipids (Hornstra, 1989). A reduction in the control of arachidonic acid (AA;20:4n-6) in tissues or cells has been shown to be beneficial in several animal disease models (Wan, 1989; Kinsella *et al.*, 1990; Kinsella and Lokesh, 1990). A popular approach to achieve a reduction of AA

involves a dietary-induced exchange of AA with other C20-22 polyunsaturated fatty acids (PUFAs), most notably the n-3 fatty acids, eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA: 22:6 n-3), both of which are abundant in marine fish oils.

However, most of the studies using fish oil or pure omega-3 fatty acids supplementation have failed to show any effect on CRP levels (Giugliano *et al.*,2006), unless the fish oil supplement was given at a high dose. Although some epidemiologic studies have shown an inverse correlation between dietary intake of fish or fish oil and circulating markers of inflammation, clinical trials have not yet confirmed these effects. Moreover, diets rich in n-3 polyunsaturated fatty acids (PUFA) compared to n-6 PUFA, monounsaturated, or saturated fatty acids have reported to enhance lipid peroxidation product levels in serum, liver, kidney, and colon (Kaur *et al.*,1997; Guimaraes and Curi,1991; Hu *et al.*,1999; Turini *et al.*,1999). On the other hand, ALA appears to have anti-inflammatory potential, and future studies should focus on this.

4.2.20 Hypercholesterolemia in animal models

Albino rats have been considered as suitable animal models for studies on the comparative biochemistry of lipid metabolism and transport especially cholesterol because it is highly sensitive to dietary cholesterol (Chandler, 1979). In an experimental study analysing the metabolism of lipids in Albino rats, cholesterol content in the rat plasma and liver sharply decreases during the first days after hatching (Aguilera JA, 1984; Castillo M, 1992). The results also showed that neonatal chick provided a suitable model to study the role of VLDL in atherogenesis and the rapid response to saturated fatty acids with 12–14 carbons (Castillo *et al.*,1996). On the other hand, another study showed that supplementation of 10% menhaden oil to the chick diet for 7 days produced a significant hypocholesterolemia and hypotriglyceridemia (Castillo, 1999). All chemical constituents of VLDL decreased by the same dietary manipulation. Because of these considerations, the workers compared the effects of diet supplementation with n-3 PUFA (menhaden oil) on plasma and lipoprotein composition from control and hypercholesterolemic chicks. Given that several biological effects of n-3 PUFA could be mediated by changes in AA, the influence of menhaden oil on fatty acid composition of chick

plasma and lipoprotein fractions were also studied. The dyslipidemia or hypercholesterolemia induced by coconut oil feeding during the first two weeks of neonatal chick life was reverted by menhaden oil supplementation to the diet. This treatment produced drastic reductions of cholesterol levels in VLDL as well as in low and high density lipoprotein (LDL and HDL) fractions. Triacylglycerol levels also were significantly reduced in plasma and VLDL. Arachidonic and linoleic acids drastically decreased by the same dietary manipulation in plasma and lipoprotein fractions.

Extensive literature is available to highlight the beneficial effects of fish oil in the reduction of inflammation in rheumatic disease, reduction of atherosclerosis, and modification of hormone-dependent tumor growth (Berry, 1986; Phillipson, 1985, Hornstra, 1985, Harris, 1983). A possible mechanism for health benefits of fish oil includes increased dietary consumption of the PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Such enrichment has been associated with reduction in proinflammatory prostanoids or cytokines in circulating mononuclear cells (Adam *et al.*,1986), particularly tumor necrosis factor α and interleukin-1β (Harris *et al.*,1983).

4.2.21 Dietary lipids and bodyweight

The type of dietary fat had no significant effect on body weight gain of rats, but significantly ($p<0.009$) affected feed intake. Rats fed diets containing coconut oil had higher average feed intake (21.86 g/day) than rats fed diets containing menhaden or olive oil, 20.76, 20.41 g/day, respectively. However, studies by Mohammed *et al.* (2002) showed that vitamin E levels had no significant effect on either body weight gain or feed intake in rats.

4.2.22 Dietary substitution of rat feed with vegetarian sources

There are reports which show that partial dietary substitution of ground flaxseed into standard rat chow to a level of 10% by weight of diet, reduced renal cystic, inflammatory, and fibrotic changes in the Han:SPRD-cy rat (Hwang *et al.*, 1988; Marshall and Johnston, 1982). The disease is characterized by epithelial proliferation and progressive dilatation of nephrons, marked interstitial inflammation and fibrosis. Both soy protein and flaxseed based diets are associated with amelioration of this disease (Marshall and Johnston, 1982; Ishihara *et al.*,1995). In both instances, this

was associated with the alteration of the renal and hepatic PUFA proportion, with evidence of a reduced conversion of linoleic acid (LA) to arachidonic acid (AA) for animals on a soy diet and an increase of n-3 FA with flaxseed. A pure animal protein-based diet is associated with more rapid disease progression than that seen with mixed vegetable and animal protein-based rat chows (Marshall and Johnston, 1982; Ishihara *et al.*,1995).

Many studies have examined the effects of dietary lipids on plasma lipoproteins and other studies have demonstrated that dietary cholesterol and triglyceride intake can also influence the plasma/serum lipid composition. The liver, of course, plays a critical role in determining how diet will ultimately affect lipid concentrations in either plasma or bile. In a study conducted by Booker *et al.* (1997), to understand the effects of dietary cholesterol and triglycerides on lipid concentrations in liver, plasma and bile it was observed that supplementation with dietary cholesterol resulted in increases in the cholesterol levels in liver, plasma, and bile and the type of dietary oil had a more profound effect on triglyceride levels than on cholesterol. His findings implied that although it was possible to increase hepatic free cholesterol above normal levels if the input of cholesterol to the liver was high enough, there is a limit above which excess free cholesterol would either be excreted or converted to a storage form. The concentrations of plasma cholesterol became elevated only in the presence of severe increases in hepatic cholesterol, suggesting that the primary response to an increased input of cholesterol into the hepatic compartment was an augmented biliary cholesterol secretion.

4.2.23 Lipid metabolism during dietary substitution of rat feed with fish oil sources

Several workers have reported the benefits of supplementing rat feeds with fish oil. This supplementation resulted in the lowest levels of plasma triglycerides (TG), similar to previous studies reporting a hypotriglyceridemic effect of marine n-3 polyunsaturated fats (Lee *et al.*,1992; Sawazaki *et al.*,1989, Al-Shurbhaji *et al.*,1991). Some controversy exists as to the mechanism by which this reduction in TG occurs. Several reports indicate that fish oil and n-3 polyunsaturated fatty acids reduce hepatic TG synthesis (Parks *et al.*,1990), while other evidence suggests that n-3 oils reduce plasma TG by inhibiting hepatic secretion (Gloria Lena *et al.*,2008; Booker *et al.*,2006). The study suggested that fish oil decreased the hepatic TG secretion,

since the 40% menhaden oil diet resulted in low plasma TG concentrations in the presence of high levels of hepatic TG. However, as TG synthesis was not measured directly in this study, the possibility exists that both synthesis and secretion were reduced, with the change in synthesis masked by the accumulation of TG which would likely occur as the result of the inhibition of secretion. Recent studies have indicated that dietary cholesterol can induce hypertriglyceridemia in the rat *via* an increased synthesis of fatty acid and secretion of TG by the liver (Basciano *et al.*,2009).

Alteration of the fatty acid profile of dietary fat has been shown to affect several metabolic processes such as those involving plasma lipids and lipoproteins (Berry *et al.*,1986; Phillipson *et al.*,1985). In fish oil, the long-chain n-3 polyunsaturated fatty acids, eicosapentaenoicacid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are active in lowering plasma lipid levels. Fish oils (e.g., salmon oil) affect platelet function by reducing platelet aggregation and adhesion (Hornstra, 1989), plasma lipids by reducing the plasma concentrations of very low density lipoprotein(VLDL), and triacylglycerols (TG) (Harris *et al.*,1983). As for plant oil, there are several reports on the effects of various n-6/n-3 ratios of dietary fatty acids on lipid metabolism (Adam *et al.*,1986; Swansen and Kinsella, 1986; Hwang *et al.*,1988; Marshall and Johnston, 1982; Takahashi, 1987). There are also several reports on the effects of animal oils on lipid metabolism (Herzberg and Rogerson, 1988). Herzberg and Rogerson (1988) reported that a tallow diet with fructose reduced serum cholesterol more than a corn oil diet with fructose. Fish oils have hypocholesterolemic activity compared with animal and plant oils (Bouziane *et al.*, 1944; Ishihara *et al.*,1995; Lee *et al.*,1992; Sawazaki *et al.*,1989). It has also been reported that administration of fish oil by intravenous_infusion resulted in a marked reduction of serum cholesterol, HMG–CoA reductase activity, and cholesterol 7α hydroxylase activity compared to intravenous infusion of_soybean oil (AL-Shurbaji *et al.*,1991).

Administration of fish oil to human has been recognized as a preventive or therapeutic approach for treatment of cardiovascular disease based on the ability of fish oil to reduce both the thrombotic risk (by modifying the metabolism of prostaglandins) and the level of circulating atherogenic lipids. Vijayapadma and Shyamala Devi (2000) reported a reduced rate of restenosis after coronary

angioplasty in humans. In humans, diets with a high fish oil content modified several risk factors of atherosclerosis. Dietary fish oil supplementation has favourable effects on hyperlipidemia and lowers blood pressure in hypertensive patients. A reduced infarct size after coronary occlusion in a rat model had also been reported.

4.2.24 Metabolism of fatty acids in the body

Consumption of marine oils rich in eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids decreases plasma levels of cholesterol and triacylglycerols in healthy (Nestel *et al.*,1984; Harris *et al.*,1983; Yamazaki *et al.*,1987) and hyperlipidemic subjects (Nestel 1986; Phillipson *et al.*,1985; Simons *et al.*,1985). While the hypotriglyceridemic effect of fish oil exceeds the capacity of most available drugs, the hypocholesterolemic effects are still doubtful except when the excess cholesterol is present in very low density lipoprotein (Nestel 1986; Phillipson *et al.*,1985). Consumption of fish oil also lowers serum levels of thromboxane (TxA2) and thus has anti-aggregatory effects on the manifestation of thrombosis (Dyerberg and Jorgensen, 1982; Herold and Kinsella, 1986; Bruckner *et al.*,1984). While the exact mechanisms for the fish oil-induced decrease in plasma lipid levels are not clear, the platelet anti-aggregatory effect is most likely mediated via changes in the balance of eicosanoids formed from their common precursor, arachidonic acid (20:4n-6) (7-9). Arachidonic acid is usually present in the SN-2 position of membrane phospholipids (Yarnashita *et al.*,1973). Eicosapentaenoic acid present in fish oil competes with 20:4n-6 for incorporation into phospholipid (Iritani and Fujikawa, 1982; Iritani and Narita, 1984) and subsequently competes with 20:4n-6 at the level of cyclo-oxygenase (Culp *et al.*,1979) in the formation of eicosanoids such as prostacyclin, prostaglandins, thromboxane and leukotrienes. In a recent study we demonstrated that fish oil reduces synthesis of 20:4n-6 by inhibiting the Ae-desaturase enzyme (Garg *et al.*,1988), a rate-limiting enzyme in the 20: 4n-6 biosynthetic pathway (Holloway *et al.*,, 1963; Stoffel, 1961).

Many reports have demonstrated that dietary n-3 fatty acids, particularly those of fish oils (20:5n-3 and or 22:6n-3), and cholesterol enrichment of the diet independently alter metabolism of essential fatty acids (Iritani and Fujikawa, 1982; Iritani and Narita, 1984; Culp *et al.*,1979; Garg *et al.*,1988). Fish oil or cholesterol

supplementation decreases 20:4n-6 content of plasma and tissue phospholipids and in turn alters the pattern of eicosanoids synthesized from 20:4n-6 (Culp *et al.*,1979). Feeding the diets with fish oil to rats for a 4-week period lowered plasma cholesterol level by 13% and 33%, respectively, compared with animals fed the beef tallow diet. These results are in agreement with several previous reports that dietary n-3 fatty acids decrease blood cholesterol levels in normal animals and human subjects (Harris *et al.*,1983; Nestel 1986; Phillipson *et al.*,1985; Simons *et al.*,1985). Some authors have reported that fish oil may increase serum cholesterol levels in man (Nestel *et al.*,1984). Because the rat is a species that has a very high density lipoprotein and low levels of low density lipoprotein under normal dietary conditions, the changes in the plasma cholesterol level may be related to disturbances in lipid transport and lipoprotein metabolism (Nestel, 1986). Feeding the fish oil diet also provided protection against cholesterol accumulation in plasma that was apparent when 2% (w/w) cholesterol was fed with diets containing beef tallow or linseed oil. These results suggest that fish oil consumption may have beneficial effects against hypercholesterolemia even under a high dietary load of cholesterol. In this regard, feeding the fish oil diet lowered cholesterol content of liver tissue, while feeding the coconut oil diet had no effect.

Following feeding of low-cholesterol diets, rats have been shown to accumulate more liver cholesterol when coconut oil is the fat source (Villarino *et al.*,1998; Ebihara *et al.*,2006). The present study demonstrates that when a high fat diet is fed, fatty acids of coconut oil affect the concentration of liver cholesterol, whereas the n-3 fatty acids of fish oil {primarily 20:5n-3 and 22:6n-3) reduce their levels.

Stangl *et al.*, (1993) showed that fish oil diets lowered MUFA level in rat liver in spite of its high MUFA content when male Sprague-Dawley rats were maintained on a high-fat hyperlipidemic diet supplemented with 7.3% coconut oil, 7.3% beef tallow, 0.4% safflower oil, and 1.5% cholesterol for 28 days and then switched to fish oil diets with 1.4%, 2.8%, and 5.6% fish oil in exchange for coconut oil and beef tallow for, respectively, 10 and 20 days. Rats fed the highly saturated hyperlipidemic diet low in 18:2 n-6 had reduced 18:2 n-6 levels in liver. Also, 20:4 n-6 level in liver was markedly reduced after the administration of the hyperlipidemic diet or the fish

oil diets. Moreover feeding fish oil increased all long-chain n-3 polyunsaturated fatty acids (PUFA) in liver.

4.2.25 Foods that lower cholesterol

Results have shown that people whose diets contained the most whole grains like oatmeal and oatbran had the thinnest carotid artery walls and showed the slowest progression in artery wall thickness. Oatmeal contains soluble fiber, which reduces low-density lipoprotein (LDL), the "bad" cholesterol. Soluble fiber is also found in such foods as kidney beans, apples, pears, psyllium, barley and prunes. Soluble fiber appears to reduce the absorption of cholesterol in the intestines. Ten grams or more of soluble fiber a day decreases the total and LDL cholesterol.

Blueberries contain a compound called pterostilbene that may help lower cholesterol as effectively as commercial drugs with fewer side effects. Studies have shown that eating pistachios, walnuts, almonds etc. significantly lowered LDL (bad) cholesterol levels. Research published in the Journal of the American College of Cardiology showed eating walnuts after a high-fat meal might protect the heart. Omega-3 fats and antioxidants in nuts work to reverse the arterial damage caused by saturated fats.

Research has supported the cholesterol-lowering benefits of eating fatty fish like mackerel, lake trout, herring, sardines, albacore tuna, salmon etc. because they contain high levels of omega-3 fatty acids. Omega-3 fatty acids also help the heart in other ways such as reducing blood pressure and the risk of blood clots. In people who have already had heart attacks, fish oil or omega-3 fatty acids significantly reduce the risk of sudden death. A study from the Norwegian University of Science and Technology found that people with type 2 diabetes who consumed high doses of fish oil over nine weeks lowered the size and concentration of several lipoprotein subclasses (cholesterol) in their bodies.

The good fats in avocados, olives, and olive oil protect against heart disease and diabetes. Flaxseed oil can lower blood pressure in men with high cholesterol. In a three-month study of 59 middle-aged men, those who took daily flaxseed oil supplements (with eight grams of the omega-3 fats, alpha-linoleic acid) experienced significantly lower systolic and diastolic blood pressure. Study from the Journal of the Science of Food and Agriculture shows that black soybeans may help prevent

obesity, lower LDL cholesterol, and reduce the risk for type 2 diabetes. A National Academy of Sciences study showed that pomegranate juice reduces cholesterol plaque buildup and increases nitric oxide production (nitric oxide helps reduce arterial plaque).

Studies conducted by Vicki Koenig, MS, RD, CDN have shown that the probiotics *Lactobacillus Acidophilus* and *Lactobacillus Reuteri* actually help lower cholesterol by preventing the reabsorption of cholesterol back in to the blood stream."

4.2.26 Atherosclerosis and hypercholesterolemia

Atherosclerosis can be defined as the development of abnormal fat deposits in the arterial wall. It is a pathologic condition that leads to several disorders including coronary artery disease, cerebrovascular disease and diseases of the aorta and peripheral arterial circulation. The process of atherogenesis is not a degenerative one and it begins as intimal lipid deposits. This is an active process, which involves the elements of chronic inflammation associated with those of repair in the arterial wall. The formation of atherosclerotic lesion is in part mediated by inflammatory and oxidative mechanisms including lipid peroxidation (Watanabe *et al.*,2001). Epidemiologic research has identified risk factors that increase the likelyhood of coronary heart disease events. Hypercholesterolemia is one of the major risk factors of atherosclerosis. Numerous prospective epidemiologic studies have identified a continuous graded, direct relationship between serum total cholesterol level and coronary heart disease incidence (Stamler *et al.*,1986). Data indicate that LDLC contributes predominantly to the relationship between total cholesterol and coronary heart disease (Lipid Research Clinic Program [a,b], Blankenhorn *et al.*,1987; Brown and Albers, 1990; Buchwald *et al.*,1990). LDL enters the normal arterial wall interstitium and accumulates in amounts that are regulated by the endothelium and likely the internal elastic lamina (Nikkila *et al.*,1978). Recent studies have suggested that oxidative modification of LDL play a crucial role in the process of atherogenesis. Oxidized LDL and certain of its other modified forms are ligands for the 'scavenger receptors' on macrophages and can therefore convert them to the cholesterol loaded foam cells characteristic of the fatty streaks (Witzhum, 1994; Duthie *et al.*, 1989). High level of plasma LDL proportionately increases the rate of LDL entry (Carroll and Khor, 1971) into the intima. If it is oxidized at some ongoing rate, the concentration of

oxidized LDL in the intima also increases. Once LDL is oxidized, injury to endothelium may lead to local increase in epithelial cell turnover and enhanced entry of lipoproteins. The development of the fatty streak and the progression of the fatty streak to the advanced lesions of atherosclerosis have been observed in a large number of experimental animals (Rosenfeld[a] *et al.*, 1987; Rosenfeld[b] *et al.*, 1987; Gerrity *et al.*, 1979; Faggioto and Ross, 1984; Masuda and Ross, 1990; Nakashima *et al.*,1994)

4.2.27 Hypercholesterolemia and the antioxidant defense

Hypercholesterolemia which is a major contributing factor for atherosclerosis, leads to lipid peroxidation (Das *et al.*,1997)which might be due to the release of excess oxygen free radicals (Prasad and Kalra, 1993). Hypercholesterolemic subjects have been reported to have a high level of circulating lipid peroxides (Mehmetcik *et al.*,1997). Defense mechanisms against oxidative damage include sequestration of reactive species by the antioxidants. Antioxidants can probably act as antiatherogens by preventing or suppressing the prooxidant states (Steinberg *et al.*,1989; Carew *et al.*,1987). Ascorbic acid and α or β tocopherol have been reported to be beneficial in prevention of up regulation of vascular endothelial growth factor and vascular endothelial growth factor receptor-2 in hypercholesterolemia induced experimental atherosclerosis (Rodriguez *et al.*,2005). Ascorbic acid is a water soluble antioxidant which is necessary for the conversion of cholesterol to bile acids. It is also required for the biosynthesis of collagen. In absence of ascorbate, collagen formed is insufficiently hydroxylated and does not form proper fibers, resulting in fragile blood vessels (Stocker and Keany, 2004). Previous experiments (Das *et al.*,1997) have demonstrated that a high dose of ascorbic acid significantly reduces the level of lipid peroxidation in hypercholesterolemic rabbits.

4.2.28 High energy diets

The composition of a high-energy diet is of outmost importance for the induction of a specific disease phenotype. Diverse 'high energy diets' with increased caloric content, have been used to induce obesity and related diseases in rodents and other animals (Reuter, 2007). Usually the fat and/or the carbohydrate source are enriched or modified, the cholesterol content can be increased or condensed milk may be

added. It is advantageous to use a purified standard diet as basis for a high-energy diet instead of chow diets, because chows are made from plant material and may contain phytoestrogens. Those can exert estrogenic or anti-estrogenic activities and thus affect the progression of metabolic disease states (Lephart, 2004). Several studies have indicated that diets rich in SFA increase the plasma cholesterol concentration, whereas diets rich in polyunsaturated fatty acids lower it (Norum, 1992; Sherperd *et al.*,1980).

4.2.29 Coconut oil induced hypercholesterolemia

Coconut oil is rich in saturated fatty acids (SFA) namely lauric (C12:0) and myristic (C14:0) acids (Ebihara *et al.*, 2006). The hypercholesterolemic action of dietary saturated fatty acids is well established (Grundy and Denke, 1990). More than thirty years ago, various authors (Ahrens *et al.*,1957; Hegsted *et al.*,1965; Keys *et al.*,1965) proposed that the C12-Cl6 saturated fatty acids were equally capable of raising serum cholesterol. By subsequent experiments McGandy *et al.* (McGandy *et al.*,1970) included 18:0 among the "saturated pool" with respect to their effects on serum cholesterol. Later reports indicated that cholesterol levels in low density lipoproteins (LDL) are raised by dietary saturated fatty acids (Mattson and Grundy, 1985; Grundy and Vega, 1988).

While this generalization was originally considered applicable to all saturated fatty acids varying in chain length from 10 to 18 carbons, recent data indicate that individual saturated fatty acids can have specific, and in some cases, opposite effects on plasma cholesterol levels in different animal species. Thus, numerous studies in the last decade seem to confirm that stearic acid (18:0) has a neutral effect on cholesterol metabolism (Hegsted *et al.*,1993) despite that Bonamone and Grundy (1988) had reported a hypocholesterolemic effect of this acid in normolipidemic subjects when compared to palmitic acid (16:O). Myristic (14:0) and lauric (12:0) acids, mainly present in palm kernel and coconut oils, have been reported as the most potent hypercholesterolemic fatty acids (Hayes *et al.*,1991). Other studies in normocholesterolemic human subjects and various animal species suggest that diets rich in 12:0 + 14:0 increase plasma total and LDL cholesterol, and that 16:0 (similar to 18:1) can be quite neutral in its impact on plasma cholesterol (Lindsey *et al.*,1990; Ng *et al.*,1992; Pronczuk *et al.*,1994; Sundram *et al.*,1994).

Myristic acid appears to be the principal saturated fatty acid that raises plasma cholesterol, while the effect of palmitic acid may vary. In normocholesterolemic subjects consuming diets containing < 300 mg/day of cholesterol, 16:0 appeared neutral, whereas a hypercholesterolemic effect of 16:0 was observed in subjects consuming > 400 mg/day of cholesterol (Hayes and Khosla, 1992). More recently, Fielding et al. (Fielding *et al.*,1995) have confirmed the dependence of "the saturated fat effect" on the presence of dietary cholesterol in normocholesterolemic subjects. In this sense, Pronczuk et al. (1995) have reported that in Mongolian gerbils and cebus monkeys, two species sensitive to manipulation in dietary fat while consuming cholesterol-free diets, 16:0 was not hypercholesterolemic relative to 12:0. Gil Villarino *et al.* (1998) studied the comparative effects of diets, supplemented with 20% coconut oil, on the fatty acid profiles of 14 day-old chicks.

In view of the cellular and mitochondrial damage produced by supplementation of 20% coconut oil to the standard diet and the undesirable effects of high intakes of saturated fatty acids, a diet containing 10% coconut oil had been adopted in the present study to induce obesity in male Albino rats. Standard diets supplemented with 10% coconut oil have been reported to have produced hypercholesterolemia in Newborn White Leghorn male chicks (Garc´ıa-Fuentes *et al.*,2002). Makdessi *et al.*,(1994) reported changes in fatty acid composition of the phospholipids of rat heart sarcolemma upon10-week feeding period of diets containing 10% coconut oil and fish oil, in comparison to a low-fat diet.

Elevations in serum total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) increase the risk of atherosclerosis and coronary heart disease. Numerous studies have demonstrated that oils containing particularly saturated fatty acids (SFA) raise serum TC, and in particular, LDL-C while those enriched in unsaturated fatty acids lower LDL-C when replacing SFA (Mendis *et al.*,2001; Reiser 1973; Kris-Etherton 1999; Parodi 2009) . More recently, there has been a renewed interest in the nontriglyceride components of dietary oils, especially on the antioxidant vitamin E, the hydrocarbon squalene and the long chain n-3 PUFAs.

4.2.30 The metabolism of cholesterol

Whole body cholesterol homeostasis is controlled by supply and removal pathways. The liver is the main organ that regulates cholesterol homeostasis (Bravo *et*

al.,1998). Cholesterol can be excreted into the bile directly or after conversion to bile acids. In rats that were fed with corn oil, olive oil, or coconut oil, bile flow and biliary cholesterol secretion were not differentially influenced by the type of dietary fat (Smit *et al.*,1994).

Bile acids are the chief constituent of bile. The conversion of cholesterol to bile acids is an irreversible and terminal process of cholesterol catabolism. Bile acid synthesis occurs exclusively in the liver, and CYP7A1 is the first and rate-limiting enzyme in this pathway. Ebihara *et al.* (2006) previously reported that taurine increased CYP7A1 activity and the fecal excretion of bile acids in OVX rats (Kishida *et al.*,2000). Cantafora *et al.*,(1994) also reported that taurine increased CYP7A1 activity and the fecal excretion of bile acids in rats. In those studies, the increases in CYP7A1 activity and fecal excretion of bile acids induced by taurine were associated with a reduction in plasma cholesterol concentration. Thus, taurine reduced the plasma total cholesterol concentration in ovariectomized rats fed with corn oil (Kishida *et al.*,2001; Kishida *et al.*,2000).

Bile acids are derived from hepatic cholesterol, which originates from plasma lipoprotein cholesterol and from de novo cholesterol synthesis (Stephan and Hayes, 1985). The liver takes up plasma lipoprotein through a receptor. The level of LDLR activity is correlated with the level of hepatic LDLR mRNA (Horton *et al.*,1993). Ebihara *et al.* (2006) found that the increased fecal excretion of bile acids induced by taurine is not due to increased recovery of plasma lipoprotein cholesterol and/or increased de novo cholesterol synthesis.

In summary, in ovariectomized rats fed with coconut oil, the levels of CYP7A1 activity, and fecal bile acids excretion logarithmically increased as the dietary level of taurine increased, although the plasma cholesterol concentration did not significantly change by the addition of taurine. Since the experimental diets used in this study did not contain cholesterol, the results suggested that taurine changed endogenous sterol metabolism in rats fed with coconut oil.

4.2.31 Hypocholesterolemic action of fish oils in animals, on coconut oil rich diets

Castillo *et al.* (2000) reported that the hypocholesterolemic action of fish oil may be mediated by the depletion of VLDL synthesis both in normal and

hypercholesterolemic chicks. Diets high in polyunsaturated and monounsaturated fatty acids promote reduced fat accumulation compared to diets high in saturated fatty acids (Ellis *et al.*,2002). Studies in animals suggest that the type of dietary oil may alter body composition and adiposity (Bell *et al.*,1997; Park *et al.*,1997; Loh *et al.*,1998; Hill *et al.*,1993). Since high plasma total cholesterol is associated with the risk of CVD, diets which include vegetable oil high in poly and monounsaturated fatty acids have been recommended to lower plasma cholesterol (Kris-Etherton *et al.*,1993). Ellis *et al.* (2002) showed that body fat content and adipose tissue depots were significantly higher in rats fed diets containing high amounts of saturated fats and that the fat cell number was greater for coconut oil fed rats than for corn or canola oil fed rats. Coconut oil had a great influence on plasma lipids, with high values for cholesterol and triglycerides, on rats fed high fat diets (Ellis *et al.*,2002). Body weight gain increased 16–35% and body fat increased 9–23% in rats fed high fat diets. Shillabeer and Lau (1994) demonstrated that diets high in saturated fat promoted adipocyte replication. Induction of adipocyte hyperplasia with saturated fat diets may have longer term effects on adiposity compared to the increase in hypertrophy of diets high in unsaturated fat.

Jones (1993) compared the effects on fat accumulation of diets high (42% kcal) in polyunsaturated fatty acids and monounsaturated fatty acids using safflower and olive oil as the fat sources in growing male Sprague-Dawley rats for 12 wk. No difference in fat accumulation was found between these two dietary sources. Parrish *et al.*, (1990) also reported that fish oil limited adipose tissue hypertrophy in rats. Mendis *et al.*, (2001) reported that replacement of coconut oil with polyunsaturated and monounsaturated fatty acids were beneficial in reducing plasma lipids.

In studies by Allan *et al.*, (2001) the total cholesterol and HDL cholesterol concentrations were significantly higher in pigs fed the diet containing coconut oil compared to those receiving carbohydrate and fish oil. This was not surprising as previous studies in animals and human beings (Zock and Katan 1999; have shown that total cholesterol concentrations rise when saturated fat (especially coconut oil) replaces monounsaturated fat, polyunsaturated fat or carbohydrate. Furthermore, substituting carbohydrate or n-6 polyunsaturated fat with saturated fat results in an increase in the concentration of HDL cholesterol in man and animals. Coconut oil has been shown to raise HDL cholesterol concentrations more profoundly than other

sources of saturated fat (Allan *et al.*, 2001). Trilaurin appears to be the triglyceride in coconut oil responsible for this effect. Contrary to these findings some workers have discovered the lack of difference in the total cholesterol concentration between the groups of rats receiving coconut oil and olive oil.

Numerous studies have shown that high fat diets containing 30% or more energy from fat increase fat accumulation in rodents (Hill *et al.*,2000). Talavera *et al.* (1997) showed that the amount of middle chain fatty acids increased when new born chicks were fed a 20% coconut oil enriched diet for 14 days. Added to this the total MUFA, PUFA and n-6 fatty acids also decreased. He also demonstrated the different behavioural patterns of lipoprotein fractions (LDL and HDL) in response to dietary saturated fat. Thus total cholesterol/phospholipids (TC/PL) ratio, considered as an inverse index of membrane fluidity, increased in LDL particles by 20% coconut oil supplementation to the diet, while no significant differences were found in this ratio in HDL fraction. These findings supported their hypothesis that coconut oil decreased the fluidity of LDL.

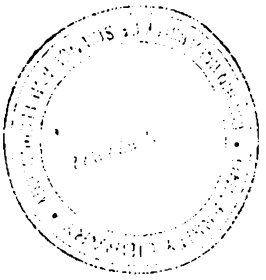
Cox *et al.*, (1995) compared the physiological effects of coconut oil, butter and safflower oil on lipids and lipoproteins in moderately hypercholesterolemic individuals and observed that both coconut oil and butter (rich in saturated fatty acids) raised the total cholesterol and LDL levels when compared with safflower oils, rich in unsaturated fatty acids. HDL-cholesterol on the other hand did not differ significantly on the three diets in the total group but in women levels were higher on butter and coconut diets than on safflower oil. Previous studies have generally found HDL-cholesterol to be lower when diets rich in polyunsaturated fatty acids were compared with those high in saturated fatty acids (Grundy and Denke, 1990).

Even though the coconut oil and butter diets resulted in increased total cholesterol and LDL cholesterol relative to the safflower oil diet, the levels of both these lipid measurements were significantly lower in the coconut oil than on the butter diet. Their data provided convincing evidence that coconut oil rich in lauric acid had a lesser effect than butter, which is high in palmitic acid, on total and LDL cholesterol in hypercholesterolemic men and women.The findings suggested that, in certain circumstances, coconut oil might be a useful alternative to butter and hydrogenated vegetable fats. However, it should be noted that in individuals and populations with a tendency to obesity, all fat sources should be restricted and that

depending upon the requirements of individuals, fats high in stearic acid or cis-monounsaturated fatty acids may be preferable to coconut oil.

Nardini *et al.* (1993) studied the effect of varying unsaturation degree of dietary lipid on the oxidative response of rat liver microsomes. Three groups of growing male rats were maintained for 6 weeks on 15% fat diets containing either soybean oil, olive oil, or coconut oil, with the same level of vitamin E. They observed that when dietary requirement for vitamin E was satisfied, the increased polyunsaturated fatty acid intake from vegetable oils did not enhance lipid peroxidation in physiological conditions, as demonstrated by similar malondialdehyde concentrations found in the three groups. However, the somewhat lower vitamin E content measured in soybean oil-fed rats confirmed an enhanced requirement for dietary antioxidant caused by the increased intake of polyunsaturated fatty acids. The susceptibility of liver microsomes to lipid peroxidation stimulated by the ADP/iron/ascorbate system was also studied. Membranes of soybean oil-fed rats exhibited the highest peroxidation rate, as shown by oxygen consumption and malondialdehyde and 4-hydroxy-2,3-trans-neonenal formation, because of the lower concentration of vitamin E and of the higher content of polyunsaturated fatty acids. Microsomes of olive oil- and coconut oil-fed rats showed highest protection against lipid peroxidation.

Oliveros *et al.* (2003) investigated the effects of a saturated fat diet on mice lipid metabolism in resident peritoneal macrophages. When male C57BL/6 mice at 21 days of age were assigned a diet, containing coconut oil as fat source, for 6 weeks, the concentration of triglyceride, total cholesterol, HDL and LDL+VLDL-cholesterol, and thiobarbituric acid-reactive substances (TBARS) increased, without changes in phospholipid concentration, compared with the controls which were fed soyabean oil. Coconut oil diet also produced an increase in myrystic, palmitic and palmitoleic acids proportion, a decrease in linoleic and arachidonic acids and no changes in stearic and oleic acids, compared with the control. Also, a higher relative percentage of saturated fatty acid and a decrease in unsaturation index ($p < 0.001$) were observed. These results indicate that the coconut oil-diet, high in saturated fatty acids, altered the lipid metabolism and fatty acid composition and produced a significant degree of oxidative stress. Jeffrey *et al.* (1996) also reported similar effects in lipid profile of rats fed saturated fat diets using coconut oil.



4.2.32 Are PUFA rich diets beneficial in reducing cholesterol levels in the body?

Prasad and Saraswathy (1996) demonstrated the effect of vitamin E on the lipid peroxidation induced by polyunsaturated fatty acids (PUFA) alone and PUFA in conjuction with cholesterol in liver and erythrocyte membrane of rats, fed a natural mixed diet normally consumed by the Indian military personnel. Increased consumption of PUFA or PUFA together with cholesterol (eg. lacto-ovo-vegetarians) was found to have deleterious effect on the integrity of the tissue membranes, in vitro, due to peroxidative damage caused by the significant increase in the tissue lipid peroxidation rate. This effect was found to be more pronounced in the latter case. However, an increase in the vitamin E to PUFA ratio in the diet resulted in a notable counter effect. Numerous workers have reported the level of peroxidation of plasma and liver lipids upon consumption of diets rich in PUFA (Allard *et al.*, 1997; Kubo *et al.*, 1997). It has been shown by some workers that the consumption of highly unsaturated fish oil leads to a compensatory increase in cellular antioxidant defences (Demoz *et al.*, 1992), although this is not always observed (Javouhey-Donzel *et al.*,1993). Studies by Suzuki *et al.*(1999) showed that there were no significant differences in the TBA values or phospholipid hydroperoxide levels of the membrane when experimental rats were fed oxidized fish oil diets. Groot *et al.*(1989) studied the levels of plasma lipoproteins and lipoprotein lipase activities in pigs fed on diets containing either 21 energy % mackerel oil or 21 energy % lard fat for 8 weeks. After 8 weeks levels of plasma triacylglycerol (-62%) and cholesterol (-55%) were lower in the mackerel oil than in the lard fat-fed animals. The triacylglycerol decline was exclusively due to the VLDL fraction, while cholesterol was reduced in all lipoprotein fractions (VLDL, IDL, LDL and HDL). The results supported their hypothesis that regular intake of fish oil reduced VLDL secretion.

Luostarinen *et al* (2001) showed that plasma triacylglycerol and total, esterified and free cholesterol concentrations were significantly lower in rats fed fish oil (FO) than in those fed the corn oil (CO) or saturated (SAT) fat – butter diets. He also showed that among the different FO diets, lipid peroxidation in the plasma, heart and liver was the greatest after feeding a fish oil diet with lower level of α-tocopherol and not stabilized with PUFANOX – an antioxidant. Indeed, the FO diets containing

the higher level of α -tocopherol and/or PUFANOX did not increase lipid peroxidation in plasma compared with the SAT and CO diets. The combination of the high level of α -tocopherol and PUFANOX had the greatest effect on enhancing heart and liver α -tocopherol concentrations.

4.2.33 Role of dietary fatty acids in immunity

The development, maintenance, and optimal functioning of the immune system are dependent on balanced and adequate nutrition. However, either a deficiency or an excess of a number of nutrients can have adverse effects. The nutrients with the most pronounced effects in humans include amount and type of dietary fatty acids (FAs), protein energy malnutrition, vitamins A, B6, B12, C, and E, and minerals including zinc, copper, selenium, and iron. Multiple rather than single nutrient deficiencies are often the cause for a compromised immune system. A number of workers have reported on the nutritional regulation of immune functions (de Pablo *et al.*,2000; McFarland *et al.*,2008; Hennig *et al.*,1996; Calder 2008). Dietary fat can have diverse effects on human health based on the amounts consumed and, more importantly, on the types consumed. Dietary fat may also differentially affect certain cells, tissues and organs depending on their stage of development. The FA composition of human tissues and organs can vary depending on the types of FAs that are consumed in the diet; that composition has been used as a biomarker for correlation with immunity and risk with disease. In addition, some dietary FAs can be transformed into potent biological mediators that have been shown to initiate or alter numerous processes in the body. For example, linoleic acid (LA, 18:2, n-6), a common component of some vegetable oils, can be converted by a number of cell types into arachidonic acid (AA, 20:4, n-6), a major precursor for the potent immunomodulatory agents, prostaglandin (PG)E_2 and leukotriene (LT)B_4, which are produced from AA by the enzymes cyclooxygenase and 5-lipoxygenase, respectively. Other 20-carbon FAs, eicosapentaenoic acid (EPA, 20:5 n-3), and dihomo-gamma linolenic (DGLA, 20:3, n-6) compete with AA as substrates for these enzymes and, thus, can decrease the production of PGE_2 and LTB_4. The eicosanoids produced from EPA and DGLA consequently have only weak effects on cells of the immune system. Docosahexaenoic acid (DHA, 22:6, n-3) is not a substrate for the cyclooxygenase and lipoxygenase; however, it can inhibit the

synthesis of the n-6 eicosanoids by inhibiting the release of membrane AA (Katan *et al.*, 1994). It can also be retroconverted to EPA. Because PGE_2 and LTB_4 have been linked to alterations in the immune system and to specific pathological processes, dietary fat intake has the potential to alter human disease. Reduced production of inflammatory eicosanoids by DHA, EPA, and DGLA, therefore, forms the basis for their use in the management of inflammatory diseases. The effects of dietary FAs on the number and activity of the cells of the immune system is a main focal point. (Kelly *et al.*,2005)

From the above review, it is evident that inclusion of fish oil in diet reduces cholesterol and triglycerde levels in body, but it is not the n3 PUFA content alone which is responsible for the observed hypocholesterolemic behaviour. PUFA concentrates on the contrary have been reported to raise the lipid peroxides in the system. Of late, several attempts have been made to find out the mechanism of action of fish oil and its components on the hypocholesterolemic behaviour. But still most of the tested or proposed mechanisms remain unclear. Literature on the role of liver oils of *C.scalpratus* and *N.raleighana* on the high fat diet induced hypercholesterolemia in Albino rats is nil. Besides, the ability of any fish oil must be experimentally tested before its consumption, rather than blind assumptions of the oils being hypocholesterolemic. Therefore, in the present study, we decided to investigate the effect of feeding liver oils of the deep sea elasmobranchs *C.scalpratus* and *N.raleighana* and compare its efficiency with PUFA concentrate, on the high fat diet induced condition and thereby analyse some of the important metabolisms undergoing within blood, liver and heart during hypercholesterolemia in Albino rats.

4.3 MATERIALS AND METHODS

Cholesterol, α-tocopherol, tetraethoxy propane, epinephrine were obtained from M/s. Sigma Chemical Company, St. Louis. MO, USA. All chemicals and reagents used were of analytical grade. Commercially available coconut oil was used for the study. Fish oil was extracted following the method of Folch *et al.*, (1957) from the liver of a *Centrophorus scalpratus* (CS) and *Neoharriotta raleighana* (NR). PUFA concentrate was prepared from cod liver oil by removing the major part of saturated and monounsaturated fractions as urea inclusion complexes, using a modified procedure of Ackman *et al.*, (1988). The fatty acid profile of the three lipid sources have been shown in Table 4d. The fatty acid (Bakes and Nichols 1995) composition was determined in a Gas Chromatograph (M/s. Thermo Electron Corporation, Milan, Italy) equipped with Perkin Elmer Elite 225 (Perkin Elmer Life and Analytical Services, Watham, MA) 50% cyanopropyl phenyl – 50% methyl capillary column (30 m × 0.25 mm i.d.) , an FID and a split/splitless injector. Peaks were quantified with Chromcard software by comparing retention time data with those obtained for authentic standards. The major fish oil constituents were determined using an Iatroscan – MK-6S (Ms Mitsubishi Kagaku Iatron Inc. Tokyo, Japan) to determine the abundance of individual lipid classes besides fatty acids, employing the procedure of Mathen *et al.*, 2008.

4.3.1 Preparation of PUFA from cod liver oil

The extraction of PUFA from cod liver oil was done by the method of Ackman *et al.*,(1988). 250g cod liver oil was taken in a 1L round bottom flask. 0.25g butyl hydroxy toluene, 62.5g potassium hydroxide, 340 ml distilled ethyl alcohol and 210ml distilled water were added into it. To prevent bumping glass beads were put. This was refluxed under nitrogen for 2 hours. After cooling, the content was transferred to a 2L separating funnel. Added 625ml distilled water and 90 ml concentrated hydrochloric acid. Shook well, to ensure thorough mixing. After that, it was kept for cooling under nitrogen. After cooling, separated the upper fatty acid layer and kept in a brown bottle under nitrogen in freezer.

Ethyl alcohol (930 ml) was taken in an amber coloured conical flask. After warming, 100g fatty acid was added. The contents were mixed thoroughly and 260g urea was added in to it. This was further mixed, until the urea was completely

dissolved. It was heated for 10 minutes. After cooling, the solution was filtered. The filtered solution was collected in a separating funnel. To this was added 18 times volume distilled water and 0.025 times volume concentrated hydrochloric acid. Kept under nitrogen for a while, until the PUFA layer separated. The upper PUFA layer was collected in an amber coloured bottle and stored at -18°C.

4.3.2 Animals and diets

Wistar strain male albino rats (n=24) weighing 160-180 g were used in the experimental study. They were housed individually in polypropylene cages under hygienic conditions and were provided food and water *ad libitum*. The animals were maintained on a 12:12 h light:dark photoperiod under standard conditions of temperature and ventilation. The experiments were performed as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and with the approval of the Institutional Animal Ethics Committee (IAEC).

All animals were fed a basal diet enriched with either coconut oil (CO), *Centrophorus scalpratus* liver oil (CS), *Neoharriotta raleighana* liver oil (NR) or poly-unsaturated fatty acids (PUFA) for a period of 8 weeks. The composition of the basal diet was as follows: corn starch (60.6%), casein (18.3%), salt mixture (4%), vitamin mixture (1%), cellulose (5%), cholesterol (1%) and methionine (0.1%). Five days after acclimatization, the rats were divided into four groups of 6 animals each and were allowed free access to the experimental diets and water. The first group of animals (Group CO) was fed, in addition to the basal diet, coconut oil at 10% feed levels. The remaining groups were also fed with the basal diets and fish oil (Group NR and CS) and polyunsaturated fatty acids (Group PUFA) at 5%, 5% and 1% feed levels respectively and made isolipidemic with coconut oil.

Body weight and feed consumption were recorded every week. Feed conversion ratio which is the ratio of the mass of food eaten to the gain in body mass was recorded every week. Animals with low FCR were considered efficient users of feed. Each diet was stored at -20°C and freshly prepared each day, to avoid auto-oxidation of the fat. At the end of the experimental period the animals were fasted overnight, thereafter, ether anesthetized and blood samples collected. They were then sacrificed; liver and heart tissues were excised, weighed and washed with

chilled isotonic saline; a portion of the tissue homogenates were prepared in 0.1 M Tris-HCl buffer (pH 7.2) and subjected to further analyses. The myocardial fatty acid composition of CO, CS, NR and PUFA rats was determined using Gas Chromatograph (M/s. Thermo Electron Corporation, Milan, Italy).

4.3.3 Estimation of proteins (Lowry *et al.*, 1951)

Reagents

1. Alkaline copper reagent

Solution A: 2% sodium carbonate in 0.1N sodium hydroxide solution.

Solution B: 0.5% Copper sulfate in water.

Solution C: 1% sodium potassium tartarate in water.

Mix 50ml of solution A with 0.5 ml of solution B and 1ml of solution C just before use.

2. Folin's phenol reagent: Diluted 1:2 with double distilled water before use.
3. Standard bovine serum albumin (BSA): Dissolved 100mg of BSA in 100ml of distilled water in a standard flask.10ml of the stock was diluted to 100ml to get a working standard of 100µg/ml.

Procedure

Pipetted out 0.01ml of plasma or 0.1 ml of tissue homogenate and standard BSA in the range of 20-100µg into test tubes and the total volume was made up to 1.0ml with distilled water. The blank contained 1.0ml of distilled water. Exactly 4.5ml of alkaline copper reagent was added to all the tubes and left at room temperature for 10min after which was added 0.5ml of Folin's phenol reagent. The blue colour developed was read after 20min at 640nm against the reagent blank, in a Shimadzu-UV-visible spectrophotometer.

The protein values are expressed as g/dl in plasma; mg/g in tissue

4.3.4 Assay of the diagnostic marker enzymes (AST, ALT, LDH)

4.3.4.a) Assay of alanine aminotransferase (EC 2.6.1.2)

The activity of alanine aminotransferase (ALT) was determined by the method of Mohur and Cook (1957).

Reagents

1. Buffered substrate solution (0.1M phosphate buffer, pH 7.4, 0.2 M DL-alanine, 2.0mM 2-oxoglutarate): 1.5g dipotassium hydrogen phosphate, 0.2g potassium

dihydrogen phosphate, 0.03g of 2-oxoglutaric acid and 1.78g DL-alanine were dissolved in distilled water. The pH was adjusted to 7.4 with 1N NaOH and made up to 100ml.

2. 20mg 2,4 dinitrophenyl hydrazine (DNPH) in 100ml of hot 1N hydrochloric acid.
3. 0.4N Sodium hydroxide.
4. Standard pyruvic acid: 12.5mg of sodium pyruvate was dissolved in 10ml of distilled water.10ml of this was diluted to 100ml with distilled water and this was prepared freshly for the calibration curve.

Procedure

To 1.0ml of the buffered substrate, 0.1ml sample was added and incubated at 37°C for 30min. The reaction was arrested by adding 1.0ml of DNPH and left aside for 20min at room temperature. Color developed by the addition of 10ml of 0.4N NaOH was read at 540nm in a Shimadzu UV-spectrophotometer against the reagent blank. The enzyme activity was expressed as μmol of pyruvate liberated/h/l for plasma and as nmol pyruvate liberated/h/mg protein for liver tissue.

4.3.4.b) Assay of aspartate aminotransferase (EC 2.6.1.1)

The aspartate aminotransferase (AST) activity was assayed by the method of Mohur and Cook (1957).

Reagents

1. Phosphate buffer: 0.15M, pH 7.5
2. Substrate: 300mg of L-aspartic acid and 50mg of α-ketoglutaric acid were dissolved in 20-30ml of the phosphate buffer and added 10% sodium hydroxide to bring the pH to 7.5 and was made up to 100ml with phosphate buffer.
3. 2, 4-dinitro phenyl hydrazine (DNPH) reagent: Dissolved 200mg of DNPH in 85ml of concentrated hydrochloric acid and made up to one litre with distilled water.
4. 0.4 N sodium hydroxide.
5. Standard pyruvic acid: 12.5mg of sodium pyruvate was dissolved in 10ml of distilled water.10ml of this was diluted to 100ml with distilled water and this was prepared freshly for the calibration curve.

Procedure

To 1.0ml of the buffered substrate, 0.1ml of the sample was added and incubated for one hour at 37°C. Then 1.0ml of DNPH reagent was added and left for 20min. At the end of incubation, 10ml of 0.4N NaOH was added and the colour developed was estimated by reading OD at 540nm in a Shimadzu UV-spectrometer after 10min. The standards were also treated similarly.

The enzyme activity was expressed as μmol of pyruvate liberated/h/l for plasma and as nmol pyruvate liberated/h/mg protein for liver tissue.

4.3.4.c) Assay of lactate dehydrogenase (EC 1.1.1.27)

The lactate dehydrogenase (LDH) activity was assayed according to the method of King (1965a) with slight modification. The amount of pyruvate formed in the forward reaction was measured colorimetrically.

Reagents

1. 0.1M glycine buffer: 7.5g of glycine and 5.88g of sodium chloride were dissolved in one litre of distilled water.
2. Buffered substrate: 2.76g of lithium lactate was dissolved in 125ml of glycine buffer containing 75ml of 0.1N sodium hydroxide to adjust the pH 10. This was prepared just prior to use.
3. 0.4N Sodium hydroxide.
4. 5.0mg of NAD^+ was dissolved in 1.0ml of distilled water. This was prepared just before use.
5. 2,4-dinitrophenyl hydrazine (DNPH): 200mg of DNPH was dissolved in one litre of 1N HCl.
6. Standard pyruvate solution: 12.5mg of sodium pyruvate was dissolved in 100ml of buffered substrate.

Procedure

To 1.0ml of the buffered substrate, 0.2ml of the sample was added and the tubes were incubated at 37°C for 15min. After adding 0.2ml of NAD^+ solution, the incubation was continued for 30min and then 1.0ml of DNPH reagent was added. And the tubes were incubated at 37°C for 15min. Then 7.0ml of 0.4N NaOH was added and the colour developed was measured at 540nm in a Shimadzu UV-spectrophotometer against the reagent blank. Suitable aliquots of the standards were also treated in the same manner.

The enzyme activity was expressed as μmol of pyruvate liberated/h/l for plasma and as μmol of pyruvate liberated/min/mg protein for liver tissue.

4.3.5 Estimation of total cholesterol

The total cholesterol present in plasma and liver was estimated according to method of Parekh and Jung (1970) with slight modifications.

Reagents

1. Standard cholesterol solution (stock): 1mg /ml in chloroform
   Working standard: 1.0ml of the stock was diluted to 10ml with chloroform.
2. $FeCl_3$ stock solution: 10g $FeCl_3$ in 100ml acetic acid.
3. $FeCl_3$ - H_2SO_4 reagent: 2.0ml of $FeCl_3$ stock solution was diluted to 200ml with conc. H_2SO_4.
4. 33% KOH (w / v): 10g of KOH was dissolved in 20ml distilled water.
5. Alcoholic KOH solution: 6.0ml of 33% KOH was made up to 100ml with distilled ethanol. This solution was prepared fresh just before use.

Procedure

0.1ml of the lipid sample was taken into a 25ml glass stoppered tube and evaporated off the chloroform. Added 5ml of freshly prepared alcoholic KOH solution. The tubes were shaken well and incubated in a water bath at 37^0C for 55min. After cooling to room temperature, added 10ml of petroleum ether and inverted the tubes once to mix the contents. Then added 5.0ml of distilled water and shaken the tubes vigorously for 1min. Took 0.5-2ml aliquots from the supernatant (petroleum ether) into test tubes. Evaporated the petroleum ether extract under nitrogen. To each of the sample as well as the standard tubes including the blank, added 3.0ml of glacial acetic acid followed by 0.1ml -distilled water. Mixed the tubes thoroughly and added 2ml of the $FeCl_3$ - H_2SO_4 reagent through the sides of the test tubes. A brown ring was formed at the interface; tap the bottom of the tubes well to effect mixing and a light colour appeared which changed to an immense purple colour, which was measured in a Shimadzu-UV spectrophotometer at 560nm.

The amount of total cholesterol was expressed as mg/dl (plasma); mg/g (liver).

4.3.6 Lipoprotein fractionation

Addition of heparin-manganous chloride to plasma caused the precipitation of VLDL and LDL. The supernatant represented the HDL fraction. In another aliquot of plasma, addition of sodium dodecyl sulphate resulted in aggregation of VLDL. The cholesterol content of each fraction was carried out in the following manner.

Total plasma cholesterol - (HDL+LDL) cholesterol = VLDL cholesterol

(HDL+LDL)- HDL = LDL

4.3.6.a) Estimation of high-density lipoprotein fraction

Total HDL was separated by the method of Burstein and Scholnick (1972).

Reagents

Heparin-Manganous chloride reagent: 3.167g of manganous chloride was added to 1.0ml of heparin containing 20,000 units/ml. This was made up to 8.0ml with water.

Procedure

2.0ml of plasma was added to 0.09ml of heparin-manganous chloride reagent and mixed well. The solution was allowed to stand at 4°C for 30min. The supernatant represented HDL fraction. Aliquots were taken from HDL fraction for the estimation of cholesterol.

4.3.6.b) Estimation of low-density lipoproteins

This differential analysis was made by the method of Burstein and Scholink (1972) using sodium dodecyl sulphate.

Reagent

Sodium dodecyl sulphate: 10% in 0.15M sodium chloride pH 9.0

Procedure

To 1.0ml of plasma, 0.75ml of sodium dodecyl sulphate solution was added, which was taken in a ploy carbonate centrifuge tube. The contents were swirled briefly and packed for 2 h in a water bath at 35°C. The contents were centrifuged in a refrigerated centrifuge at 10,000g for 30min. VLDL got aggregated as a pellicle at the top. The supernatant was a mixture containing HDL and LDL cholesterol was estimated in 0.05ml aliquot of the supernatant as described above.

4.3.7 Estimation of triglycerides

The level of triglycerides in plasma and liver were determined by the method of Rice (1970) with slight modifications.

Reagents

1. Activated silicic acid.
2. Saponification reagent: 5.0g of potassium hydroxide was dissolved in 60ml distilled water and 40ml isopropanol.
3. Sodium metaperiodate reagent: To 77g of anhydrous ammonium acetate in 700 ml distilled water, added 60ml glacial acetic acid and 650mg of sodium metaperiodate and was dissolved and diluted to 1litre with distilled water.
4. Acetyl acetone reagent: To 0.75ml of acetyl acetone, 20ml of isopropanol was added and mixed well.
5. Stock solution: 400mg of triolein was dissolved in 100ml chloroform.
6. Working standard: 1.0ml of the stock solution was diluted to 10ml.

Procedure

0.2ml of the lipid sample was taken into a test tube and evaporated off the chloroform, added 4.0ml isopropanol. It was mixed well and added 0.4g of activated silicic acid. It was shaken in a vortex mixer for 15min and centrifuged at 4000 rpm for 5min. To 2.0ml of the supernatant and standards ranging from 20-100 mg made up 2.0 ml with isopropanol, 0.6 ml of saponifying reagent was added and incubated at 60-70^{0}C for 15min. After cooling, 1.0ml sodium metaperiodate solution was added and mixed. To this, 5ml acetyl acetone was added, mixed and incubated at 50^{0}C for 30min. After cooling, the colour was estimated by measuring OD at 405 nm in a Shimadzu-UV spectrophotometer.

The value of triglyceride in plasma was expressed as mg/dl and in liver as mg/g tissue.

4.3.8 Estimation of free fatty acids

Free fatty acids in plasma and liver were estimated by the modified method of Horn and Menahan (1981) with colour reagent of Itaya (1977).

Reagents

1. Activated silicic acid

2. Chloroform, heptane, methanol (CHM) solvent mixture: It was prepared by mixing chloroform, heptane and methanol in the ratio of 200:150:7(v/v)

3. Copper-triethanolamine solution: 50ml of 0.1M copper nitrate and 50ml of 2M triethanolamine were mixed with 33g of sodium chloride. The pH of the solution was adjusted exactly to 8.1.

4. Diethyldithiocarbomate (DDC) solution: 0.1% DDC in butanol was prepared.

5. Standard Stock: A solution containing 2mg/ml of palmitic acid was prepared in CHM solvent. For working standard, the stock was diluted 1:10 in CHM to give a concentration of 200μg/ml.

Procedure

To 1.0ml of the lipid sample, 6.0ml of CHM solvent and 200mg of silicic acid were added. The mixture was shaken well, centrifuged at 4000 rpm for 5min and 3.0ml of the supernatant taken. Standard solution in the range of 25-100μg were taken and made up to 3.0ml with CHM solvent. The blank contained 3.0ml of CHM solvent. To all these samples, 2.0ml of copper triethanolamine solution was added and then mixed on a mechanical shaker for 10min. The tubes were centrifuged at 4000 rpm for 5min. To the 2.0ml of the supernatant taken, 1.0ml of DDC solution was added and shaken well. The colour intensity was read immediately at 430nm in a Shimadzu UV-spectrophotometer.

Values are expressed as mg/dl plasma and mg/g wet tissue.

4.3.9 Estimation of inorganic phosphorous

Inorganic phosphorus was estimated by the method of Fiske and Subbarow (1925). The method is based on the formation of phosphomolybdic acid by the reaction between a phosphate and molybdic acid and its subsequent reduction to a dark blue phosphomolybdic acid, the intensity of which is proportional to the phosphate ion concentration.

Reagents

1. Ammonium molybdate reagent : 2.5 g of ammonium molybdates was dissolved in 100 ml of 3N sulphuric acid.
2. Amino naphthol sulphonic acid (ANSA)

Standard Phosphorus: 35.1 mg of potassium dihydrogen phosphate was accurately weighed, dissolved and made upto 100 ml with distilled water. One ml ontained 80 µg phosphorus.

Procedure

To suitable aliquots of the supernatant, 1.0 ml of ammonium molybdate reagent was added 0.4 ml of ANSA was added after 10 minutes incubation at room temperature, standards and blank were also treated in the above manner. The blue colour developed was read 20 minutes at 640 nm in a Shimadzu-UV- spectrophotomer.

The values are expressed as nmoles of Pi liberated per min mg protein.

4.3.10 Lipid peroxidation and tissue antioxidant status

4.3.10.a) Estimation of lipid peroxides (LPO)

Lipid peroxides content was determined by thiobarbaturic acid reaction as described by Ohkawa *et al.* (1979) in liver tissue.

Reagents

1. Acetic acid 20%: 20ml of glacial acetic acid dissolved in 100ml distilled water.
2. 8% Thiobarbaturic acid (TBA) in 20% acetic acid.
3. 8% Sodium dodecyl sulphate (SDS).
4. Standard: 41.66mg of tetraethoxy propane (TEP) dissolved in 100ml distilled water. 1.0ml of above was made up to 10ml with distilled water.

Procedure

To 0.2ml of sample, 1.5ml of 20% acetic acid, 0.2ml of SDS and 1.5ml of TBA were added. The mixture was made upto 4.0ml with distilled water and heated in a boiling water bath for one hour. After cooling the mixture was centrifuged at 3000g for 10min. Supernatant was taken and absorbance was read at 532nm in a Shimadzu-UV spectrophotometer.

The lipid peroxides content was expressed as nmol of malondialdehyde/g tissue.

4.3.11 Antioxidant defense system

4.3.11.I) Non-enzymatic antioxidants

4.3.11.a) Determination of total reduced glutathione (GSH)

The total reduced glutathione was determined by the method of Ellman (1959). The method is based on the reaction of reduced glutathione with 5, 5'-dithiosbis (2-nitrobenzoic acid) (DTNB) to give a compound that has absorbance at 412nm.

Reagents

1. DTNB: 0.6mM in 0.2M Phosphate buffer pH 8.0
2. 0.2M Phosphate buffer, pH 8.0.
3. 5% Trichloroacetic acid
4. Standard: 61.4mg of reduced glutathione was dissolved in 100ml 0.02M EDTA, 0.1ml of this is made up to 10ml with 0.02M EDTA.
5. Working standard: 2.0ml of the above was made up to 10ml.

Procedure

0.5ml of liver homogenate was precipitated with 5% of TCA. The contents were mixed well for complete precipitation of proteins and centrifuged at 4000 rpm for 5 min. To an aliquot of clear supernatant, 2.0ml of DTNB reagent and 0.2M phosphate buffer were added to make a final volume of 4.0ml. The absorbance was read at 412nm against a blank containing TCA instead of sample, series of standards treated in a similar way were also run to determine the reduced glutathione content. The amount of glutathione was expressed as nmol/mg protein.

4.3.11.b) Vitamin E (α-Tochopherol)

α-Tochopherol (Vitamin E) was determined in liver tissue by the method of Baker *et al.* (1980).

Reagents

1. 2% pyrogallol (or epinephrine) in ethanol
2. Saturated KOH in ethanol
3. Hexane
4. Ethanol
5. 0.2% Bathophenanthroline in ethanol
6. 0.001M $FeCl_3$ in ethanol
7. 0.001M H_3PO_4 in ethanol
8. Standard: 1mg/ml in ethanol.

Procedure

100mg heart sample was homegented with 2.0ml of ethyl alcohol, centrifuged and took 1.0ml of the supernatant into a test tube. To this added 1ml of 2% epinephrine and incubate at 70^{O}C for 2min. Added 0.3ml of of saturated KOH and kept in a water bath at 70^{O}C for 2min. Cooled in ice bath and added 1ml H_2O and 4.0ml hexane. Centrifuged and took 3ml of the upper hexane layer and evaporated to dry. To the residues and standards (0.1-0.5ml) added 3.0ml ethyl alcohol, 0.2ml bathophenanthroline, 0.2ml $FeCl_3$ and 0.2ml H_3PO_4. Mixed well and read OD at 550nm.

The content of vitamin E was expressed as mg/g tissue

4.3.11.II) Antioxidant enzymes

4.3.11.c) Assay of glutathione-S-transferase (EC 2.5.1.18)

Glutathione-S-tranferase (GST) activity was determined by the method of Habig *et al.* (1974).

Reagents

1. 0.3M Phosphate buffer, pH 6.5
2. 30mM 1-chloro-2, 4-dinitrobenzene (CDNB).
3. 30mM reduced glutathione (GSH).

Procedure

The reaction mixture containing 1.0ml of buffer, 0.1ml of CDNB and 0.1ml of tissue homogenate was made up to 2.5ml with water. The reaction mixture was pre-incubated at 37°C for 5min. 0.1ml of GSH was added and the change in the absorbance was measured at 340nm for 3min at 30sec intervals.

The enzyme activity was expressed as μmol of CDNB-GSH conjugate formed/min/mg protein.

4.3.11.d) Estimation of glutathione peroxidase (EC 1.11.1.9)

The method of Paglia and Valentine (1967) was adopted for assay of glutathione peroxidase (GPx).

Reagents

1. 0.4 M Phosphate buffer, pH 7.
2. 0.4M Tris buffer, pH 8.9
3. 0.4mM EDTA

4. 2mM GSH
5. 10mM NaN_3
6. 10% TCA
7. DTNB: 99mg in 25ml of methanol.
8. H_2O_2: 1mM was prepared freshly from commercial 30%solution.
9. GSH standard: 61.4mg GSH was dissolved in 100ml distilled water. 1.0ml of this solution was made up to 10ml with distilled water.

Working standard: 2ml of the stock was made up to 10ml with distilled water.

Procedure

0.2ml of tissue homogenate was added to a mixture containing 0.2ml of buffer, 0.2ml of EDTA and 0.1ml of sodium azide. Mixed well and added 0.1ml reduced glutathione and 0.1ml of hydrogen peroxide. Incubated in a water bath at 37°C for 10min. At the end of incubation period, 0.5ml of 10%TCA was added and centrifuged at 10000 rpm for 5min. 1.0ml of the supernatant was taken into a separate test tube and added 2.0ml Tris buffer and 50µl DTNB. Immediately read the OD at 412nm.

The enzyme activity was expressed as nmol of glutathione oxidized/min/mg protein.

4.3.11.e) Assay of superoxide dismutase (EC 1.15.1.1)

The superoxide dismutase was assayed according to the method of Misra and Fridovich (1972) based on the oxidation of epinephrine-adrenochrome transition by the enzyme.

Reagents

1. 0.1M Carbonate-bicarbonate buffer, pH 10.2 containing 57mg/dl EDTA.
2. 3mM Epinephrine

Procedure

50µl of sample was taken into the cuvette and added 1.5ml buffer and 0.5ml epinephrine. Mixed well and immediately read the change in optical density at 480nm for 2min in a Shimadzu-UV spectrophotometer.

One unit of SOD activity was the amount of protein required to give 50% inhibition of epinephrine auto oxidation.

4.3.11.f) Assay of catalase (EC 1.11.1.6)

Catalase (CAT) was assayed according to the method of Takahara *et al.* (1960).

Reagents

1. 50mM Phosphate buffer, pH 7.0
2. 30mM Hydrogen peroxide solution in the above buffer.

Procedure

To 2.45ml of the phosphate buffer, 50µl of the liver homogenate was added and the reaction was started by the addition of 1.0ml of H_2O_2 solution. The decrease in absorbance was measured at 240nm at 30sec intervals for 2min. The enzyme blank was run simultaneously with 1.0ml of distilled water instead of hydrogen peroxide.

The enzyme activity was expressed as nmol of H_2O_2 decomposed/min/mg protein.

4.4 RESULTS AND DISCUSSION

Reports by several workers have shown that there are multiple mechanisms by which diet-induced obesity leads to atherosclerotic coronary artery disease. Obesity is associated with multiple factors which are themselves major risk factors for atherosclerosis, including abdominal obesity, insulin resistance (and its ultimate form, type 2 diabetes), atherogenic dyslipidemia (i.e. high plasma triglyceride and low high-density lipoprotein [HDL]-cholesterol concentrations) and hypertension. These risk factors often cluster in what has been termed the 'metabolic syndrome.' There is considerable controversy as to whether the metabolic syndrome itself is a distinct risk factor for CVD, or if it simply confers the risks associated with its parts. However, it is helpful to recognize that separate components of the metabolic syndrome often accompany obesity and may cluster together. The metabolic syndrome is associated with increased risk of death from CVD even in the subset of patients without frank disorders.

4.4.1 Feed and bodyweights

In the present study we compared the effects of four isolipidemic diets on the diagnostic marker enzyme levels, the lipid profile and the antioxidant status in Albino rats. Rats fed CO diets gained a significant increase ($P<0.05$) in body weight towards the end of the experimental period (from the 6^{th} to the 8^{th} week). However there was no significant difference among rats fed either of the CS, NR or PUFA diets (Table 4a). Rats fed CS and PUFA diets showed a comparatively higher ($P<0.05$) feed conversion ratio than those on CO and NR diets, from the sixth week of feeding (Fig. 4.1). The results are in close agreement with the studies of fish oil supplementation in cattle diets by Nicholson *et al.*, (1992) who reported an improved feed efficiency for cattle. Similarly, though rats fed on NR and PUFA diets showed increase in both liver and heart weights, no significant differences were observed in their weights, in rats fed on any of the other experimental diets.

4.4.2 Protein Contents

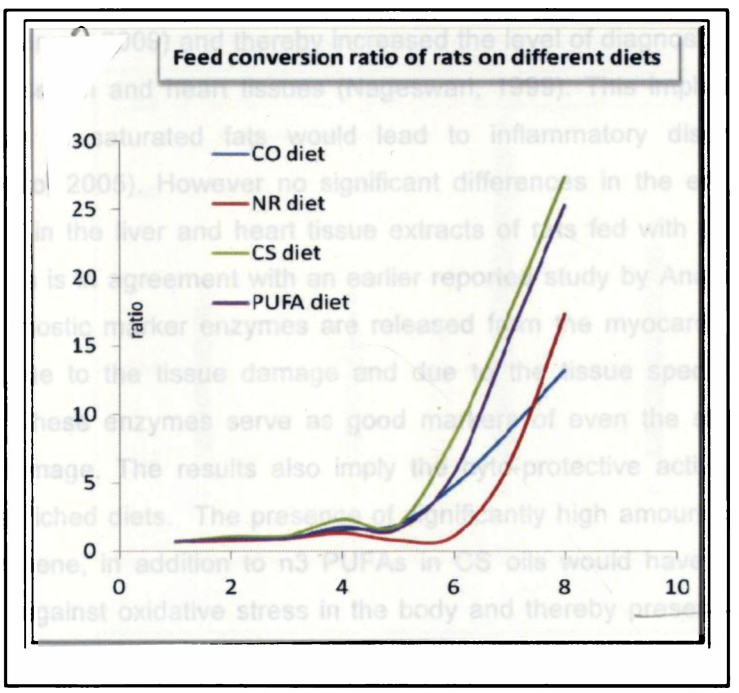
Significant ($p<0.05$) elevations in protein contents were observed in liver and heart tissue of CS and PUFA fed rats compared to their levels in CO or NR fed rats. (Fig.

Table 4a. Effect of isolipidemic diets on body weights of rats

Weeks	CO diet	CS diet	NR diet	PUFA diet
1	197.6 ± 2.4	196.0 ± 2.7	192.0 ± 3.1	194.0 ± 2.2
2	219.7 ± 3.1	220.3 ± 3.6	220.2 ± 1.6	218.5 ± 3.5
3	236.3 ± 2.3	235.5 ± 4.7	236.5 ± 2.4	233.6 ± 2.9
4	249.7 ± 2.6	247.3 ± 2.9	250.1 ± 2.1	247.8 ± 3.5
5	256.8 ± 2.4	252.8 ± 3.3	253.3 ± 2.3	255.7 ± 3.1
6	275.3 ± 2.7 [b]	264.8 ± 3.1 [a]	267.8 ± 2.2 [a]	267.8 ± 2.6 [a]
7	283.7 ± 2.4 [b]	274.8 ± 2.9 [a]	272.8 ± 1.5 [a]	275.8 ± 3.6 [a]
8	284.5 ± 2.8 [b]	275.6 ± 2.4 [a]	276.2 ± 2.5 [a]	277.2 ± 3.1 [a]

Results are mean ± SD for n=6 animals, For each week, the weights of animals with different letters[a,b] are significantly different.

Figure 4.1 Feed conversion ratio of animals on different isolipidemic diets



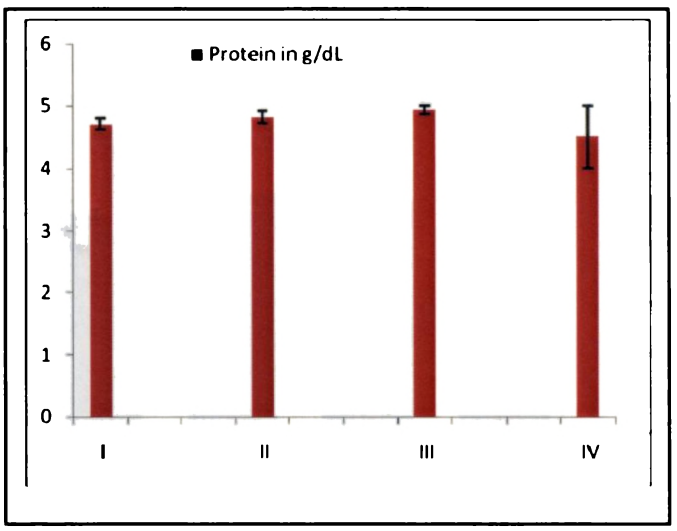
4.2). However there was no significant difference in the level of protein contents in the blood sera of rats on any of the isolipidemic diets.

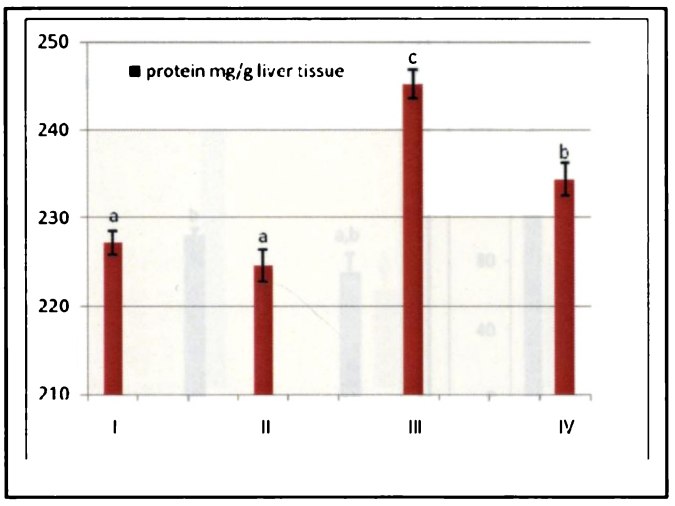
4.4.3 Diagnostic marker enzyme levels

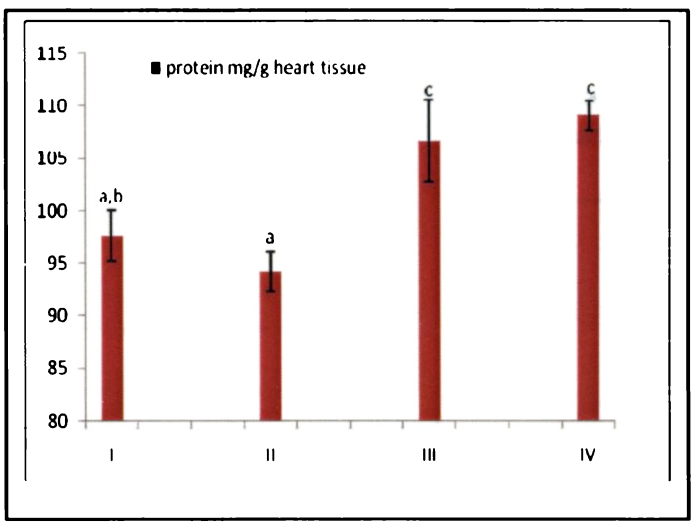
Rats fed with the high energy CO diets showed significantly high ($P<0.05$) levels of diagnostic marker enzymes (AST, ALT, LDH) in the blood than those fed with NR, CS or PUFA enriched diets (Fig. 4.3). This is in accordance with previously reported works which showed that supplementation of coconut oil to the diet induced cellular damage (Gil-Villarino, 1997). However, there were no significant differences in marker enzyme levels in the liver and heart tissues among CO and NR groups. This could be so owing to the significantly higher amounts of saturated fatty acids in NR compared to CS oil. Moreover, diets rich in saturated fatty acids increase oxidative stress (Harvey, 2009) and thereby increased the level of diagnostic marker enzymes in blood serum and heart tissues (Nageswari, 1999). This implies that consuming diets rich in saturated fats would lead to inflammatory disorders and CVDs (Fachinetto, 2005). However no significant differences in the enzyme levels were observed in the liver and heart tissue extracts of rats fed with either CS or PUFA diets. This is in agreement with an earlier reported study by Anandan *et al.* (2007). The diagnostic marker enzymes are released from the myocardium into the blood stream due to the tissue damage and due to the tissue specificity and catalytic activity. These enzymes serve as good markers of even the slightest myocardial tissue damage. The results also imply the cyto-protective activity of the CS and PUFA enriched diets. The presence of significantly high amounts of alkyl glycerols and squalene, in addition to n3 PUFAs in CS oils would have contributed to the defence against oxidative stress in the body and thereby preserved the membrane integrity of the animal cell.

Squalene, alkyl glycerols, vitamin E contained in significantly high amounts in CS oil being liphophilic in nature, could be compared to any other lipophilic agents such as antipyrin and nifedine. The lipophilic β-blocking drugs intercalate into the lipid matrix and impart stabilization to cell membranes in relation to the degree of their lipophilicity (Cruickshank and Neil Dweyer, 1985). Hence, it is possible that likewise fish oil components may also prolong the viability of cell membranes from

Figure 4.2 Effect of isolipidemic diets on protein contents in blood sera, liver and heart tissues of rats

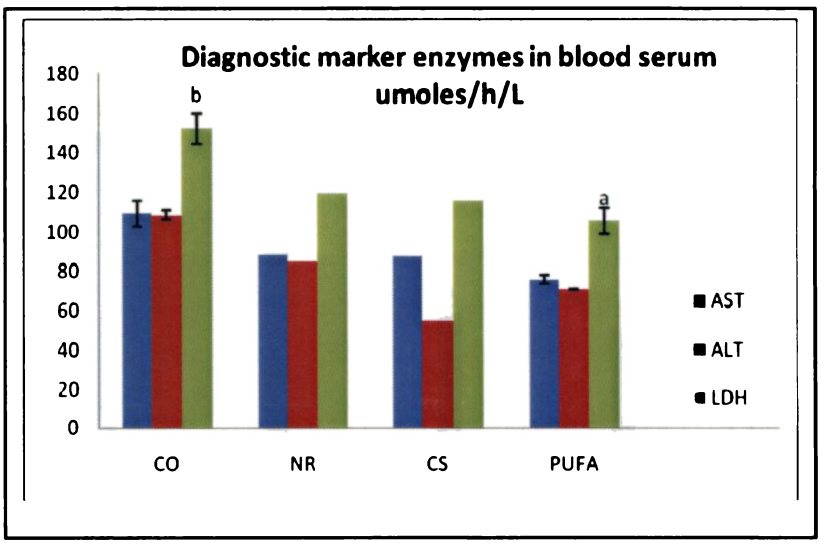


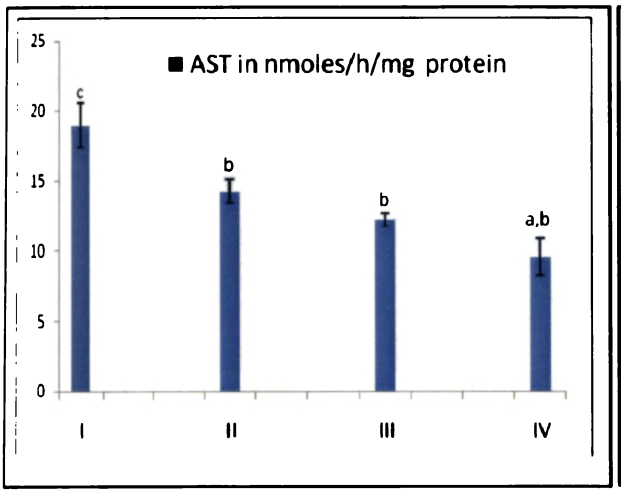


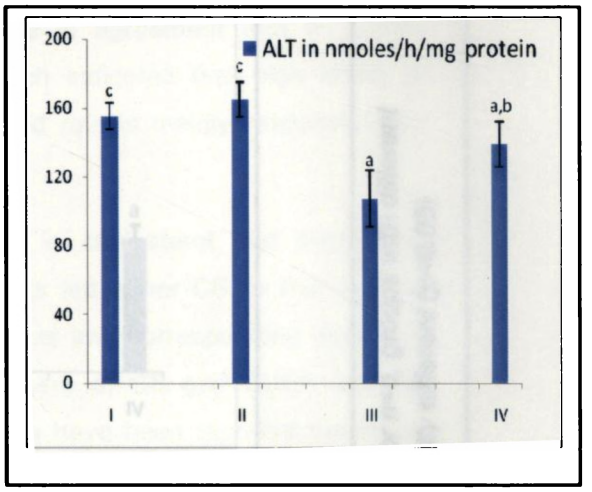


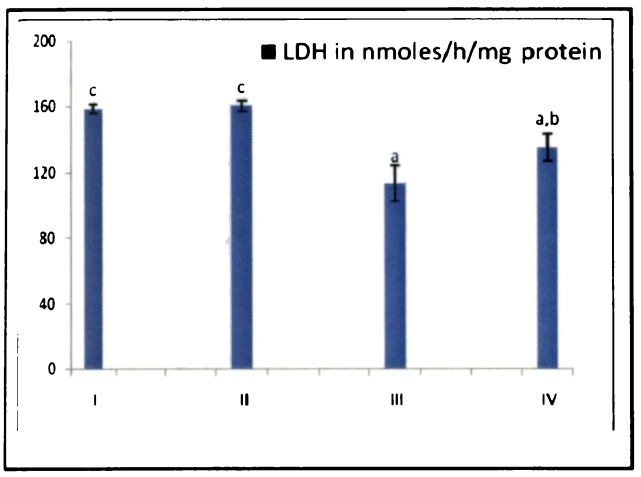
Values are mean±SD for n=6, groups with different letters[a,b,c] are significantly different (p<0.05)

Figure 4.3 Effect of isolipidemic diets on diagnostic marker enzyme levels in blood and liver of rats



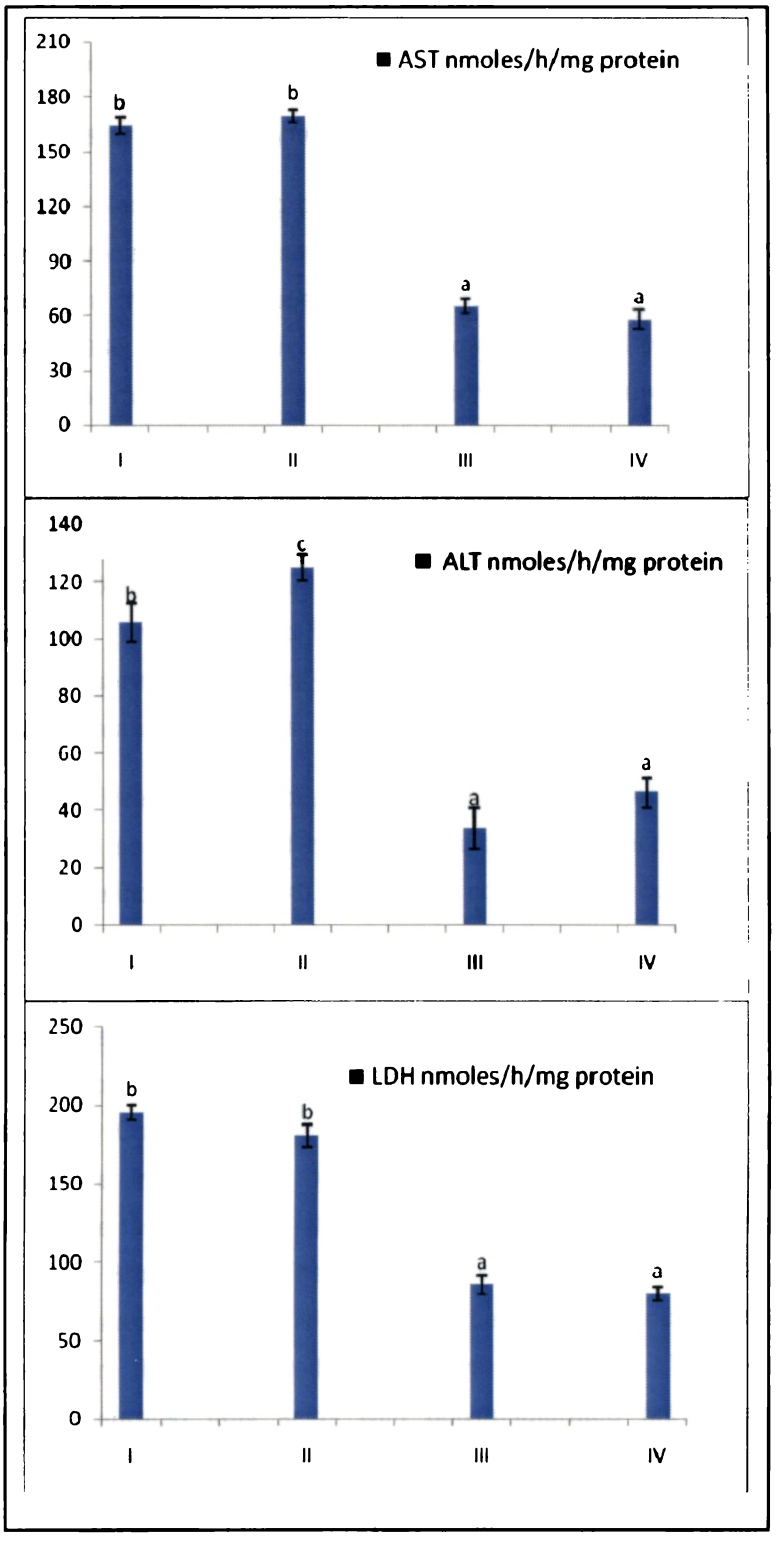






Values are mean±SD for n=6, groups with different letters[a,b,c] are significantly different ($p<0.05$)

Figure 4.3 Effect of isolipidemic diets on diagnostic marker enzyme levels in heart tissues of rats



Values are mean±SD for n=6, groups with different letters^{a,b,c} are significantly different ($p<0.05$)

necrotic damage by its membrane stabilizing action (Ivashkevich et al., 1981). The role of CS oil as a membrane stabilizer is hence displayed.

4.4.4 Lipid profile

4.4.4.a) Cholesterol and triglyceride levels

Hypercholesterolemia has long been recognized as one of the major reversible risk factors for coronary heart disease. High level of circulating cholesterol and its accumulation in the heart tissue are well associated with cardiovascular damage (Joan *et al.,* 1984; Buring *et al.,* 1992).Lipid composition of the blood sera, liver and heart tissues of rats changed rapidly in response to the supplemented diets (Table 4b). A significant increase ($P<0.05$) in the levels of cholesterol and triglyceride was observed in sera, liver and heart tissues of CO and NR rats compared to CS and PUFA fed rats, indicating the severity of the high saturated fat diet in CO and NR rats.(Denke, 2006; Stone, 1990) Our findings are in agreement with an earlier reported study (Ithayarasi and Devi, 1997b) which indicated that high levels of cholesterol in serum and heart of stressed-induced rats is mainly responsible for altered cardiovascular functions.

No significant differences were observed in cholesterol and triglyceride contents in the sera, liver and heart tissues of rats fed either CS or PUFA diets. However, a significant rise ($P<0.05$) in HDL contents and corresponding decline in LDL and VLDL contents were observed in the sera of CS and PUFA fed rats compared to CO and NR rats. Similar observations have been reported previously (Ebenezar *et al.,*, 2003). Low-density lipoprotein (LDL) is the major cholesterol-carrying lipoprotein in plasma and is the causal agent in many forms of coronary heart disease. Coconut oil mainly increases the low-density lipoproteins (LDL) cholesterol level in the blood, which in turn and leads to the build up of harmful deposits in the arteries, and thus favors induction of coronary heart diseases (Goldstein and Brown, 1984) The elevated cholesterol level observed in the hepatic and cardiac tissues of CO and NR rats might be due to the increased uptake of LDL-cholesterol from the blood by the respective membranes. The results imply that substituting saturated fatty acids with polyunsaturated fatty acids results in a decrease in serum total and LDL cholesterol concentrations (Alan *et al.*,2001).

Table 4b. Effect of isolipidemic diets on cholesterol, triglyceride, phospholipid, free fatty acids and lipid peroxides in blood sera, liver and heart tissues of rats

Lipid Components	CO diet			NR diet		
	Serum	Liver	Heart	Serum	Liver	Heart
Triglycerides	354.71 ± 13.19 b	7.51 ±0.4b	65.34± 5.96d	305.8± 21.9 b	6.27± 0.26a	44.51± 2.90c
Cholesterol	110.88 ± 10.01b	28.4± 3.1c	76.25± 1.12d	93.0± 1.9 b	21.2± 1.8 a,b	55.67± 2.58c
Phospholipids	1.88 ± 0.15a	0.82± 0.01a	0.41± 0.01a,b	3.6± 0.69 b	0.8± 0.03a	0.40± 0.01a
Free Fatty Acids	32.40 ± 1.54b	3.64± 0.4d	11.11± 1.74	19.96± 1.76 a	1.7 ± 0.15 b,c	10.07± 2.01 b,c
Lipid Peroxides	7.01 ± 0.25	250.4± 22.5a	1.67± 0.32a	6.7± 0.2	842.9± 59.8 b,c	3.51± 0.39c

Lipid	CS diet			PUFA diet		
Components	Serum	Liver	Heart	Serum	Liver	Heart
Triglycerides	205.51 ± 3.97a	6.1± 0.8a	22.47± 5.25a,b	221.42± 13.76a	6.8± 0.5a	19.83± 0.87a
Cholesterol	62.92 ± 2.29a	15.3± 3.3a	32.58± 2.22a,b	69.35± 6.70a	22.7± 3.4a,b	37.95± 2.44a,b
Phospholipids	4.19 ± 0.42b	0.91± 0.01b	0.49± 0.05c	4.14± 0.27b	0.94± 0.0b	0.49± 0.03c
Free fatty acids	14.4± 2.5a	1.32± 0.18a	7.99± 1.32a	14.32± 2.14a	1.52 ± 0.12a,b	9.46 ± 0.89
Lipid peroxides	6.8± 0.1	533.4± 145.4b	2.34± 0.07b	6.98 ± 0.07	1871.6 ± 129.8d	3.17 ± 0.18c

Values are mean±SD for n=6, groups with different letters[a,b,c,d] are significantly different ($p<0.05$); Cholesterol(mg/dl in blood, mg/g in liver/heart tissues) triglycerides(mg/dl in blood, mg/g in tissues); phospholipid(mg/ml in blood, mg/g in tissues);free fatty acids(mg/dl in blood, mg/g in tissues); lipid peroxides (nmoles MDA/ml, ug/g in tissues)

In human beings, this is believed to be partly due to the effect of acyl-coenzyme A: cholesterol acyltransferase (ACAT), a key enzyme in cholesterol metabolism. This enzyme esterifies free cholesterol to cholesteryl ester within the cytoplasm of cardiocytes but it prefers unsaturated fatty acids, rather than saturated fatty acids, as the substrate for esterification (Levy 1995). Diets high in unsaturated fats result in increased ACAT activity and decrease in the cardiac pool of free cholesterol (Levy 1995). Low levels of free cytosolic cholesterol increases the transcription of the LDL receptor gene. The net result is an increase in the number of cardiac LDL receptors and a concomitant decrease in the serum LDL concentration (Goldstein and Brown, 1990).

Hypertriglyceridemia has been reported to be associated with cardiovascular disturbances (Freedman *et al.*, 1988). In the present study, rats fed with CS oil significantly ($p<0.05$) prevented the coconut oil-induced elevation in the levels of triglycerides and free fatty acids in plasma, liver and heart tissue of CS rats as compared to that of NR rats. CS oil comprises lipid lowering components, which probably inhibited the HMG CoA activity, the rate-limiting step in cholesterol biosynthesis (Davignon *et al.*, 1992), as observed in the present study. These compounds are potent LDL cholesterol lowering agents; however, relative to the LDL response, the ability of the fish oil components in NR, to reduce triglyceride concentration, is modest as observed in the present study. The cardio and hepato-protective effect of CS oil compared to NR is probably related to the former's ability to inhibit the increased accumulation of lipids in the respective tissues by its antilipidemic property (Qureshi *et al.*, 1996) as shown in Table 4b.

The exact mechanisms involved in the antilipidemic property of dietary fish oil are not fully known. Squalene is a key intermediate in the biosynthesis of cholesterol, bile acids and sterols. It is possible that the observed inhibitory effect of dietary squalene in the present study may be due to the modulation of the cholesterol biosynthetic pathway. Qureshi *et al.*, (1996) have shown that fish oils rich in squalene is effective in lowering the plasma total cholesterol, VLDL cholesterol and LDL cholesterol levels in experimental animals. The present result confirms this finding and show reduction in the level of total cholesterol and LDL-cholesterol in Group III animals, establishing the anticholesterolemic property of CS oil. Significant increase noticed in the HDL cholesterol also exhibits the beneficial action of the fish

Figure 4.4 Effect of isolipidemic diets on lipoprotein levels in blood of rats

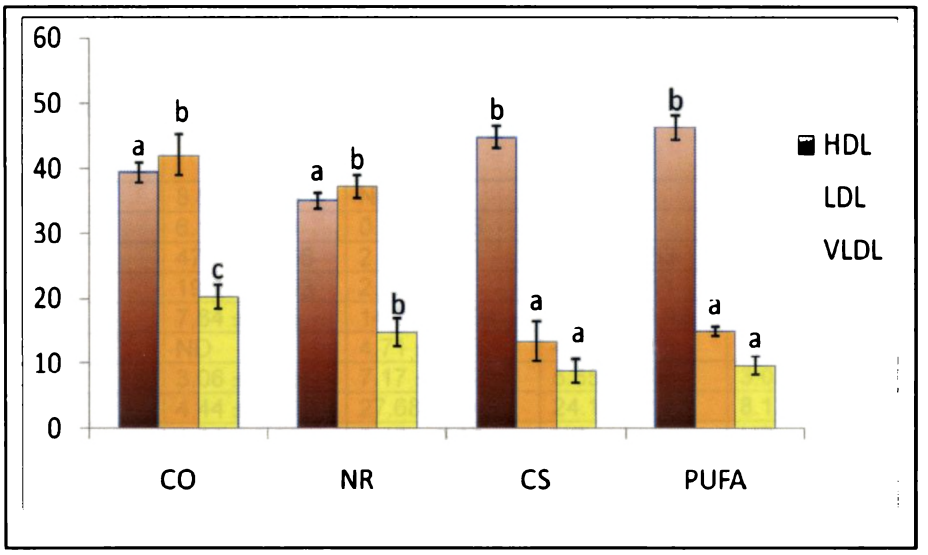


Table 4c. Effect of isolipidemic diets on the antioxidant defence parameters in the heart tissues of rats

Antioxidant parameters	CO	NR	CS	PUFA
GSH	3.56 ± 0.32a	3.84 ± 0.73a	4.83 ± 0.10b	3.37 ± 0.28a
Vitamin E	3.80 ± 1.12a	7.27 ± 2.61b	9.72 ± 1.08b	4.2 ± 0.47a
GPx	118.32 ± 2.83a	124.46 ± 3.91a	153.91 ± 3.22b	157.22 ± 3.89b
GST	475.62 ± 17.83a	547.35 ± 22.31b	612.74 ± 24.85c	711.42 ± 19.46d
CAT	47.36 ± 1.14a	51.73 ± 2.03b	50.29 ± 1.03b	55.81 ± 3.89b
SOD	2.09 ± 0.32a	3.32 ± 1.2a	6.53 ± 1.86b	5.87 ± 0.73b

Values are mean±SD for n=6, groups with different letters[a,b,c,d] are significantly different ($p<0.05$);GSH (reduced glutathione)in nmoles/mg; vitamin E in mg/g; GPx (glutathione peroxidise) in nmoles GSH oxidised/min/mg; GST (glutathione S transferase) umoles conjugate/min/mg; CAT (catalase) nmoles H_2O_2 decomposed/min/mg; SOD (superoxide dismutase) enzyme units.

Table 4d. Fatty acid (as % total fatty acid) composition of lipid sources used in the study

Fatty Acid	Coconut oil	*N.raleighana* oil	*C.crepidater* oil	Polyunsaturated Fatty Acid concentrate
8:0	8.86 ± 0.66	ND	ND	ND
10:0	6.17 ± 0.04	0.53 ± 0.06	ND	ND
12:0	47.53 ± 1.56	2.64 ± 0.11	2.64 ± 0.53	1.31 ± 0.49
14:0	19.97 ± 1.03	2.02 ± 0.53	1.17 ± 0.11	ND
16:0	7.84 ± 0.35	14.36 ± 0.94	12.43 ± 0.92	5.86 ± 0.68
16:1	ND	4.71 ± 0.86	4.00 ± 0.53	3.62 ± 0.33
18:0	3.06 ± 0.73	7.17 ± 0.52	5.65 ± 0.85	3.03 ± 0.53
18:1n9	4.44 ± 0.79	27.68 ± 2.43	24.11 ± 2.43	8.12 ± 1.05
18:2n6	1.76 ± 0.05	2.46 ± 0.06	ND	ND
20:0	0.10 ± 0.34	4.40 ± 0.12	0.47 ± 0.12	0.43 ± 0.72
20:1	ND	11.35 ± 1.20	16.02 ± 1.19	ND
20:4n6	ND	5.20 ± 1.39	4.17 ± 1.42	9.49 ± 1.13
20:5n3	ND	4.53 ± 0.98	10.88 ± 0.95	27.53 ± 2.76
22:6n3	ND	10.03 ± 1.00	16.08 ± 1.02	38.71 ± 3.52
Others	0.27 ± 0.57	2.92 ± 0.97	1.90 ± 0.97	1.90 ± 0.16
Σsat	93.53 ± 0.51	31.12 ± 0.35	22.36 ± 0.39	10.63 ± 0.11
Σmono-unsat	4.44 ± 0.79	43.74 ± 0.82	44.13 ± 0.96	11.74 ± 0.51
Σpoly-unsat	-	19.76 ± 0.56	31.13 ± 0.25	75.73 ± 1.22
Σn6	1.76	7.66	4.17	9.49
Σn3	-	14.56	26.98	66.24
Σ(n6/n3)	1.76	0.52	0.15	0.14

Table 4e. Major constituents of the fish oils (as % total lipid)

Fish Oil constitutents	*N.raleighana* oil (as % total lipids)	*C.crepidater* oil (as % total lipids)
Squalene	58.37 ± 4.12	47.81 ± 2.84
Cholesterol	7.21 ± 1.06	4.72 ± 1.24
Glycerol Ethers	11.72 ± 1.65	12.31 ± 1.79
Vitamin E	2.19 ± 0.52	3.65 ± 1.13
Vitamin A	1.03 ± 0.29	1.58 ± 1.02

Values are mean±SD for n=6 determinations

Table 4f. Diet composition (as % total feed) of rats

Diet Components	Coconut oil (C) diet	Fish Oil (NR/CC) diet	Polyunsaturated fatty Acid (PUFA) diet
Corn starch	60.6	60.6	60.6
Casein	18.3	18.3	18.3
Salt Mixture	4.0	4.0	4.0
Vitamin mixture	1.0	1.0	1.0
Cellulose	5.0	5.0	5.0
Cholesterol	1.0	1.0	1.0
Methionine	0.1	0.1	0.1
Coconut oil	10.0	5.0	9.0
Fish oil (NR/CC)	-	5.0	-
Polyunsaturated fatty acid concentrate	-	-	1.0

Table 4g. Level of Fatty acids (as % total fatty acids) in the heart tissues of Albino rats fed CO, NR, CC and PUFA supplemented diets

Fatty acid	CO	NR	CC	PUFA
12:0	16.82 ± 0.20b	2.34 ± 0.43a	1.53 ± 0.43a	2.47 ± 0.54a
14:0	11.66 ± 0.26c	4.36 ± 0.51b	2.77 ± 0.34a	4.20 ± 0.15b
16:0	28.01 ± 1.89b	24.89 ± 0.89a	23.02 ± 1.27a	23.14 ± 1.18a
18:0	12.52 ± 0.47c	7.81 ± 0.76a	7.90 ± 0.65a	9.25 ± 0.74b
Σ saturated	**69.01 ± 0.79c**	**39.4 ± 0.58b**	**35.22 ± 0.41a**	**38.86 ± 0.42b**
16:1	4.68 ± 0.35a	9.33 ± 1.30b	8.97 ± 1.30b	7.31 ± 1.22b
18:1n9	7.37 ± 1.61a	23.56 ± 1.14c	23.71 ± 2.19c	18.45 ± 2.30b
Σ mono-unsaturated	**12.05 ± 0.18a**	**32.89 ± 0.63c**	**31.68 ± 0.62c**	**25.76 ± 0.76b**
18:2n6	5.12 ± 0.35a	4.89 ± 0.84	4.90 ± 0.72	5.36 ± 0.91
20:2	0.36 ± 0.10a	4.08 ± 2.88b	7.32 ± 1.32b	6.82 ± 0.94b
20:4n6	10.91 ± 1.83b	4.14 ± 0.52a	4.87 ± 0.33a	3.42 ± 1.64a
20:5n3	0.86 ± 0.04a	6.05 ± 1.54b	6.92 ± 1.45b	8.82 ± 1.62b
20:6n3	1.46 ± 0.82a	7.44 ± 0.93b	8.76 ± 0.78b	10.57 ± 1.72c
Σ poly-unsaturated	**18.71 ± 0.73a**	**26.6 ± 0.46b**	**32.77 ± 0.46c**	**34.99 ± 0.48d**
Σ n6	16.03 ± 1.04c	9.03 ± 0.63b	9.77 ± 0.27b	8.78 ± 0.51a
Σ n3	2.32 ± 0.55a	13.45 ± 0.67b	15.68 ± 0.47b	19.39 ± 0.07c
Σ n6/n3	6.91 ± 0.35b	0.67 ± 0.53a	0.62 ± 0.14a	0.45 ± 0.31a

Values are mean±SD for n=6 determinations

oil in preventing the cardiovascular complications. Dietary squalene and n3 fatty acids were shown to inhibit the activity of 3-hydroxy-methylglutaryl coenzyme A (HMG CoA) reductase, an endoplasmic reticulum (ER) protein, which catalyzes the rate-determining reductive deacylation of HMG CoA to mevlonate in cholesterol biosynthesis (Stansberg *et al.*, 1989). The hypocholesterolemic effect of CS oil observed in the present study is probably related to its ability to modulate cholesterol metabolism either by regulating HMG CoA reductase activity through a feedback inhibition mechanism or by increasing cholesterol esterification process, as reported earlier by Khor and Chieng (1997). Furthermore oral supplementation of fish oil has been previously reported to upregulate the fecal excretion of cholesterol and its non-polar derivatives as bile acid conjugates in experimental animals (Stansberg *et al.*, 1990; Nakamura *et al.*, 1997).

In the cardiovascular system, platelet activating factor (PAF) or plasmalogens or ether lipids may have a role in embryogenesis because it stimulates endothelial cell migration and angiogenesis and may affect cardiac function because it exhibits mechanical and electrophysiological actions on cardiomyocytes. PAF cooperates in the recruitment of leukocytes in inflamed tissue by promoting adhesion to the endothelium and extravascular transmigration of leukocytes. In the present study, the significantly high amount of ether lipids observed in the liver oils of CS compared to NR would have contributed to the lipid lowering effects of the same and boosted the immune system of the body. The finding that human heart can produce PAF, expresses PAF receptor, and is sensitive to the negative inotropic action of PAF suggests that this mediator may have a role also in human cardiovascular pathophysiology.

4.4.4.b) Level of Free fatty acids and phospholipids

Increased lipolysis of depot triglycerides liberates free fatty acids from adipose tissue stores (Kruger *et al.*, 1967; Steinberg 1976). In the present study, a significant increase ($P<0.05$) was observed in the level of free fatty acids in the serum, liver and heart tissues of CO fed rats compared to NR, CS and PUFA fed rats (Table 4b). The significant rise noticed in the levels of free fatty acids in serum and tissues of CO rats may be due to the enhanced breakdown of membrane phospholipids both in the adipose tissue and myocardium by the lipolytic action of phospholipase A2 which

could likely be the reason for cell injury and ischemia in patients with cardiovascular disorders (Farvin *et al.*,2006).

Phospholipid, measured as the levels of total inorganic phosphorus contents, varied significantly in the blood sera of CO, NR, CS and PUFA rats. The levels were significantly higher ($P<0.05$) in the CS and PUFA fed groups compared to CO and NR fed rats. CS and PUFA administration in the diets would have prevented the degradation of membrane phospholipids and hence improved membrane stability (Nev *et al.*,2009).

Intracellular calcium ions (Ca^{2+}), an inducer of phospholipase A_2, which degrades membrane phospholipids, has been reported to rise in stress-induced coronary heart diseases (Maier *et al.*, 2002; Zhang *et al.*, 1995) This presumption is further supported by studies in cultured cardiomyocytes in which inhibition of fatty acid accumulation by phospholipase inhibitors protected the cells from calcium overload and morphological damage (Jones *et al.*, 1989). Furthermore, the protective effect of phospholipase inhibitors, apart from blocking calcium influx, can also be due to its anti-oxidant activity (Freedman *et al.*, 1991; Jenkins *et al.*, 1992). In the present study CS oil and PUFA effectively raised the concentration of phospholipids in the respective animals which may be due to the phospholipase inhibitory activity of the lipids . The role of squalene, n3 fatty acids and alkyl glycerols as potential phospholipase inhibitors has been documented. (Freedman *et al.*, 1991; Jenkins *et al.*, 1992)

4.4.5 Lipid peroxides and the antioxidant defences in the heart

Biological membranes are sensitive to lipid peroxidation induced by reactive oxygen species. The oxidation of unsaturated fatty acids in biological membranes may cause impairment of membrane function, decrease in membrane fluidity, inactivation of membrane receptors and enzymes, increase of non-specific permeability to ions and disruption of membrane structure. Oxygen free radicals are implicated as mediators of tissue injury in cardiovascular pathology (Kekreja and Hess, 1992). It has been proposed earlier that free radical generation and lipid peroxidation could be involved when rats are fed high saturated fat diets which inturn lead to cardiac damage (Singal *et al.*, 1982; Sathish *et al.*, 2003).

Free radical scavenging enzymes such as catalase, superoxide dismutase, glutathione peroxidase and glutathione-S-transferase are the first line cellular defense against oxidative injury, decomposing O_2 and H_2O_2 before interacting to form the more reactive hydroxyl radical ($OH^{\bullet}$). The equilibrium between these enzymes is an important process for the effective removal of oxygen stress in intracellular organelles. The second line of the defense consists of non-enzymatic scavenging viz. sulfhydryl group containing compounds, which scavenge residual free radicals escaping decomposition by the antioxidant enzymes (Ganesan *et al.*,2008).

In the present study lipid peroxide levels varied significantly ($P<0.05$) in heart tissues of rats fed with the different diets. Their levels were significantly higher in the ($P<0.05$) PUFA rats than in NR, CS or CO rats. When PUFA and NR are compared, significantly higher ($P<0.05$) contents of peroxides were noted in the PUFA compared to NR diet, which may be attributed to the high extent of unsaturation in the PUFA (Table 4b).

Lipid peroxidation *in vivo* has been identified as one of the basic deteriorative reactions in the cellular mechanisms of aging (Nev 2009). Lipid peroxidation of membranes is regulated by the availability of substrate in the form of polyunsaturated fatty acids, the availability of inducers such as free radicals and excited state molecules to initiate propagation, the antioxidant defence status of the environment and the physical status of the membrane lipids (Farvin *et al.*, 2006). In the present study the polyunsaturated fatty acids in the biological membranes would have undergone oxidation causing impairment of membrane function, decrease in membrane fluidity, disruption of membrane structure and hence inactivation of membrane receptors and enzymes.

The antioxidant effect of CS and NR oil in the present study is probably due to the presence of isoprenoid unit in the structure of squalene and of the ether linkages in alkyl glycerols. The unpaired electron present in the hydroxyl radical ($OH^{\bullet}$) generated during stress might have been trapped for dismutation by its free radical scavenging isoprenoid units in squalene. Earlier reports (Miyachi *et al.*, 1983) indicate that subsequent to oxidative stress, such as sunlight exposure, squalene functions as an

efficient quencher of singlet oxygen and prevents the corresponding lipid peroxidation in human skin surface. The rate constant of quenching of singlet oxygen by squalene is much larger than those of other lipids, and to be comparable to 3,5-di-t-butyl-4-hydroxytolene (BHT). Reports by Kohno *et al.*, (1995) indicate that squalene is not particularly susceptible to peroxidation and is stable against attacks by peroxide radicals.

Antioxidants are well known to alleviate inflammatory processes mediated by allergic substances. The presence of natural antioxidants like vitamin E and squalene in the FO diets would have been responsible for the observed low levels of peroxides in the heart tissues of the respective animals. Numerous workers have proved the protective effects of vitamin E (Kaybay *et al.*,2009; Kobayashi *et al.*, 2009, Montilla *et al.*,2001; Vittala and Newhouse 2004) and squalene (Farvin *et al.*,2004; Farvin *et al.*,2007; Surendraraj *et al.*,2009) against oxidative stress in animals.

In the present study, significant ($p<0.05$) changes were observed in the activities of the antioxidant enzymes in the heart tissues of male albino rats fed on CO, NR, CS and PUFA enriched diets. Administration of CS and PUFA diets had a significant increase ($P<0.05$) in the levels of glutathione dependent antioxidant enzymes, glutathione peroxidise (GPx) and glutathione-S-transferase (GST) and antiperoxidative enzymes, catalase (CAT) and superoxide dismutase (SOD), in the heart tissues of the animals (Table 4c). These results are in agreement with previously reported works (Erdogan *et al.*,2004). Both CAT and GPx have complimentary roles in hydrogen peroxide detoxification Faria *et al.*, (2009). However no significant differences were observed in the vitamin E and reduced glutathione (GSH) contents between CO and PUFA fed rats, in the myocardial tissues. CS supplementation however, significantly elevated ($P<0.05$) the vitamin E and GSH contents in the respective rats. The glutathione antioxidant system plays a fundamental role in cellular defence against reactive free radicals and other oxidant species. The cellular tripeptide GSH thwarts peroxidative damage by neutralizing the free radicals. D'aquino *et al.*, (1991) observed a decrease in glutathione peroxidise activities in liver tissues of rats fed 15% coconut oil diets.

The reduction noticed in the level of GSH in CO and PUFA rats was either due to increased degradation or decreased synthesis of glutathione. New GSH may be

ecovered from the oxidized form (GSSG) by glutathione reductase, with the consumption of NADPH. The amount of NADPH may be reduced during stress, contributing to a reduction in the effectiveness of mechanism for recovering GSH. Depletion of GSH results in enhanced lipid peroxidation, and excessive lipid peroxidation can cause increased GSH consumption (Comporti, 1985), as observed in the present study. Tappel and Tappel (1997) have reported that GSH protects the mitochondrial membrane from the damaging action of lipid peroxide.

The reduction in the activity of GPX and GST in CO and NR rats may be due to the reduced availability of GSH. GPX offers protection to the cellular and sub cellular membranes from the peroxidative damage by eliminating hydrogen peroxide and lipid peroxide. Inhibition of this enzyme leads to the accumulation of these oxidants and makes myocardial cell membranes more susceptible to oxidative damage. GST is a member of a complex supergene-encoded family of detoxification enzymes found in wide variety of animal tissues. GST binds to many different lipophilic drugs (Seishi *et al.*, 1982); so it would be expected to bind to saturated fats, significantly higher in CO rats and act as an enzyme for GSH conjugation reactions. Reduction in the activity of GST ($p<0.05$) in diet induced hypercholesterolemia may be due to down regulation of glutathione-S-transferase subunits.

SOD and CAT, responsible for the destruction of peroxides, have a specific role in protecting tissues against oxidative damage (Wohaieb and Godin, 1987; Anandan *et al.*, 1998). Reduction in the activities of these enzymes can lead to the formation of O_2^- and H_2O_2, which in turn can form hydroxyl radical (OH $^\bullet$) and bring about a number of reactions harmful to the cellular and subcellular membranes (Kalra *et al.*, 1988). Reduction in the activities of the antiperoxidative enzymes in diet induced hypercholesterolemia may be due to the increased generation of reactive oxygen radicals such as superoxide and hydrogen peroxide, which in turn leads to the inactivation of these enzymes. SOD contains arginine and histidine residues in its active site. The free radicals formed during stressed conditions may attack on these highly reactive amino acids, which might result in the chemical modification of the amino acids with loss of activity.

The animals fed with CS oil and NR oil in the present study showed a significant ($p<0.05$) reduction in the level of lipid peroxidation along with a marked rise in the activities of superoxide dismutase and catalase, thus indicating the antioxidant

nature of the fish oil in experimentally induced oxidative stress condition. There are several molecules that directly scavenge free radicals, including many ingested antioxidants such as vitamin E, alkylglycerols, squlaene and n3 fatty acids. Of particular relevance to this present study, recent research has revealed that squalene and alkylglycerol, both endogenous and exogenous, may help to neutralize free radicals (O' Sullivan *et al.*, 2002). Squalene is highly lipophilic and, when administered exogenously, it can readily pass across the cellular and subcellular membranes (Kamimura *et al.*, 1989). The ability of squalene to diffuse into intracellular compartments helps in the capabilities of this isoprenoid as a potent antioxidant (Kohno *et al.*, 1995).

Within the last decade a large number of articles appeared dealing with various aspects of the antioxidative activity of alkyl glycerols or plasmalogens. Again their vinyl ether bond is target of oxidative attack by reactive oxygen species (ROS), e.g. singlet oxygen, superoxide anion, hydrogen peroxide and peroxy radicals. In the present study, the presence of unsaturated double bonds in n3 PUFAs raised the level of attacks by free radicals and oxidative species and the absence of antioxidant vitamins and alkylglycerols and squalene to counter act their attacks led to elevated levels of peroxides in PUFA group.

4.4.6 The myocardial fatty acid composition of rats on different isolipidemic diets

Fatty acids play an integral role in determining the structural and functional properties of cellular and subcellular membranes of the myocardium. Their influence on fluidity and stability of membrane structure markedly impacts on membrane functions such as transport of the ions and substrates, and elctrophysiology, which is intrinsic to cardiac function and cardiac excitability (Van Bilsen *et al.*, 1998). In addition to the structural and functional roles played within the myocardial cell membrane, fatty acids and associated lipids are also recognized as regulatory molecules with roles in cell signaling, as second messengers in transduction, and as effectors in apotosis (programmed cell death) in response to oxidative and ischemic damage (Van der Vusse *et al.*, 1992; Diep *et al.*, 2000). Apoptosis plays a prominent role in the myocyte loss that occurs in myocardial infarction (Narula *et al.*, 1996; Olivetti *et al.*, 1996 & 1997). It is also involved in the extensive cardiac remodeling

that encompasses the transition from cardiac hypertrophy to heart failure (Li *et al.*, 1997).

Table 4g depicts the myocardial fatty acid composition of rats fed CO, FO and PUFA enriched diets. Significant changes were observed in the fatty acid composition of the heart tissues of rats fed CO diets compared to those on NR, CS and PUFA diets. Significant rise ($P<0.05$) in saturated fatty acids (12:0, 14:0, 16:0, 18:0) were observed in the heart tissues of rats on CO diets compared to those on the other diets. The monounsaturated fatty acid composition of NR, CS and PUFA ($P<0.05$) fed rats were significantly higher than CO rats, the former two groups being richer in oleic acid contents. Oleic acid is potential hypotensive agent and may hinder the progression of adrenoleukodystrophy, a fatal disease that affects the brain and adrenal glands (Rizzo 1986).

Rats fed on NR, CS and PUFA diets showed increased levels of polyunsaturated fatty acids in their heart tissues. There was a significant increase in the levels of arachidonic acid (20:4n6), in the CO fed group compared to CS, NR and PUFA groups. EPA (20:5n3) and DHA (22:6n3) levels in CS, NR and PUFA groups rose significantly ($P<0.05$) than in the CO group; EPA and DHA levels being higher in the PUFA group than in the others. The results are in close agreement to the reports of Castillo *et al.*, 1999 who observed reduction in arachidonic acid levels and cholesterol in plasma of chicks fed menhaden oil.

The increase observed in the levels of unsaturated fatty acids might be either due to the residual uptake of fatty acids from extracellular sources such as blood and adipose tissue and their accumulation caused by reduced use in the mitochondrial oxidation (van der Vusse *et al.*, 2000) or due to the action of phospholipases on membrane lipids (Van der Vusse *et al.*, 1992). During restriction of flow (ischemia), fatty acid homeostasis is severely disturbed because of oxygen deprivation. Accumulation of nonesterified fatty acids and their metabolites occur because of diminishing mitochondrial oxidation and respiratory chain activity. Moreover the accumulation of nonesterified arachidonic acid signals the beginning of a chain of events that include eicosanoid synthesis (Van der Vusse *et al.*, 1997). Reports by Nair *et al.*, (1997) show that prostaglandins and leukotrienes derived from arachidonic acid influence arrhythmogenesis and the electrophysiological properties

of ischemic tissue. Arrhythmia occurs when certain ion (sodium and calcium) channels in the mocyte become hyperactive and fatty acids can act as messenger molecules that regulate these ion channels (Hallaq *et al.*, 1992; Kang and Leaf, 1996).

Epidemiological studies indicate that EPA and DHA have been reported to possess cardioprotective action (Harper *et al.*, 2006; Hirafuji *et al.*, 2003; Singh *et al.*, 2001; Leaf and Kang, 1997). The protective effects of n-3 PUFA are attributable to their direct effects on vascular endothelial and smooth muscle cell (VSMC) functions (Abeywardena and Head, 2001). The greater effect of DHA was considered to be due to its greater ability to decrease membrane cholesterol content and/or the cholesterol/phospholipids molar ratio and also to its greater ability to evaluate the unsaturated index in the plasma membrane (Dusserre et al., 1995). These physicochemical alterations in the membrane properties may directly or indirectly influence functions of membrane-bound proteins such as receptors, GTP binding proteins, ion channels and various enzymes. Persistant cellular oxidative stress and enhanced peroxidation of PUFAs, leading to to macromolecular damage and disruption of signaling pathways are known to to stimulate CVD (Meerson et al., 1993). Thus the decline in the level of these fatty acids in CO diets might have rendered the myocardium more susceptible to stress related injury.

4.4.7 The significance of n6/n3 fatty acid ratio

Clinical studies (Okuyama 2001, Griffin 2008) have indicated that the ingested ratio of *n*−6 to *n*−3 (especially Linoleic vs Alpha Linolenic) fatty acids is important in maintaining cardiovascular health. Both *n*−3 and *n*−6 fatty acids are essential, i.e. humans must consume them in the diet. *n*−3 and *n*−6 compete for the same metabolic enzymes, thus the *n*−6:*n*−3 ratio will significantly influence the ratio of the ensuing eicosanoids (hormones), (e.g. prostaglandins, leukotrienes, thromboxanes etc.), and will alter the body's metabolic function (Tribole 2006). Metabolites of *n*−6 are significantly more inflammatory (esp. arachidonic acid) than those of *n*−3. This necessitates that *n*−3 and *n*−6 be consumed in a *balanced proportion*; healthy ratios of *n*−6:*n*−3 range from 1:1 to 4:1 (Lands and William 1992).

In the present study n6:n3 ratio in CO animals was 6.9:1 as compared to 0.7:1,0.6:1 and 0.4:1 in NR, CS and PUFA animals respectively. This implied that NR, CS and PUFA diets provide balanced amounts of n6 and n3 fatty acids compared to CO diets.

4.4.8 Correlations

Pearson's correlation coefficient test (Table 4h) was done to analyse the effect of feeding a fish oil diet on the various lipid and antioxidant components and peroxide levels in the blood serum of Albino rats. Significant ($P<0.05$) and positive correlations were observed between saturated fatty acid and triglyceride/cholesterol and between lipid peroxides and n3 PUFA whereas the correlations were found to be significant ($P<0.05$) and negative between n3 PUFA and triglyceride/cholesterol, lipid peroxides and vitamin E and between phospholipids and cholesterol. These results are in partial agreement with reports by other workers. (Ali *et al.*,2002; Nagyova *et al.*,2003; Leen *et al.*,2005).

Table 4h. Pearson's Correlation tests

CS oil diet	TG	Cho	P	SFA	n3PUFA	Vit E	SOD	LPx
TG	1.00	0.50	-0.50	0.50#	-1.00*	-1.00*	-0.50#	0.50
Cho	0.50	1.00	-1.00*	0.50*	-1.00*	-0.50#	-0.50#	0.50
P	-0.50	-1.00*	1.00	-0.50	1.00	0.50	0.50	1.00
SFA	0.50#	0.50*	-0.50	1.00	-0.50	-0.50	-0.50	-1.00
n3PUFA	-1.00*	-1.00*	1.00	-0.50	1.00	1.00#	0.50	1.00*
Vit E	-1.00*	-0.50#	0.50	-0.50	1.00#	1.00	1.00*	-1.00*
SOD	-0.50#	-0.50#	0.50	-0.50	0.50	1.00*	1.00	-1.00*
LPx	0.50	0.50	1.00	-1.00	1.00*	-1.00*	-1.00*	1.00

TG=triglyceride, Cho=cholesterol, P=phospholipids,SFA=saturated fatty acids, n3pufa= n3 polyunsaturated fatty acids,vit E = vitamin E, SOD=superoxidedismutase, LPx=lipid peroxides

N=6, #correlation significant at 0.05 level.*correlation significant at 0.001 level.

4.5 SUMMARY AND CONCLUSION

The role played by liver oils of *Centrophorus scalpratus* and *Neoharriotta raleighana* on the lipid profile and antioxidant defence status in blood, liver and heart tissues of male Albino rats was evaluated. The results of the present study indicated that consuming a coconut oil diet with a partial replacement by fish oil can significantly lower the LDL or bad cholesterol and lipid peroxide contents and can significantly improve the antioxidant defence status in the blood and heart tissues of Albino rats. The reduced levels of diagnostic marker enzymes, triglycerides, LDL cholesterol, lipid peroxides and enhanced levels of phospholipids, HDL cholesterol, antioxidants such as vitamin E and GSH, in the blood serum and heart tissues of albino rats upon consumption of FO diets could be attributed to the presence of anti-inflammatory LC-PUFAs and vitamin E in the fish oils. Hence the protective effects of fish oil on the risk of CVD may be due to the synergistic effects of the nutrients in fish and not solely to the presence of LC-PUFAs. These results support the notion that fish oils may be effective dietary supplements in the management of various diseases in which oxidant/antioxidant defense mechanisms are decelerated. The salient findings of the study were as follows.

There was no significant difference in body weights of animals fed any of the isolipidemic diets however animals on CS and PUFA diets had better feed conversion ratios compared to those on CO and NR diets. Though there was an increase in liver and heart weights among animals on NR and PUFA diets, the results were not significant.

Diagnostic marker enzyme levels AST, ALT and LDH were significantly higher among animal groups fed CO diets compared to others. CO diets rich in saturated fats would have raised the oxidative stress in the body and hence a corresponding increase in the levels of the marker enzymes was observed.

There was a significant rise in the cholesterol and triglyceride levels in the blood of CO and NR rats compared to the others which might be due to the increased uptake of LDL-cholesterol from the blood by the hepatic membranes.

The animals fed with CS oil and NR oil in the present study showed a significant ($p<0.05$) reduction in the level of lipid peroxidation along with a marked rise in the

activities of superoxide dismutase and catalase, thus indicating the antioxidant nature of the fish oil in experimentally induced oxidative stress condition.

Further there was a significant rise in the saturated fatty acid content in the myocardial membranes of CO rats compared to the others but a simultaneous rise in mono-unsaturated fatty acid content in the tissues of NR and CS rats. Thus the protective effects of the studied fish oils against inflammation and injury may be partly due to the protective effects of the mono-unsaturated fatty acids.

Since diet-induced hypercholesterolemia is one of the major cardiovascular diseases affecting about 2 million people in India alone, it is high time that we switch on to a better and healthy lifestyle. Avoiding or limiting saturated fats in the diet not only decreases the risk of atherosclerosis and other CVDs but also increases the longevity of the individual. These days, more emphasis has been laid on the use of fish oils as agents in reducing cholesterol levels. But one must be cautious on the use of fish oils for pharmaceutical purposes as not all fish oils have the same functional values. The fish oils which may serve as anti-inflammatory and analgesic agents may not be effective in lowering cholesterol or triglyceride levels in the body. On the contrary they may even raise these bad fats which may lead to fatal consequences. Moreover, polyunsaturated fatty acid concentrates must not be consumed above the prescribed or recommended dosages since these too may have unwanted complications like elevations in peroxide levels owing to presence of unsaturated bonds in their structures. Care must also be taken to ensure that PUFA concentrates are consumed within the shelf life period. To conclude it is advisable to consume whole fish rich in PUFA sources or marine fishes for health benefits rather than isolating components of fish oil since the pharmacologic action of the fish oil results from the synergistic contribution of the lipid bioactives and not solely to the presence of any particular component.

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Chapter 5.

Effect of Centrophorus scalpratus liver oil against CFA-induced arthritis in Albino rats

Chapter 5. Effect of *Centrophorus scalpratus* liver oil against CFA-induced rheumatoid arthritis in Albino rats

5.1 INTRODUCTION

Rheumatoid arthritis is a systemic autoimmune disease that causes chronic inflammation of the joints. Autoimmune diseases are illnesses that occur when the body's tissues are mistakenly attacked by their own immune system (Shlotzhauer and McGuire, 2003). The immune system contains a complex organization of cells and antibodies designed normally to "seek and destroy" invaders of the body, particularly infections. Patients with autoimmune diseases have antibodies in their blood that target their own body tissues, where they can be associated with inflammation. Because it can affect multiple other organs of the body, rheumatoid arthritis is referred to as a systemic illness and is sometimes called rheumatoid disease.

The joint inflammation of rheumatoid arthritis causes swelling, pain, stiffness, and redness in the joints. The inflammation of rheumatoid disease can also occur in tissues around the joints, such as the tendons, ligaments, and muscles. In some people with rheumatoid arthritis, chronic inflammation leads to the destruction of the cartilage, bone, and ligaments, causing deformity of the joints. Damage to the joints can occur early in the disease and be progressive. Moreover, studies have shown that the progressive damage to the joints does not necessarily correlate with the degree of pain, stiffness, or swelling present in the joints.

Rheumatoid arthritis is one of the most potential debilitating inflammatory disorders that affect more than 2.5 million Americans with 200,000 new cases every year and over 7 million cases/year in India, according to current census data. The disease is three times more common in women than in men. It afflicts people of all races equally. The disease can begin at any age, but it most often starts after 40 years of age and before 60 years of age. In some families, multiple members can be affected, suggesting a genetic basis for the disorder. It has been reported that 33 to 75% of rheumatoid arthritis patients believe that food plays an important role in their symptom severity and 20 to 50% try dietary manipulation as an attempt to relieve suffering (Salminen *et al.,* 2002).

Even though non-steroidal anti-inflammatory drugs (NSAIDS) and disease modifying anti-rheumatic drugs (DMARDS) are available today for treatment of

inflammatory disorders they have their side effects. These drugs react by blocking the activity of cyclooxygenases (COXs) during an inflammation. COX is a bifunctional enzyme exhibiting both COX and peroxidase activites. While the COX component converts arachidonic acid to a hydroperoxy endoperoxide, the peroxidase component reduces the endoperoxide to the corresponding alcohol, the precursor of thromboxanes and prostaglandins. There are two isoforms of COX. COX I is expressed in a variety of cell types and is involved in normal cellular homeostasis. A variety of mitogenic stimuli such as phorbol esters, lipopolysaccharides and cytokines lead to the induced expression of a second isoform of COX – COX II. COX II is responsible for the biosynthesis of prostaglandins under acute inflammatory conditions. This inducible COX II is believed to be the target enzyme for the anti-inflammatory activity of NSAIDS/DMARDS. However prolonged use of NSAIDS can bring about anemia, dyspepsia, erosions, gastric ulcers and renal dysfunction, adverse reactions of DMARDS can lead to nausea & stomatitis, bone marrow suppression, liver disease & interstitial pneumonitis.

Scientists throughout the world are studying many promising areas of new treatment approaches for rheumatoid arthritis. These areas include treatments that block the action of the special inflammation factors, such as tumor necrosis factor (TNFalpha) and interleukin-1 (IL-1). Many other drugs are being developed that act against certain critical white blood cells involved in rheumatoid inflammation. The need for natural anti-inflammatory agents that can selectively block the activity of COX II during an inflammation is increasing, owing to the enhanced disease resistance and little or no side effects upon prolonged usage.

Natural remedies for arthritis are safer and better tolerated by the body than conventional arthritis drugs. These medications go beyond pain relief and look at the whole body with the aim of healing and reversing disease, rather than simply masking the symptoms. Clinical trials on rheumatoid arthritis patients who relied on fish oil as a supplement of their diet showed decreased joint tenderness, joint pain, swelling and morning stiffness (Kremer *et al.,* 1990). The oleoresin from ginger, gingerol, has long been used in traditional medical practices to reduce inflammation. In fact, many health care professionals use ginger to help treat health problems associated with inflammation, such as arthritis and ulcerative colitis.

The use of such natural remedies for arthritis is being promoted world wide on the basis of their traditional use as an anti-inflammatory agents in Chinese and Ayurvedic medicine. However, scientific evidence of the antiarthritic effects of liver oils of *C.scalpratus* and gingerol is sparse. In view of this, the present study was designed to study the efficacy of *Centrophorus scalpratus* liver oil against Complete Freund's Adjuvant (CFA)-induced arthritis in male Albino rats and to compare the anti-inflammatory activity of the fish oil with a traditionally used anti-inflammatory substance gingerol. The brief objectives of the study and the parameters selected for the experiment are as below.

1. To study the effect of feeding *C.scalpratus* liver oil in comparison to gingerol on paw-sizes in CFA-induced arthritis in male Albino rats.
2. To compare the effects of feeding *C.scalpratus* liver oil and gingerol extracts on the levels of inflammatory mediators, of myeloperoxidases, nitric oxides (as sum total of nitrite and nitrate levels) and cyclooxygenases, in the inflamed paw tissues of both treated and untreated groups of animals.
3. To study the effect of feeding fish oil and gingerol extracts on changes in liver during CFA-induced arthritis in Swiss albino mice :
    a) To determine the changes in food intake, body weight, liver weight, levels of albumin, globulin, glucose, creatinine, urea, glycoproteins and activities of marker enzymes and electrophorectic pattern of plasma proteins, as secondary indices to measure inflammation.
    b) To study the activities of membrane bound ATPase enzymes and levels of cell surface glycoprotein components to assess the impact of fish oil and gingerol extracts on cell membrane integrity during inflammation.
    c) To estimate the activities of enzymes such as glucose-6-phosphatase, fructose -1, 6- diphosphatase, aldolase, hexokinase and levels of tissue glycogen, protein and free amino acids to assess the changes in the carbohydrate and protein metabolisms during inflammaiton and role of the *C.scalpratus* liver oil and gingerol extracts on these metabolisms.
    d) To assess the content of adenosine triphosphate (ATP) which serve as an important source of energy during inflammation.

e) To determine the levels of triglycerides, free fatty acids, cholesterol, lipoproteins in plasma and liver tissues, to assess the effect of fish oil and gingerol extracts on lipid metabolism during inflammation.

f) To evaluate the levels of lipid peroxides, antioxidants such as glutathione, tocopherol, ascorbic acid etc and activities of antioxidant enzymes to assess the effect of fish oil and gingerol extracts on oxidant and antioxidant status in inflamed tissues.

4. To determine the effect of *C.scalpratus* liver oil and gingerol extracts on architecture of liver tissues during CFA-induced inflammation, by conducting histopathological studies.

5.2 REVIEW OF LITERATURE

5.2.1 Arthritis

Arthritis is the most common kind of inflammatory disorder seen in majority of the human race. It is an inflammation of the joints accompanied with pain and stiffness in the affected areas (Rostamian *et al.*, 2007). Pain is generally defined as either "acute" or "chronic" with acute pain being the short-term variety. After three months, the pain is considered to be chronic. Both these types of pain can be mild to severe and for many people suffering from arthritis, chronic pain is present in their activities of daily living. Indeed, many people deal with pain for the rest of their lives. The majority of the human population does not know that there is more than one type of arthritis. Most people think that arthritis is a disease in itself that exhibits all joint disorders. Not only are there many other kinds of joint disorders but also, there are many known types. The only common characteristic amongst all the different types is that they all feature pain (Shlotzhauer and McGuire, 2003). The pain itself may vary in terms of the location of the joints affected. Another characteristic of the types that vary is that it may be progressive or non-progressive. Progressive means that the disease affects the person on a continuing basis during the person's whole lifetime. Non-progressive, on the other hand, means that it can be cured by treatment or surgery.

The different types are subdivided into three: primary form, secondary groups and disease that mimic arthritis. Examples of primary form of arthritis are: Still's disease, ankylosing spondylitis, septic arthritis, osteoarthritis, juvenile arthritis, gouty arthritis, and rheumatoid arthritis. These diseases directly affect the joints and surrounding area. The secondary groups are: psoriatic arthritis, reactive arthritis, systemic lupus, erythematosus, Hencoch-Schonlein Purpura, hermachromatosis, and others. These diseases occur when preceded by another illness. Diseases that mimic arthritis are as follows: hypertrophic osteoarthropathy, multiple myeloma, osteoporosis, and fifth disease. Some causes of these diseases are already known and studied while others are still unknown. Treatment varies with the different types of arthritis. In worst cases, affected people will have to undergo surgery; others just might be treated with simple and traditional therapy. Studies have shown that physical exercise of the affected joint may bring forth improvement in a long-term basis. Cure of the disease is the primary goal for others while some may just opt for

relief from the pain. Some may be lucky enough to have their ailment cured while others will have to endure the disease itself for the remainder of their days. Whatever the case, the affected people will have to make changes with their lifestyle.

5.2.2 Chronic arthritis

Chronic pain is a major health problem in the United States and is one of the most weakening effects of arthritis. More than 40 million Americans suffer from some form of arthritis, and many have chronic pain that limits daily activity. Osteoarthritis is by far the most common form of arthritis, affecting about 16 million Americans, while rheumatoid arthritis, which affects about 2.1 million Americans, is the most crippling form of the disease.

Unfortunately, these numbers are on the rise as the population ages. Many people may find themselves dealing with arthritic moments along with their senior moments. However, just because a body is aging, it does not follow that that body must have joint pain. Healthcare providers, from primary care physicians to physical therapists and rheumatologists, are recognizing the value of exercise as part of a plan to relieve arthritis pain, even in fairly severe, chronic cases! It is a natural inclination to want to rest and move around as little as possible when we are in pain, when in fact, it can actually make matters worse for those with arthritis pain. Joint pain relief is gaining attention in the supplement aisle. It is probably safe to assume that most people are aware of glucosamine and chondroitin and the difference that these two substances are making in the lives of joint pain sufferers. One of the newer choices hitting the shelves is made from cetylated fatty acids, and is most commonly found under the brand name of Celadrin. This alternative has been shown to work well all on its own or as an addition to a glucosamine/chondroitin regimen. As with so many supplements it appears to become more effective as use continues. These supplements work to restore lubrication and reduce inflammation in the joints, providing pain relief and better range of motion.

5.2.3 Causes of rheumatoid arthritis

The cause of rheumatoid arthritis is a very active area of worldwide research. Even though infectious agents such as viruses, bacteria, and fungi have long been suspected, none has been proven as the cause. It is believed that the tendency to

develop rheumatoid arthritis may be genetically inherited. It is also suspected that certain infections or factors in the environment might trigger the activation of the immune system in susceptible individuals. This misdirected immune system then attacks the body's own tissues. This leads to inflammation in the joints and sometimes in various organs of the body, such as the lungs or eyes. Regardless of the exact trigger, the result is an immune system that is geared up to promote inflammation in the joints and occasionally other tissues of the body. Immune cells, called lymphocytes, are activated and chemical messengers (cytokines, such as tumor necrosis factor/TNF, interleukin-1/IL-1, and interleukin-6/IL-6) are expressed in the inflamed areas (Rubin J, 2007). Environmental factors also seem to play some role in causing rheumatoid arthritis. For example, scientists have reported that smoking tobacco increases the risk of developing rheumatoid arthritis.

5.2.4 Symptoms and signs

The symptoms of rheumatoid arthritis come and go, depending on the degree of tissue inflammation. When body tissues are inflamed, the disease is active. When tissue inflammation subsides, the disease is inactive (in remission). Remissions can occur spontaneously or with treatment and can last weeks, months, or years. During remissions, symptoms of the disease disappear, and people generally feel well. When the disease becomes active again (relapse), symptoms return. The return of disease activity and symptoms is called a flare. The course of rheumatoid arthritis varies among affected individuals, and periods of flares and remissions are typical.

When the disease is active, symptoms can include fatigue, loss of energy, lack of appetite, low-grade fever, muscle and joint aches, and stiffness. Muscle and joint stiffness are usually most notable in the morning and after periods of inactivity. Arthritis is common during disease flares. Also during flares, joints frequently become red, swollen, painful, and tender. This occurs because the lining tissue of the joint (synovium) becomes inflamed, resulting in the production of excessive joint fluid (synovial fluid). The synovium also thickens with inflammation (synovitis).

In rheumatoid arthritis, multiple joints are usually inflamed in a symmetrical pattern (both sides of the body affected). The small joints of both the hands and wrists are often involved. Simple tasks of daily living, such as turning door knobs and opening jars, can become difficult during flares. The small joints of the feet are also

commonly involved. Occasionally, only one joint is inflamed. When only one joint is involved, the arthritis can mimic the joint inflammation caused by other forms of arthritis, such as gout or joint infection. Chronic inflammation can cause damage to body tissues, including cartilage and bone. This leads to a loss of cartilage and erosion and weakness of the bones as well as the muscles, resulting in joint deformity, destruction, and loss of function.

Since rheumatoid arthritis is a systemic disease, its inflammation can affect organs and areas of the body other than the joints. Inflammation of the glands of the eyes and mouth can cause dryness of these areas and is referred to as Sjogren's syndrome. Rheumatoid inflammation of the lung lining (pleuritis) causes chest pain with deep breathing, shortness of breath, or coughing. The lung tissue itself can also become inflamed, scarred, and sometimes nodules of inflammation (rheumatoid nodules) develop within the lungs. Inflammation of the tissue (pericardium) surrounding the heart, called pericarditis, can cause a chest pain that typically changes in intensity when lying down or leaning forward. The rheumatoid disease can reduce the number of red blood cells (anemia) and white blood cells. Decreased white cells can be associated with an enlarged spleen (referred to as Felty's syndrome) and can increase the risk of infections (Pemberton and Osgood RB, 2009). Firm lumps under the skin (rheumatoid nodules) can occur around the elbows and fingers where there is frequent pressure. Even though these nodules usually do not cause symptoms, occasionally they can become infected. Nerves can become pinched in the wrists to cause carpal tunnel syndrome. A rare, serious complication, usually with long-standing rheumatoid disease, is blood vessel inflammation (vasculitis). Vasculitis can impair blood supply to tissues and lead to tissue death (necrosis). This is most often initially visible as tiny black areas around the nail beds or as leg ulcers.

5.2.5 Cancer and inflammation

An association between the development of cancer and inflammation has long-been appreciated (Rakoff-Nahoum, 2006). The inflammatory response orchestrates host defenses to microbial infection and mediates tissue repair and regeneration, which may occur due to infectious or non-infectious tissue damage. Epidemiological evidence points to a connection between inflammation and a predisposition for the

development of cancer, i.e. long-term inflammation leads to the development of dysplasia. Epidemiologic studies estimate that nearly 15 percent of the worldwide cancer incidence is associated with microbial infection (Kuper *et al.*,2000). Chronic infection in immunocompetent hosts such as human papilloma virus or hepatitis B and C virus infection leads to cervical and hepatocellular carcinoma, respectively. In other cases, microbes may cause cancer due to opportunistic infection such as in Kaposi's sarcoma (a result of human herpes virus (HHV)-8 infection) or inappropriate immune responses to microbes in certain individuals, which may occur in gastric cancer secondary to *Helicobacter pylori* colonization or colon cancer because of long-standing inflammatory bowel disease precipitated by the intestinal microflora. In many other cases, conditions associated with chronic irritation and subsequent inflammation predispose to cancer, such as the long-term exposure to cigarette smoke, asbestos, and silica.

Observing signs of inflammation, such as leukocyte infiltration, at tumors infected with microbes or sites of chronic irritation is expected. However, as first observed by Virchow in the middle of the 19th century, many tumors for which infection or irritation are not necessarily a predisposing factor, such as mammory adenocarcinoma, show a "lymphoreticular infiltrate." Many tumors of this type contain activated fibroblasts and macrophages, in addition to a gene expression profile with an inflammatory signature. Quantitative aspects of wound repair or inflammatory gene expression often correlate negatively with cancer stage and prognosis (Wang *et al.*, 2006). Further evidence for the role of inflammation has come from the use of non-steroidal anti-inflammatory drugs (NSAIDs) in the prevention of spontaneous tumor formation in people with familial adenomatous polyposis (FAP) (Ulrich *et al.*, 2006) Thus, cancer and inflammation are related by epidemiology, histopathology, inflammatory profiles, and the efficacy of anti-inflammatory drugs in prophylaxis. These observations have provided impetus for investigation and hypothesis on the mechanisms and semantics of the relationship between cancer and inflammation.

There is evidence to suggest the inflammatory and immune systems may inhibit the development of cancer. This may occur by two cancer-associated recognition events. In tumor immunosurveillance, the host may have a dedicated mechanism to perceive and eliminate transformed cells. Adaptive immune recognition of tumor-associated and specific antigens also may be an important

means by which the immune system controls the development of cancer. However, it seems the net effect of the inflammatory system is to positively affect tumor development. The relationship between cancer and inflammation is not simple and cannot be reduced to one grand theory. Previous reviews have focused on various aspects of the relationship between cancer and inflammation, such as the role of various inflammatory cells, mediators, or signaling pathways in cancer (Rakoff-Nahoum, 2006).

5.2.6 Inflammation can cause cancer

As stated, long-standing inflammation secondary to chronic infection or irritation predisposes to cancer. In the case of some types of viral infection, it is clear that virally encoded genes can contribute to cellular transformation. An example of this are the transformative abilities of high risk HPV mediated by the oncoproteins E6 and E7 (Munger and Howley, 2002). However, many microbes associated with cancer cannot transform cells. For example, while certain strains of *H. pylori* contain factors that affect host cell signaling, they are not classical oncogenes (Peek and Blaser, 2002).

The chronic inflammatory states associated with infection and irritation may lead to environments that foster genomic lesions and tumor initiation. One effector mechanism by which the host fights microbial infection is the production of free radicals such as reactive oxygen intermediated (ROI), hydroxyl radical (OH•) and superoxide (O_2-•) and reactive nitrogen intermediates (RNI), nitric oxide (NO•) and peroxynitrite (ONOO-). Primarily thought to be anti-microbial, these molecules form due to the activities of host enzymes such as myeloperoxidase, NADPH oxidase, and nitric oxide, which are regulated by inflammatory signaling pathways. Importantly, ROI and RNI lead to oxidative damage and nitration of DNA bases which increases the risk of DNA mutations (Hussain *et al.*,2002). Cells have intrinsic mechanisms by which to prevent unregulated proliferation or the accumulation of DNA mutations. These include tumor suppressor pathways that mediate DNA repair, cell cycle arrest, apoptosis and senescence. In the face of DNA damage or oncogenic activation, cells will either repair their DNA and prevent mutations or initiated cells will undergo cell death.

In the face of massive cell death as occurs in infection or non-infectious tissue injury, lost cells must be repopulated by the expansion of other cells, often undifferentiated precursor cells such as tissue stem cells. There are two requisites for this: Some cells must survive the injury, and cells must expand to maintain cell numbers for a proper functioning tissue. Many inflammatory pathways function to mediate these two prerequisites of tissue repair (Rakoff-Nahoum, 2006). In an extension of its physiologic role in mediating tissue repair or as a strategy in host defense to infection, the inflammatory response may play a role in providing survival and proliferative signals to initiated cells, thereby leading to tumor promotion.

Tumor promotion requires not only the survival of initiated cells, but also their expansion. Many inflammatory mediators such as cytokines, chemokines, and eicanosoids are capable of stimulating the proliferation of both untransformed and tumor cell proliferation. Mice deficient in TNF have fewer skin tumors upon administration of the phorbol ester TPA and the mutagen DMBA. Investigation into how TNF regulates tumor progression in this model suggested that this inflammatory mediator acts as a tumor promotor, since soon after application of TPA/DMBA, the characteristic hyperproliferation of keratinocytes was shown to be dependent on TNF. What induces this production of inflammatory mediators, such as TNF, which leads to expansion of tumor initiated cells? NF-κB activation in myeloid cells recently was shown to play a critical role in the production of inflammatory mediator in both the AOM/DSS model of CAC and mutagen-induced hepatocellular carcinoma upon administration of diethylnitroseamine (DEN). In both of these models, when myeloid cells were defective in activating NF-κB via the classical pathway, there was impaired production of inflammatory mediators, proliferation of dysplastic epithelium, and a decrease in both the frequency and size of tumors compared to WT mice (Maeda *et al.*,2005). Many of these factors, such as IL-6, are required for hepatic regeneration after injury. An important finding of the DEN study was that when NF-κB was impaired in hepatocytes, there was increased epithelial cell death, yet a increased tumor burden. This finding suggests that myeloid cells may lead to proliferation of initiated cells by detecting epithelial cell death. Thus, in the presence of initiation and both tissue injury and massive cellular death, activation of an inflammation dependent tissue repair/compensatory proliferative response leads to tumor promotion.

Recent data have expanded the concept that inflammation is a critical component of tumour progression. Many cancers arise from sites of infection, chronic irritation and inflammation (Coussens and Werb, 2002). It is now becoming clear that the tumour microenvironment, which is largely orchestrated by inflammatory cells, is an indispensable participant in the neoplastic process, fostering proliferation, survival and migration. In addition, tumour cells have co-opted some of the signalling molecules of the innate immune system, such as selectins, chemokines and their receptors for invasion, migration and metastasis. These insights are fostering new anti-inflammatory therapeutic approaches to cancer development.

5.2.7 The inflammatory mediators: COX, NO and free radicals

5.2.7.a) The cylcooxygense (COX) enzyme

Cyclooxygenase (COX) is an enzyme (EC 1.14.99.1) that is responsible for formation of important biological mediators called prostanoids (Patrico, *et al.*, 2001), including prostaglandins, prostacyclins and thromboxanes. Pharmacological inhibition of COX can provide relief from the symptoms of inflammation and pain. Non-steroidal anti-inflammatory drugs such as aspirin and ibuprofen, exert their effects through inhibiton of COX. The names "prostaglandin synthase (PHS)" and "prostaglandin endoperoxide synthetase (PES)" are still used to refer to COX. Prostaglandins are produced following the sequential oxidation of arachidonic acid (AA), gamma linolenic acid (GMA) or eicosapentaenoic acid (EPA).

5.2.7.b) The Discovery of Two Isoforms of COX

In the early 1990s, evidence emerged from two lines of investigation suggesting that there were two different COX enzymes (Xie *et al.*, 1991; Masferrer *et al.*,1990 ; Kubuju *et al.*, 1991). Investigators studying cell growth discovered a new gene product that was induced in vitro and that exhibited great similarity to COX. At the same time, other investigators were discovering that COX activity could be induced by cytokines such as interleukin-1 (IL-1) and inhibited by corticosteroids. Steroids inhibited the interleukin-1 induced COX activity but not basal COX activity. These observations led to the hypothesis that there are two COX isoenzymes. One COX enzyme was theorized to be constitutively (constantly) expressed and responsible for basal prostaglandin(PG) production, while the second COX enzyme was induced by

inflammatory stimuli such as interleukin-1 and suppressed by glucocorticoids. Indeed, these two distinct COX genes have now been cloned and localized to different chromosomes.

COX-1, the name assigned to the constitutively expressed enyzme, is found in nearly all tissues and cells. COX-2, the inducible enzyme, is responsible for the profound increase in prostanoid synthesis and release that occurs at sites of inflammation. COX-2 expression is tightly regulated and its mRNA transcript appears to be highly unstable.

5.2.7.c) Differential Expression of COX-1 and COX-2

COX-1 and COX-2 serve identical functions in catalyzing the conversion of arachidonic acid to prostanoids. The specific prostanoid(s) generated in any given cell is not determined by whether that cells expresses COX-1 or COX-2, but by which distal enzymes in the prostanoid synthetic pathways are expressed. For example, stimulated human synovial cells synthesize small amounts of PGE_2 and prostacyclin but not thromboxanes. Following exposure to interleukin-1, synovial cells make considerably more PGE_2 and prostacyclin, but they still do not synthesize thromboxanes. (Bathon *et al.*,1996). The IL1-induced increase in PGE_2 and prostacyclin is mediated through COX-2 (Crofford *et al.*,1994).

Thus, while the *species* of prostanoid synthesized in a cell is dependent upon the specific distal synthetic enzyme(s) expressed, the *amount* synthesized is determined by the amount of COX-1 and -2 activities expressed. COX-1 is expressed in nearly all cells (except red cells) in their basal (unstimulated) states, suggesting that low levels of prostanoids are important in serving critical physiological (homeostatic) functions. COX-1 mediated production of prostaglandins in the stomach serves to protect the mucosa against the ulcerogenic effects of acid and other insults (the so-called "cytoprotective" role of prostaglandins). COX-1 mediated production of thromboxane in platelets promotes normal clotting. And COX-1 mediated synthesis of prostaglandins in the kidney appears to be responsible for maintaining renal plasma flow in the face of vasoconstriction. COX-1 levels remain relatively stable in most cells, although mild increases (2-4 fold) have been reported in response to growth factors.

COX-2 levels, in contrast, are dramatically upregulated in inflamed tissues. For example, COX-2 expression and concomitant PGE_2 production are greatly enhanced in rheumatoid synovium compared to the less inflamed osteoarthritic synovium, and in animal models of inflammatory arthritis (Crofford *et al.*,1994; Anderson *et al.*,1996). This is undoubtedly the result of excessive production of interleukin-1, tumor necrosis factor and growth factors in the rheumatoid joint. Initially, it was thought that COX-2 was not constitutively expressed (i.e., present in the basal state) in any tissues. However, recent work has demonstrated constitutive COX-2 expression in several organs, such as kidney, brain and ovary. This may have implications for treatment.

5.2.7.d) The Rationale for the Development of Specific COX-2 Inhibitors

Currently available NSAIDs are nonselective - that is, they inhibit both COX-1 and COX-2. It is likely that inhibition of COX-2 is responsible for the anti-inflammatory effects of these drugs, while inhibition of COX-1 is responsible for the recognized toxicities of NSAIDs, including: a) peptic ulcers and the associated risks of bleeding, perforation and obstruction; b) prolonged bleeding time; and, c) renal insufficiency. NSAIDs that would selectively inhibit COX-2 are thus highly desirable since inflamed tissues could be targeted without disturbing the homeostatic functions of prostaglandins in noninflamed organs. Theoretically, then, selective COX-2 inhibition should preserve the anti-inflammatory efficacy without causing the associated toxicities of NSAIDs.

5.2.7.e) Does COX-2 serve a physiological function(s)? Does COX-1 serve an inflammatory function(s)?

Low levels of COX-2 have been observed in some non-inflamed tissues, suggesting that COX-2, like COX-1, may also serve some normal physiological functions (Dubois *et al.*,1998). In the kidney, COX-2 expression was enhanced in the macula densa in response to sodium restriction (a physiological, not inflammatory, stress), suggesting that COX-2 plays a role in salt and volume regulation. Interestingly, mice in which COX-2 was "knocked out" have a moderately high rate of still births due to severe renal dysplasia, indicating that COX-2 is also critical for development of the kidney (Lagenbach *et al.*,1995). COX-2 also plays a role in ovulation and fertility, as

evidenced by its enhanced expression in the ovary prior to ovulation, and in the uterus prior to parturition. Furthermore, COX-2 knockout mice that survive exhibit markedly decreased fertility.

The COX-2 isoform has also been observed under basal conditions in rat brain in the hippocampus, pyramidal cells and amygdale (Dubois *et al.*,1998). Its function in the brain is not yet defined but is likely to include temperature control. Interestingly, epidemiological studies have demonstrated that individuals taking chronic NSAIDs have a lower incidence of Alzheimer's disease than controls. The exact function of COX-2 in the brain remains to be elucidated. In summary, COX-2 appears to play a role in the development of the kidney, in salt and water regulation by the kidney, and in ovulation and parturition, and perhaps in an unknown brain function(s).

Even though it is a well established fact now, that COX-1 mediates important physiological functions, it still remains to be determined whether it contributes at all to inflammatory responses. The mechanisms of many animal models of inflammation are not well defined, making interpretation of currently available data difficult. In COX-1 knock out mice, however, homozygous mutant animals had a reduced inflammatory response to the application of exogenous arachidonic acid to the ear (Lagenbach *et al.*,1995). Surprisingly, these COX-1 knockout mice did not exhibit spontaneous gastric ulcers or bleeding, as expected.

5.2.7.f) COX II selectivity

If a drug is able to selectively inhibit the activity of COX II during an inflammatory response, it is said to be COX II selective (Pairet, 1995). Currently, the more accepted definition of COX-2 selectivity is by ex vivo assay in whole blood. In these assays, COX-1 activity is measured by the release of thromboxane B2 (metabolite of thromboxane A2) from platelets during clotting. COX-2 is measured by release of prostacyclin from monocytes in response to an inflammatory stimulus such as LPS. Specific COX-2 inhibitors should inhibit prostacyclin, but not thromboxane B2, release over a range of assay times and drug doses (Kurumbail, 1996). Using these assays, celecoxib (Celebrex™) and rofecoxib (VIOXX™), but not traditional NSAIDs, are considered to be COX-2 selective. However the Federal Drug Administration

(FDA) has not yet approved the designation of these drug as "COX-2 specific". Celecoxib and rofecoxib have the same general class labelling as traditional NSAIDs. FDA approval of a drug as "COX-2 selective" is likely to depend on its demonstrated ability to reduce serious NSAID-induced GI complications such as bleeding, perforation and obstruction.

5.2.7.g) NO and NO Synthases

The simple gas NO has multiple important physiologic and pathologic functions (Moncada and Higgs, 1993;Nathan, 1992; Beckman and Crow, 1993). These include roles in host resistance to tumors and microbes, regulation of blood pressure and vascular tone, neurotransmission, learning, and neurotoxicity, carcinogenesis, and control of cellular growth and differentiation (Magrinat *et al.,*1992; Punjabi *et al.,*1992). In the presence of oxygen, NO rapidly (in seconds) is converted to nitrite and nitrate, substances that are generally not bioactive (Stampler *et al.,*1992). NO binds with high affinity to iron in heme groups of proteins such as hemoglobin (Hb), myoglobin (Mb), and guanylyl cyclase; Hb and Mb are very effective quenchers of NO action. NO also reacts with O2–, and SOD and prolongs NO life by eliminating O2–. On reacting with O2–, NO may form peroxynitrite (OONO–), a very toxic/reactive molecule that may be the most important effector toxic molecules when one thinks of NO toxicity in oxygenated systems.

There are three forms of the enzyme nitric oxide synthase encoded by three different genes. Neural NOS (nNOS or NOS1) and endothelial cell NOS (eNOS or NOS3) are constitutive enzymes, demonstrating low level, constant transcription of mRNA. The enzymatic actions of NOS1 and NOS3 are modulated by regulation of cytoplasmic calcium levels, with agents inducing increases in calcium (e.g., calcium ionophores or ligands such as acetylcholine, or mechanical stress), with subsequent binding to calmodulin and activation of the enzyme. Although inducible NOS (iNOS or NOS2) was described initially in mononuclear phagocytes, it also is found in synoviocytes, chondrocytes, smooth muscle cells, hepatocytes, and others including B lymphocytes *(*Moncada and Higgs, 1993;Nathan, 1992; Nathan, 1994; Palmer *et al.,*1993; Charles *et al.,*1993; Stefanovic-Racic *et al.,*1994; Gross *et al.,*1992; Rosenkranz-Weiss *et al.,* 1994*)*.

5.2.7.h) The free radicals

Inflammatory joint tissue in RA is characterized by infiltration and accumulation of (ROS,O2–, mononuclear phagocytes, lymphocytes, and plasma cells; proliferation of synovial cells; and expression of proinflammatory cytokines. Although some cytokines are undetectable or are present in only low levels in rheumatoid synovia, synovial macrophages and fibroblasts in this disease are a good source of cytokines such as IFN-α, IL-1, TNF-α. IL-6, IL-8, and GM-CSF (Suzuki *et al.,* 1999). In addition to these protein mediators, arachidonic acid metabolites (e.g., PGE2, 15d-PGJ2, leukotrienes, and others), reactive oxygen species (hydrogenperoxide and hydroxyl radical), and reactive nitrogen species (NO and related molecules such as peroxynitrite) likely contribute to the pathology.

NO, O_2, peroxynitrite, and PG appear to be central to the inflammatory process. Various cells in the joint may participate in the inflammation. These include mononuclear phagocytes, chondrocytes, and lymphocytes. The combined actions of these mediators (along with certain inherent and induced anti-inflammatory mediators) contribute to the eventual pathology: accumulation of inflammatory cells, modification of synovial vascular cells, proliferation of synovial fibroblasts, disruption of the general synovial architecture, and destruction of cartilage and bone.

5.2.8 Treatment options

The diagnosis of arthritis is based on the pattern of symptoms like bone deformities, the distribution of the inflamed joints, the blood and X-ray findings. Several visits may be necessary before the doctor can be certain of the diagnosis. Abnormal antibodies can be found in the blood of people with rheumatoid arthritis. An antibody called "rheumatoid factor" can be found in 80% of patients. Citrulline antibody (also referred to as anticitrulline antibody, anticyclic citrullinated peptide antibody, and anti-CCP) is present in most people with rheumatoid arthritis. It is useful in the diagnosis of rheumatoid arthritis when evaluating cases of unexplained joint inflammation. A blood test called the sedimentation rate (sed rate) is a measure of how fast red blood cells fall to the bottom of a test tube. The sed rate is used as a crude measure of the inflammation of the joints. The sed rate is usually faster during disease flares and slower during remissions. Another blood test that is used to measure the degree of inflammation present in the body is the C-reactive protein. Blood testing may also

reveal anemia, since anemia is common in rheumatoid arthritis, particularly because of the chronic inflammation. Joint X-rays may be normal or only show swelling of soft tissues early in the disease. As the disease progresses, X-rays can show bony erosions typical of rheumatoid arthritis in the joints. Joint X-rays can also be helpful in monitoring the progression of disease and joint damage over time. Bone scanning, a radioactive procedure, can also be used to demonstrate the inflamed joints. MRI scanning can also be used to demonstrate joint damage.

An accurate diagnosis increases the chances for successful treatment. Treatments available include physical therapy, splinting, cold pack application, paraffin wax dips, antiinflammation medications, immune-altering medications, and surgical operations.

5.2.9 NSAIDS and DMARDS

Two classes of medications are used in treating rheumatoid arthritis: fast-acting "first-line drugs" (NSAIDS or the non-steroidal anti-inflammatory drugs) and slow-acting "second-line drugs" (also referred to as disease-modifying antirheumatic drugs or DMARDs). The first-line drugs, such as aspirin and cortisone (corticosteroids), are used to reduce pain and inflammation. The slow-acting second-line drugs, such as gold, methotrexate, and hydroxychloroquine (Plaquenil), promote disease remission and prevent progressive joint destruction, but they are not anti-inflammatory agents. However adverse reactions of DMARDS can lead to nausea & stomatitis, bone marrow suppression, liver disease & interstitial pneumonitis.

5.2.9.a) NSAIDS

NSAIDs (nonsteroidal anti-inflammatory drugs) are a large class of medications used to treat pain and inflammatory disorders. They are highly efficient in the treatment of arthritis owing to their analgesic (pain relieving), anti-inflammatory and anti-pyretic (fever reducing) properties. There are three categories of NSAIDs: Salicylates (acetylated, such as aspirin, and non-acetylated), the traditional NSAIDs, and COX-2 selective inhibitors. NSAIDs commonly used in the treatment of arthritis include Ansaid, Arthrotec, Aspirin, Cataflam, Celebrex, Ibuprofen, Indomethacin, Fenoprofen, Trilisate, Voltaren etc.

5.2.9.b) How NSAIDs work?

The mechanism of action of NSAIDs is the inhibition of the enzyme cyclooxygenase, which catalyzes arachidonic acid to prostaglandins and leukotrienes. Arachidonic acid is released from membrane phospholipids as a response to inflammatory stimuli. Prostaglandins establish the inflammatory response. NSAIDs interfere with prostaglandin production by inhibiting cyclooxygenase (Boothe, 2001). This mechanism may relate to the variation in response between patients. Scientific studies have shown a correlation between concentration of the drug and effect, but do not explain the differences in individual patient responses. It is thought that the pharmacokinetic (process by which a drug is absorbed, distributed, metabolized, and eliminated) differences among the various NSAIDs may account for the variability in response.

5.2.9.c) Adverse effects of NSAIDs

All NSAIDs induce undesirable and potentially life threatening side effects. The most commonly consumed NSAIDs in accidental poisoning include ibuprofen, aspirin, acetaminophen and indomethacin. The most common clinical signs of toxicosis in one study were vomiting, diarrhea, central nervous system depressions and circulatory manifestations. The majority of adverse reactions reflect the inhibitory effects of NSAIDs on prostaglandin activity. In addition, acute intoxication by several drugs can be fatal. The major toxicities associated with NSAIDs affect the gastrointestinal (GI), hematopoietic and renal systems. Gastrointestinal damage is the most common and serious side effect of the NSAIDs. Although not completely understood several mechanisms have been hypothesized. Gastroduodenal erosion and ulceration reflect inhibition of prostaglandin E_2 mediated bicarbonate and mucus secretion, epitheliazation and blood flow. Control of gastric acid secretion is consequently decreased as is mucus and bicarbonate secretion, epithelization of mucosa and mucosal blood flow. Breakdown of small blood vessels, due to a deficiency of mucus may be the initiating lesion. Direct irradiation by acidic drugs may be important. In addition salicylates cause local damage due to "back diffusion" of the acid, which causes injury to mucosal cells and sub mucosal capillaries. Impaired platelet activity may contribute to mucosal bleeding. Treatment for GI toxicity should include prostaglandin replacement and use of a cytoprotectant such as sucralfate.

5.2.9.d) Haematopoietic: All NSAIDs are able to impair platelet activity due to impaired thromboxane synthesis. At pharmacologic doses, aspirin selectively and irreversibly acetylates a serine residue of a platelet cyclooxygenase.

5.2.9.e) Renal: Analgesic nephropathy is a relatively common adverse effect of NSAIDs in human beings. Since NSAIDs bind to plasma proteins they may be displaced by or may displace other plasma-bound drugs such as coumadin, methotrexate, digoxin, cyclosporine, oral antidiabetic agents, and sulfa drugs. This interaction can enhance either therapeutic or toxic effects of either drug. Due to their different chemical properties some NSAIDs have substantial biliary (bile ducts, gallbladder) excretion (i.e. indomethacin, sulindac) and others are metabolized pre-excretion, while a few are excreted in the urine unchanged.

NSAID studies which have shown a variation in patient response attribute a lower rate of adherence to one NSAID when other NSAIDs are known to be available. The response to and preference of an NSAID may relate to more than just symptom control. About 60% of patients will respond to any single NSAID. A trial period of three weeks should be given for anti-inflammatory effectiveness to be observed. About 10% of rheumatoid arthritis patients would not respond to any NSAID. Antipyretic and anti-inflammatory effects of NSAIDs can mask the signs and symptoms of infection. Adverse effects of NSAIDs which can occur at any time include renal (kidney) failure, hepatic (liver) dysfunction, bleeding, and gastric (stomach) ulceration. NSAIDs (particularly indomethacin) can interfere with the pharmacologic control of hypertension and cardiac failure in patients who take beta-adrenergic antagonists, angiotensin-converting enzyme inhibitors, or diuretics. Moreover, Long-term use of NSAIDs may have a damaging effect on chondrocyte (cartilage) function.

5.2.10 Diet and rheumatoid arthritis

The dietary connection between rheumatoid arthritis and food sensitivities was first noted by Michael Zeller in 1949 in *Annals of Allergy*. Elimination /provocation diets are the best way to determine if this is the problem. The foods most likely to provoke symptoms after an elimination diet are, in order of most to least: corn, wheat,

bacon/pork, oranges, milk, oats, rye, eggs, beer, coffee, malt, cheese, grapefruit, tomato, peanuts, sugar, butter, lamb, lemon, and soy (Calder *et al.,* 2009). Cereals were the most common food, with wheat and corn causing problems in over 50 percent of people.

The concept of food sensitivity and increased intestinal permeability is gaining acceptance as more physicians see the clinical changes in their patients when they use this approach. Testing for food and environmental sensitivities, parasites, toxic metals, Candidiasis, intestinal permeability, and comprehensive digestive and stool analysis often provides an understanding of an underlying cause of the disease. Candidiasis frequently plays a role in arthritis and is a possible aggravator in rheumatoid arthritis.

Yeast in the gastrointestinal system may be the result of antibiotics, oral contraceptives, steroid medications, increased use of alcohol or sugar, or a stressed immune system. Treatment of Candida infections in the digestive system has improved rheumatoid symptoms in many cases. Nutrients such as glutamine, quercetin, gamma-oryzanol and beneficial flora can help heal the leaky cells in conditions of increased intestinal permeability. An elimination diet or fasting can significantly reduce joint inflammation, pain, and stiffness and increase mobility.

The use of natural products in the treatment of arthritic cases is fast emerging (Mahajan *et al.,* 2007; Khanna *et al.*,2007). A few of the natural products which have been come to use for inflammatory disorders are discussed below.

1. Shark liver oil is a natural product that has great success with arthritis and other inflammatory disorders because it contains different substances that combat inflammation. It boosts the immune system with alkylglycerols, squalene a potential antioxidant, vitamin E and omega 3 oils and combats Leaky Gut Syndrome which is at the bottom of so many cases of arthritis.
2. Deer Velvet. For centuries, deer velvet has been used to relieve the pain and inflammation associated with arthritis, support joint care and mobility, and assist muscle tone and general wellbeing. Mountain Red™ velvet contains over 30 Amino Acids, including ten of the most essential, together with proteins, omega 3 & 6 lipids, glycosaminoglycans (Glucosamine and Chondroitin), minerals, and trace elements.

3. Green Lipped Mussel. A unique remedy for arthritis has been developed from the green lipped mussel, native of New Zealand and has proved to be an effective anti-inflammatory agent.
4. Multivitamin with minerals: People with arthritis are often deficient in many nutrients. Aging, poor diet, medications, malabsorption, and illness all contribute to poor nutritional status. Sources of Vitamin E: Vitamin C: Alfalfa or Yucca may also be added in the diet to combat inflammation.
5. Omega-3 fatty acids/fish oils. Research has shown fish oils are really helpful for some people with arthritis. Fish oil capsules reduce morning stiffness and joint tenderness. Because fish oils increase blood clotting time, they should not be used by people who have hemophilia or who take anticoagulant medicines or aspirin regularly.
6. Ginger: Ginger is an old Ayurvedic remedy that was given to people with rheumatoid and osteoarthritis.
7. Powdered Cetyl myristoleate: Cetyl myristoleate (CM) was discovered by Harry Diehl, a researcher at the National Institute of Health. He found that mice did not develop arthritis when CM was given. When he himself developed arthritis, Diehl took CM and his arthritis resolved. CM appears to actually "cure" arthritis in many instances.
8. Superoxide dismutase: (SOD) plays an important role in reducing inflammation and has been used alone, with copper, manganese or copper, and zinc for various arthritic conditions. Oral SOD doesn't seem to work as well, except when used in a copper/zinc preparation. Some physicians are using SOD in injections. Barley grass extracts of SOD can be purchased at health food stores. Most people who try them experience benefits, but there is little scientific research to date. Some veterinarians are using wheat grass SOD with arthritic animals with excellent results.
9. Probiotics. By helping to correct disbiosis probiotics can make a big improvement to the quality of life and arthritis in particular.
10. S-adenosylmethionine: (SAMe) is a synthetic version of a chemical that is naturally made in our bodies from methione. Research on SAMe has shown it to have powerful antidepressant effects without the side effects of pharmaceutical antidepressant medications. SAMe has also been shown to

be as potent an anti-inflammatory drug as indomethacin without the negative side effects in people with arthritis.

11. Bromelain: Bromelain is an enzyme derived from pineapple that acts as an anti-inflammatory in much the same way that evening primrose, fish, and borage oils do.
12. Black Cohosh: A Herb that has long been used by European and American herbalists to reduce muscle spasm, pain, and inflammation. It can be used as either a tincture or in capsules.
13. Cider vinegar: Jarvis, in his book, Vermont Folk Medicine, popularized the use of apple cider vinegar and honey for the relief of arthritis. Vinegar and honey are alkalizing to the body, which helps dissolve calcification in the joints. Many people have found relief on this program, although there has been no scientific documentation.

5.2.11 Fish oil and arthritis

There is considerable evidence that fish oil supplementation can alleviate the symptoms of rheumatoid arthritis (RA). Omega-3 fatty acids are known to benefit patients with cardiovascular disease and arthritis, although the mechanism for these actions is not yet fully understood. Discovery of mechanisms help support the use of nutritional ingredients (Curtis *et al.*,2004) for prevention of disease and could add to evidence of omega-3's benefit to joint health. Resolvins are made from the omega-3 fatty acids by cellular enzymes and can reduce inflammation in mice. COX-2 is involved in making resolvin E1 and the inhibition of vascular COX-2 by the anti-inflammatory drugs might block the synthesis of resolvin E1, which would eliminate an important anti-inflammatory pathway.

5.2.12 Fish oil versus vegetable oil

Scientists have provided new evidence that using more fish oil than vegetable oil in the diet decreases the formation of chemicals called prostanoids, which, when produced in excess, increase inflammation in various tissues and organs. Both fish and vegetable oils are converted into prostanoids through chemical reactions that are aided by the cyclo-oxygenases (COXs), of which both COX-1 and COX-2 are involved in the reactions. Studies have shown that, in reactions involving COX-1,

when more fish oil is present, it preferentially binds to COX-1, thus limiting vegetable oil's access to this enzyme. So the chances of converting the n-6 fatty acids of vegetable oils to pro-inflammatory mediators are greatly reduced.

In a study conducted in Belgium aimed at determining the long-term effects of fish oil supplementation in rheumatoid arthritis patients, fifty-three per cent of the patients in the fish oil group showed significant overall (global) improvement as compared to 10% in the placebo group and 33% in the fish oil plus olive oil group. Forty-seven per cent of the patients in the fish oil group were also able to reduce their intake of NSAIDs and disease-modifying anti-rheumatic drugs as compared to 15% in the placebo group and 29% in the olive oil plus fish oil group. The researchers conclude that long-term supplementation with fish oils benefits rheumatoid arthritis patients significantly and may lessen their need for NSAIDs and other RA medications. (Geusens, Piet, *et al.*, 1994)

5.2.13 Ginger extracts and arthritis

Ginger (*Zingiber officinale*) supplements are being promoted for arthritis treatment in western societies on the basis of ginger's traditional use as an anti-inflammatory in Chinese and Ayurvedic medicine. Young *et al.* 2005 demonstrated the analgesic and anti-inflammaotry activities of gingerol when Intraperitoneal administration of [6]-gingerol (25 mg/kg–50 mg/kg) produced an inhibition of acetic acid-induced writhing response and formalin-induced licking time in the late phase. [6]-Gingerol (50 mg/kg–100 mg/kg) also produced an inhibition of paw edema induced by carrageenin. Researchers at the University of Michigan observed destruction in ovarian cancer cells when gingerol extracts were applied at 5% levels. Researchers believed that it was the anti-inflammatory action of gingerol that kept cancer cells from growing. However, scientific evidence of ginger's antiarthritic effects is sparse.

5.2.14 Complete Freund's Adjuvant-induced arthritis in rats

Adjuvant arthritis in rats probably resembles human rheumatoid arthritis not only clinically and histologically but also aetiologically (Mizushima *et al.*, 1970). Adjuvant arthritis, studied in detail by Pearson (1959) and described by Mizushima *et al.*, (1970), is a syndrome induced in rats by the intradermal injection of a suspension of dead mycobacteria in liquid paraffin. The syndrome is characterized by the

development of inflamed lesions followed by an intensive and chronic arthritis (so-called secondary lesions) remote from the injection site after a delay of approximately 12 days. Most of the investigations of the mechanisms involved in the production of the disease were made by immunological approaches. Extensive studies by Pearson and Wood (1964), Newbould (1964) and other investigators seemed to indicate that adjuvant arthritis is an immunological disease, probably due to delayed hypersensitivity.

For several decades Freund's adjuvants have been considered the most effective adjuvants available for raising antibodies in test animals. "Complete Freund's Adjuvant" (CFA) contains heat-killed mycobacteria which are primary agents responsible for stimulating antibody production (Joe and Wilder, 1999). Changes in rear paw size, or maximum tail thickness, are readily measured thereby providing non-invasive indices of disease onset and progression with or without the benefit of drug treatment. In some rat strains, and more notably males than females, reductions in body weight gain reflected first the initial recognition of the adjuvant and then continuing disease development (Whitehouse, 2007). Collection of hard data to monitor arthritis progression was not restricted to non-invasive physical measurements. Periodic sampling of the blood of pre- and post-arthritic animals allowed the determination of a host of biochemical and cellular responses to an adjuvant inoculation eg. Reduction of serum albumin, increased plasma ceruloplasmin, orosomucoid, numbers of granulocytes, etc. (Billingham and Davies, 1979; Lowe, 1964; Baumgartner *et al.*,, 1974) Mechanical stress on a joint in rheumatoid patients often aggravates or exacerbates the inflammation. It is considered, therefore, that a non-specific stress or a minor inflammation induced by stress influences the process of joint inflammation.

For half a century, the adjuvant disease in rats has enormously aided research on drugs to control arthritis and other chronic infl ammatory disorders. The adjuvant also triggers many systemic responses beyond the articular tissues. Simultaneously, a window to explore the converse phenomenon: namely, how a chronic disease can affect drug efficacy and toxicity can be explored.

5.2.15 MPO levels in arthritis

Several workers have studied the effects of myeloperoxidase (MPO) levels under stress conditions in living systems. MPO is an enzymatic mediator of several

inflammatory cascades and higher serum levels of MPO have been associated with increased risk of adverse cardiovascular events. Nieto *et al.*, (2002) showed that the activity of MPO was lower in rats fed fish oil rich diets rather than those fed on olive oil rich diets Chiu *et al.*, (2006) investigated the effect of n-3 fatty acids on adhesion molecules and tissue MPO activity in diabetic mice with sepsis. They observed that the fish oil fed group had lower organ MPO activities at various time-points after caecal ligation and puncture, when compared with those of the soyabean oil fed group. Vaisar *et al.*, (2007) demonstrated that myeloperoxidase targets HDL for oxidation and blocks the lipoprotein's ability to remove excess cholesterol from cells, raising the possibility that the enzyme provides a specific mechanism for generating dysfunctional HDL in humans. Thus oxidative modifications to HDL and changes in its protein composition would lead to other cardio vascular diseases.

5.2.16 Protein levels in inflammation

Moshage *et al.*, (1987) determined albumin level in patients with inflammatory diseases using [14C] carbonate technique. They reported that, in rats with turpentine induced inflammation, a decline in the albumin levels and a decreased albumin synthesis could not be related to a reduced amino acid supply but it may be associated with a reduced concentration of albumin mRNA in the liver. They even proposed the role played by monocytic products to reduce the hepatocytic albumin mRNA content in vivo and in vitro. In another study conducted by Barly *et al.*, (2006) a reduction in plasma albumin concentration among cholecystitis patients was strongly related to an increase in albumin efflux, i.e. redistribution of albumin to the extravascular space, as well as to changes in the size of the intravascular fluid space. Pekarek and Powanda (1976) recorded the effects of zinc deficiency on protein synthesis in rats during tularemia and observed a significant decrease in albumin content in infected rats.

Contrary to these findings many workers have even reported an increase in serum proteins during an inflammation or infection [4-6]. Saso *et al.* (2000) reported a marked rise of two prominent acute phase proteins, α-2 macroglobulin and hemopexin in streptozotocin-induced diabetic rats. Glycoproteins (GP) are one of the many acute phase proteins (APP) present in serum. The presence and levels of G-

reactive protein, transferrin, ceruloplasmin, albumin, a1-antitrypsin etc. have been used to diagnose the extent of inflammation in patients with arthritis.

5.2.17 Glycoprotein components

Lombart *et al.* (1980), reported significant elevation in the levels of liver sialyl transferase activities in turpentine induced inflammation in rats. Reddy and Dhar (1988) studied the metabolic changes in the carbohydrate components in adjuvant induced arthritis in rats. They observed that in both acute and chronic phases of the disease, the adjuvant arthritis caused a significant increase in the levels of carbohydrate moieties of tissue glycoproteins viz. total hexose, hexosamine, fucose, sialic acid, total neutral sugar content and neutral sugar monosaccharides. In addition, the urinary excretions of hexosamine and uronic acid in arthritic rats were found to be elevated significantly. Kulkarni *et al.*, (1986) showed that both glycoprotein levels as well as erythrocyte sedimentation rate (ESR) significantly increased in patients with Rheumatic arthritis. As against this, sera from patients with Osteo arthritis showed minimal but statistically insignificant elevations. Glycoproteins (GP) may, thus be able to differentiate an inflammatory arthritis from a degenerative, non-inflammatory arthritis. Kulkarni (1986) suggested that rise of GPs in the absence of any other obvious sign of tissue destruction may be the earliest arthrogenic index. Carter *et al.*, (1962) found increased sialic acid levels in rheumatoid arthritis (RA) Coburn *et al.*, (1953) found that sialic acid estimation was helpful both in estimating the degree of rheumatic activity and also in evaluating the efficacy of therapeutic agents.

5.2.18 The Glycolytic and gluconeogenetic pathways in inflammation

Inflammatory cells use glucose as a primary source of metabolic energy, and thus increased uptake of glucose and high rates of glycolysis are characteristics of inflamed cells. Chichelnitskiy E., et al (2009) showed that glucose levels dropped down significantly in patients with acute sepsis. Normally, the blood glucose level is very tightly regulated. Inflammations disrupt the balance of the tightly regulated blood sugar level. If it drops only slightly, the liver produces new glucose in a process called gluconeogenesis to ensure that the brain and the muscles can continue functioning. In the opposite case, an elevated blood sugar level is immediately

lowered by the secretion of insulin from the pancreas to prevent long-term damage to the blood vessels. The key enzyme for gluconeogenesis in the liver is called phosphoenolpyruvate carboxykinase, or PEPCK for short. The more active it is, the more glucose is produced. Its activity is regulated by various hormones: Stress hormones glucagon and cortisol boost its activity, while insulin slows it down. Chichelnitskiy E., et al (2009) found out that in an inflammation of the kind that occurs in sepsis the molecular cooperation of the two hormones glucagon and cortisol is prevented and thus blocks PEPCK and, consequently, gluconeogenesis.
The high rates of glucose uptake and metabolism often displayed during cases of malignant tumors are reported to be associated with increased expression of glucose transporter (Younes *et al.*, 1996; Flier *et al.*, 1987) and increased activity of glycolytic enzymes (Gennari *et al.*, 2000; van Veelen *et al.*, 1978).

The glycolytic enzyme, hexokinase (HK) can play a key role in sugar metabolism of inflammatory cells and it has been suggested that hexokinases, hexokinase II in particular, regulate glucose metabolism in arthritic cells (Fedatto *et al.*, 2000). Enhanced activity of hexokinase II has also been reported in various malignant tumors and cancer cell lines (Shinohara *et al.*, 1991; Adams *et al.*, 1995; Rempel *et al.*, 1994). In highly proliferative cancer cells there is an increased expression of type II hexokinase, an isoform with high affinity to the outer mitochondrial membrane. This is a very important feature of hexokinase regulation since it overcomes product inhibition (glucose 6- phosphate), and favors the utilization of recently formed ATP through oxidative phosphorylation, thereby increasing enzyme activity and maintaining an elevated glycolytic rate (Mathupala *et al.*, 2006). Phosphoglucoisomerase, which acts as a catalyst in the conversion of glucose-6-phosphate to fructose-6-phosphate, is elevated significantly in animals with induced stress (Sundravel *et al.*, 2006). Aldolase that catalyses the reversible change of fructose 1,6 diphosphate to glyceraldehyde 3 phosphate and dihydroxy acetone phosphate plays at central position of glycolytic pathway. Aldolase was found elevated in inflammation-induced animals (Premalatha *et al.*, 1997) and in metastatic conditions (Hennipman *et al.*, 1987).

The progressive failure of gluconeogenesis, manifested most extensively in inflammatory disorders, is explained partly by a decrease or the complete absence of glucose-6-phosphatase and fructose-1,6-diphosphatase activities in stress induced

conditions (Sugapriya *et al.*, 2008). The activity of fructose-1,6-biphosphatase, the entry enzyme for the synthesis of glucose-6-phosphate from pyruvic acid, has been found to be reduced in arthritis bearing animals. Weber and Cantero (1960) have shown that in novikoff hepatoma, there appears to be an absence of fructose 1,6-diphasphatase in the tumour and consequently a block in the pathway leading to the synthesis of glucose 6 phosphate from pyruvate. The enzyme glucose 6 phosphatase plays a key role in the maintenance of blood glucose homeostasis (Werve *et al.*, 2000). Glucose-6-phosphatase also act as a marker enzyme for liver microsomal activity, is reported to be greatly inhibited in animals induced with stress (Premalatha *et al.*, 1997). Elevated levels of DME- which are precursors for the generation of pyruvate at the beginning of gluconeogenesis and lower activities of the above enzymes in the later stage of gluconeogenesis indicate the difficulty of inflammatory cells to convert pyruvate to glucose due to lack of energy.

5.2.19 Antioxidant Metabolism

In normal physiological condition a delicate balance is maintained between the level of reactive radicals and antioxidant defense system. Oxygen free radicals have been implicated as mediators of tissue damage in patients of rheumatoid arthritis. Rheumatoid arthritis (RA) is a chronic relapsing immuno-inflammatory multisystem disease of unknown etiology, characterized by predominant synovial proliferation, bone destruction and degradation of articular cartilage. Reactive oxygen species (ROS) are produced by neutrophils, the main cells of inflamed synovial fluid in RA. Activation of neutrophils and macrophages results in the generation of superoxide radicals, H_2O_2 and highly reactive hydroxyl radicals. Another source of ROS is hypoxic re-perfusion injury from elevated synovial cavity pressure during joint movement. These ROS have been implicated as mediators of tissue damage in RA. ROS are formed during oxidative processes that normally occur at relatively low levels in all cells and tissues. Circulating human erythrocytes possess the ability to scavenge O_2^- and H_2O_2 by SOD, CAT and GSH Px dependent mechanisms. If ROS are not scavenged; these species may lead to widespread lipid, protein and DNA damage.

Seven *et al.*, (2008) reported significantly elevated levels of MDA in both the serum and synovial fluid of RA patients. Human synovial fluid is poor with respect to

antioxidant components such as GSH, GSH Px and SOD. Thus ROS, generated by phagocytes in the inflamed rheumatoid joint, are not efficiently scavenged, resulting in increased levels of lipid peroxidation products both in the synovial fluid and blood as a result of transportation by the circulatory system. Added to this Significantly low thiol levels and sulphydryl groups were observed in RA patients, the decrease being greater in active RA patients (Oztürk, 1999). DNA is a particular target for oxidation. 8-oxodG is a marker of inflammation and an indicator of cellular injury in the joints of RA patients. It has been demonstrated that oxidative stress in the RA joint not only damages cellular DNA, but also suppresses DNA mismatch repair system, leading to both an increase in the overall load of DNA adducts in the joint and to an exacerbation of the disease (Oztürk, 1999).

In inflammatory arthritis, polymorphonuclear leukocytes and macrophages are responsible for an impaired antioxidant status. Decreased SOD activity indicates a degradation process in which SOD is degraded by ROS. Alteration in whole body lipid levels; serum triglycerides levels, and the overall distribution of serum lipids are the unique manifestation of cancer lipid metabolism. Hypertriglyceridaemia in serum and tissue are the important features observed both in patients (Cohn *et al.*, 1981; Shike *et al.*, 1984) and experimental animals (Argiles and Azcon-Bieto, 1988; Tessitore *et al.*, 1993) during growth of a wide variety of neoplasms during the detoxifying processes. Impaired GSH defence system is either due to GSH conjugation with ROS or to inactivation GSH Px by H_2O_2 (Oztürk, 1999). Circulating human erythrocytes possess the ability to scavenge O_2^- and H_2O_2 generated extracellularly by activated neutrophils. However, Antioxidants and antioxidant enzymes work independently and in concert to protect against oxygen toxicity.

5.2.20 Lipid metabolism

Alteration in whole body lipid levels; serum triglycerides levels, and the overall distribution of serum lipids are unique manifestations of lipid metabolism during CFA-induced arthritis. Hypertriglyceridaemia in serum and tissue are the important features observed both in patients (Haroon, M and Devlin, J, 2009) and experimental animals during inflammatory disorders (Falasca *et al.*, 2006). Heliovaara, M. *et al* (1996) reported high levels of serum total cholesterol and LDL levels in patients with RA. However a corresponding decrease in HDL cholesterol and triglyceride levels

was reported. In a similar study conducted in Finland (Viikari *et al.*,1980), Synovial fluid from the inflamed knee joints of 30 patients with rheumatoid arthritis (RA) contained 2.75 ±0.81 mmol/1 cholesterol (51% of the corresponding mean serum level) and 0.32 ±0.13 mmol/1 triglycerides (35% of the corresponding mean serum level).

Hyperlipidemia often observed during inflammation has been associated with both decreased lipoprotein lipase (LPL) activity in adipose tissue (Noguchi *et al.*, 1991) and an increased activation of liver lipogenesis (Vosper *et al.*, 2001). Numerous investigators have attributed the loss of adipose fat and decreased food intake during adjuvant induced arthritis to a decreased adipose lipogenesis (Martin etal., 2008). Taylor *et al.* (1992) demonstrated that certain tumours express and release LPF and this factor can mobilize stored fatty acids from normal adipocytes, both *in vitro* and *in vivo*. Other factors thought to be involved in lipid metabolism during arthritis are alteration of hormonal balance and presence of elevated cytokines in inflammed tissues. Inflammtion elicits profound perturbations of the hormonal homeostasis, together with release of circulating TNF and increased levels of PGE_2 (Tessitore *et al.*, 1993). *In vivo* administration of TNF resulted in a decrease of adipose tissue LPL activity in rat, mouse and guinea pig (Semb *et al.*, 1987). This decreased activity has been shown to depress the uptake of exogenous ^{14}C lipids by adipose tissue and to increase circulating triglycerides in the rat (Evans and Williamson, 1988).

5.2.21 Diagnostic marker enzymes in arthritis

Clinical evidence for liver disease in rheumatoid arthritis is limited. In a study, liver scintigraphy had been used to assess liver size when 22% out of 32 RA patients were diagnosed with hepatomegaly (Tiger *et al.*, 1976). The concept of rheumatoid liver was based on the finding that 26% of RA patients had raised serum alkaline phosphatase levels which were confirmed to be of hepatic origin by correlation with the elevated serum-5 nucleotidase concentrations Walker *et al.*, 2002.

Abnormal biochemical tests of liver function have been reported in a large number of patients (Mills and Sturrock, 1982). Alanine aminotranferease (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) are enzymes

located in liver cells that leak out into the general circulation when liver cells are injured. In addition to its function as clinical marker to tissue damage, AST, ALT and LDH participate in different metabolic processes. AST and ALT are capable of converting glutamate, aspartate and alanine to ketoacids essential for energy and other metabolism. LDH catalyse the conversion of pyruvate to lactate and vice versa during glycolysis.

Altered activities of AST, ALT and LDH in serum have been noticed in several studies pertaining to liver disorders. The elevated activities of these enzymes can be dependent on the inflammatory disorder. Activities of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) increased significantly following the treatment of nitrosamine to rats with or without vitamin E pretreatment (Bansal *et al.*, 2005). Tarao *et al.* (1997) observed an elevated level of ALT in all patients with adjuvant induced arthritis. A possible factor contributing to the elevated LDH activity in the CFA-induced rats might be the enhanced glycolysis during progression of the inflammatory disorder. The elevated LDH activity may also have resulted from difference in the rates of synthesis, degradation or excretion of the enzyme in the rats with induced inflammations. Berruti *et al.* (1997) reported a significant elevation in the activity of serum alkaline phosphatase in prostate cancer patients with bone metastases. Similarly Patel *et al.* (1994) found significant rises in serum alkaline phosphatase activity in patients with cervical carcinoma. Increased acid phosphatase activity has also been reported in patients during stress induced disorders (Palani *et al.*, 1998). Enhanced activity of the marker enzymes discussed above during hepatotoxicity can be explained on the basis of liver damage and subsequent release of these enzymes from injured liver to circulatory system.

5.2.22 Bone & Mineral Metabolism

Patients with rheumatoid arthritis showed a reduced bone volume and decreased bone turnover, which is further aggravated by microarchitectural deterioration stressing the severe osteoporosis associated with the disease (Pérez-Edo *et al.*, 2002). Minerals are the essential elements in cellular metabolism. They are involved in many biological functions, such as the electron transport chain, and in biological

oxidation in mitochondria and in antioxidant enzyme action. Alteration of cell mineral metabolism has been proposed to be an important pathogenic step in the development of arthritis and to be related to their biological and biochemical functions.

Inflammatory cells require high concentration of intracellular calcium, which is essential for activity of glycolytic enzymes and production of ATP to meet their high energy demand for growth. Cellular calcium concentration has been found to be directly proportional to mitotic activity of tissue (Ferris, 1971). Excessive influx of calcium across the cell membrane and consequent rise in intracellular calcium content has been reported to increase DNA synthesis which is required for cell division (Gelfand *et al.*, 1984). Hickie and Galent (1967) have reported higher calcium content in rats with Morris hepatoma than in normal liver tissue. Higher concentration of Ca^{2+} in inflammatory cells could compete with sodium at sodium specific sites at the inner surface of the membrane (Vincenzi, 1971) and this may lead to the decrease in sodium content. Liver and serum samples collected from adjuvant-induced rats showed higher levels of calcium and magnesium and lower levels of sodium and potassium when compared with samples from control rats (Premalatha and Sachdanandam, 1998).

Equilibrium of minerals in normal physiological condition is maintained by membrane bound ATPases. Alteration of ATPase activity in arthritis condition may result in abnormal mineral contents. The other mechanism that regulates mineral requirement of inflammatory cells is activation of ion channel present in plasma membrane of cell. As minerals are responsible for various cellular activities, maintenance of mineral homeostasis in circulatory system as well as in cell is of important concern in normal cellular metabolic process. The membrane bound enzymes such as Na^{+}/K^{+} ATPase, Mg^{2+}ATPase and Ca^{2+} ATPase are responsible for the transport of sodium/potassium, magnesium and calcium ions across the cell membranes (Stekhoren and Bonting, 1981) and play key role in maintaining cellular mineral equilibrium. The activities of the ATPases have been found to be inhibited in adjuvant-induced inflammations in animals (Randak *et al.*, 1999).

5.2.23 Lysosomal enzymes in arthrits

Lysosomal enzymes play an important role in inflammation (Chayen and Bitensky 1971; de Duve and Wattiaux, 1966; Weissmann, 1967; Houck, 1968; Fell, 1969; Dingle, 1969). Increased activities have been reported in human rheumatoid synovia and synovial membrane, and in other inflamed tissues (Vainio 1970, Anderson, 1970). Recent work suggests that cells which migrate to inflammatory sites, which include blood polymorphonuclear leucocytes and tissue macrophages, may partially discharge their lysosomal enzymes into the extracellular spaces, probably as a result of excessive endocytosis (Weissmann, 1967; Fell, 1969).

The Complete Freund's Arthritis (CFA)-induced arthritic model of systemic chronic inflammation (Pearson, 1956; Newbould, 1963), some symptoms of which resemble those in human rheumatoid arthritis, is used extensively for detecting and evaluating compounds with anti-inflammatory properties. It is concluded that the extensive tissue breakdown in adjuvant arthritis is due to the release and degradative action of lysosomal enzymes on connective tissue components.

The role of acid phosphatase in inflammation is unknown, but it is probable that β-glucuronidase, in conjunction with lysosomal hyaluronidase, degrades synovial hyaluronic acid and the chondroitin sulphate moiety of cartilage chondromucoprotein (Anderson, 1968), thus contributing to joint inflammation. Less is known of mammalian enzymes which degrade collagen, and their effect on the inflammatory process. They have been detected in cirrhotic liver and subcutaneous granuloma tissue (Bazin and Delaunay, 1966), in rheumatoid synovial membrane (Harris and Krane, 1969), and in other tissues as discussed elsewhere (Anderson, 1969a).

Increased lysosomal enzyme activity in homogenates of adjuvant-injected hind paws, amputated at various time intervals from rats with adjuvant arthritis, paralleled increases in paw volume (oedema). The observed correlation by Weissmann between paw oedema and lysosomal enzyme activity, in both the injected and the un-injected hind paws, suggested increased vascular permeability associated with the accumulation of leucocytes in connective tissue, the subsequent oedema becoming established as blood proteins escape. It is probable that leucocytes liberate lysosomal enzymes as a consequence of intense endocytosis (Weissmann, 1967).

5.2.24 Dietary doses of fish oils and gingerol for health benefits

In most of the inflammation experiments fish oils had been used between 3 % and 30% of calorie intake. When female Lewis rats with CFA-induced inflammation, were fed semi-purified diets containing 7.5% lipid as either beef tallow or corn oil in one study, or 5% lipid as either olive oil, corn oil or fish oil in a second study, corn oil fed rats showed earlier onset of inflammation and significantly greater arthritic index compared to beef fat fed rats in the first study. In the second study corn oil and olive oil fed rats had significantly greater arthritic scores than fish oil fed rats. These results suggested that the omega-6 polyunsaturated fatty acids in the corn oil and olive oil diets may exacerbate adjuvant-induced arthritis (Lawrence,1990) Feeding 5.9 %, 11.8 % or 17.6% fish oil combined with corn oil to total 23.5 % of the diet resulted in significantly fewer animals with cancer than did feeding corn oil alone (Reddy and Sugie, 1988).

A diet containing 5% total fat and supplemented with 4.7% EPA together with 0.3% linoleic had inhibited azoxymethane-induced colon carcinogenesis in rats compared with tumorigenesis in rats fed a 5% linoleic control diet (Minoura *et al,*. 1988). Oshima *et al.* (1995) fed a 3% DHA ethyl ester-containing diet to female and male ApcD716 heterozygotes. The females showed a 69% reduction in polyp formation and a significant suppression of polyp growth.

Beneficial effect derived from dietary fish oil against any chronic disease including arthritis is dose-dependent. Limited dietary supply of linolenic acid and limited ability of the body to produce EPA, DHA from linolenic acid have led to the recommendation to increase direct consumption of long chain n-3 PUFA which are mainly found in seafood (Simopoulos,1991). Earlier studies on dietary pattern of Eskimos and low incidence rate of coronary heart disease among them have resulted in major recommendations to increase the intake of fish oil (especially EPA and DHA) approximately 1g/day, especially for high-risk and 500 mg/day for normal risk individuals (Kris-Etherton *et al.*, 2001). Recommended daily allowances (RDA) for n-3 PUFA has not yet been fully established. In 1990 Canadian Nutritional Board recommended a daily n-3 PUFA intake of 1.4 g for boys and 1.2 g for girls aged 13-15 years, with optimal intake of 800-1100 mg/day for linolenic acid and 300-400 mg/day for long chain n-3 PUFA (Simopoulos, 1991). Considering the varied nature of health benefits derived from supplementation of n-6 and n-3 fatty acids, WHO

(1993) and British Nutrition Foundation have recently recommended a ratio of between 3:1 and 4:1 for n-6 to n-3 fatty acids in diet. In 2003, WHO/FAO Expert Consultation on Diet, Nutrition and the Prevention of Chronic Diseases recommended for the general population a diet containing 5-8 % of calories from n-6 fatty acids and 1-2 % of calories from n-3 fatty acids (WHO, 2003a and 2003b) for various health benefits.

The quantity of fish oil, EPA or DHA to be added to diet of arthritis patients depends on its tolerance to the patient as overdose may lead to various discomforts such as abdominal pain, nausea and diarrhea (Gogos *et al.*, 1998; Burns *et al.*, 1999). Diets with 50% of its total energy contributed by fish oil have shown to reverse weight loss associated with inflammatory disorders in rats (Dagnelie *et al.*, 1994). Burns *et al.* (1999) on their attempt to determine maximum tolerated dose (MTD) of fish oil fatty acids among patients with inflammatory disorders, established a dose of 0.3g/kg/Day of n-3 concentrate as MTD. The authors showed that at MTD of 0.3g/kg/day of n-3 concentrate capsule, a 70 kg patient would take 21.1 g capsule per day and thereby would ingest 7.9g of EPA, 5.2g of DHA and 15.3g of total n-3 fatty acid everyday.

Ginger (*Zingiber officinale*) supplements are being promoted for arthritis treatment in western societies on the basis of ginger's traditional use as an anti-inflammatory in Chinese and Ayurvedic medicine. However, scientific evidence of ginger's antiarthritic effects is sparse, and its bioactive joint-protective components have not been identified. Young *et al.* (2005) demonstrated the analgesic and anti-inflammaotry activities of gingerol when Intraperitoneal administration of [6]-gingerol (25 mg/kg–50 mg/kg) produced an inhibition of acetic acid-induced writhing response and formalin-induced licking time in the late phase. [6]-Gingerol (50 mg/kg–100 mg/kg) also produced an inhibition of paw edema induced by carrageenin. Researchers at the University of Michigan observed destruction in ovarian cancer cells when gingerol extracts were applied at 5% levels. Researchers believed that it was the anti-inflammatory action of gingerol that kept cancer cells from growing.

From the above review, it is evident that inclusion of fish oil in diet reduces inflammatory disorders, suppresses the formation of inflammatory mediators and improves the quality of life of patients with arthritis during post treatment period.

Inflammation impairs the normal homeostasis process leading to several metabolic changes. Of late, several attempts have been made to find out the mechanism of action of fish oil and its components to reduce inflammation. But still most of the tested or proposed mechanisms remain unclear. Literature on the role of liver oil of *C.scalpratus* on various metabolisms associated with rheumatoid arthritis is scarce. In the present study, we decided to investigate the effect of feeding liver oil of the deep sea elasmobranch *C.scalpratus* and compare its efficiency with gingerol concentrate, on growth and progression of CFA-induced inflammation and thereby analyse some of the important metabolisms undergoing within blood and liver during arthritis.

5.3 MATERIALS AND METHODS

5.3.1 Chemicals

Complete Freund's Adjuvant, epinephrine, tetraethoxy propane, bovine serum albumin, phenobarbital, amino acid standards were obtained from M/s. Sigma Chemical Company, St. Louis. MO, USA. Sodium, potassium and calcium standards were purchased from Merck, Germany. All the other chemicals used were of analytical grade. The fish oil used in the study was extracted from the liver of *Centrophorus scalpratus* by the method of Folch *et al.*, (1957) and gingerol extract was obtained as a gift from the Spices Board, Cochin extracted from ginger (Zingiberaceae).

5.3.2 Animals & Diets

Healthy Wistar strain male albino rats (n=30) weighing 150 ± 4 g were used for the study. They were housed in spacious polyurethane cages in an air conditioned room, controlled for temperature (27±1° C), humidity (60±5%), lighting 12h (light-dark cycle). The experiment was carried out according to the guidelines of Committee for the Purpose of Control and Supervision of experiments on Animals (CPCSEA), New Delhi, India, and approved by the Institutional Animal Ethics Committee (IAEC).

The animals were fed standard laboratory diet, ad libitum, purchased from M/s Sai Feeds, Bangalore and water. The composition of the diet is shown in table 5a. The liver oil of *Centrophorus scalpratus* (CS) was divided into small portions, packed in airtight glass flasks under atmosphere of nitrogen and stored at -20°C. The peroxide value was estimated at regular intervals to check the oxidation of the fish oil. Details of the non-saponifiable matter and fatty acid composition of CS lipids is given in chapter 2, table 2c, table 2d. Gingerol extracts were also stored in amber colored bottles at -20°C, under nitrogen.

The animals were divided into 5 groups of six animals each. Group I animals served as the normal control and were not induced with any inflammation. They were however fed with the vehicle dimethylsulfoxide. The arthritic syndrome was induced in groups II, III and IV animals by subcutaneous injection of 0.1 ml of Complete Freund's Adjuvant (10 mg of heat killed Mycobacterium tuberculosis per ml of paraffin oil) into the plantar surface of the right hind paw (Guglielmotti *et al.*, 2002; Sterman *et al.*,1997; Mythilypriya *et al.*, 2007). The paw sizes were measured at

every 5 day intervals and maximum paw sizes were recorded on the 14th day. Group II animals were induced with inflammation and untreated. However Groups III and IV animals were orally fed with liver oil of *Centrophorus scalpratus* (1g/kg body weight) and gingerol extracts (0.5g/kg body weight) respectively, as a suspension in dimethylsulfoxide [oil:DMSO=4:1 (v/v), gingerol:DMSO=150:1(w/v)] , from the 15th day to the end of the experiment on the 36th day. Group V animals served as normal control rats for the fish oil group and were fed with the CS liver oil orally from the beginning till the end of the experiment.

The changes in paw size were measured by using a calibrated screw guage. The inflammation in joints was evaluated by an independent observer of no knowledge of the treatment protocol. The severity of arthritis was also quantified by a clinical score measurement as described by Lewis et al., (1998). The swelling that occurred in the nose, ears, fore paws, hind limbs and tail was scored macroscopically using the following scale: 0 – no macroscopic changes; 1– mild; 2 – moderate; 3 – severe swelling of the affected tissues. Body weight changes were also recorded at every 5 day intervals throughout the period of study.

Animals were sacrificed on day 36 by ether anaesthesia, the right hind paws were amputed and sent for X-ray analysis of the joint and histopathological evaluation of the bone tissues. Paw exudates were collected for monitoring the levels of inflammatory mediators:cyclooxygenases, myeloperoxidases and nitric oxides (as sum total of nitrites and nitrates.) Blood was collected with and without EDTA for plasma and serum separation. Liver tissues were dissected out, washed and transferred to an ice cold saline solution (0.9% w/v). The livers were weighed and tissue homogenates were prepared by homogenising weighed amounts of tissues (approximately 100mg) in 0.1 M Tris-HCl buffer, pH 7.4.

5.3.3 X-ray analysis

At the time of sacrifice, radiographs of the adjuvant-injected side of hind paws were taken with a soft X-ray instrument (SR-M50, Softex, Tokyo, Japan) and X-ray film (Konica CS 100E, Tokyo, Japan). The X-ray apparatus was operated with a 40-kV peak, 4 mA, and a 2-min exposure. The severity of bone and joint damage was scored blindly according to the method previously described (Harada *et al.*, 2004). The right hind paws were used for X-ray evaluation. Joint destruction was scored on

a scale from 0 to 3, as mentioned earlier. 0 score for no bone damage. 1 for minor bone destruction observed in one enlightened spot; 2 for moderate changes, two to four spots in one area; 3 for severe erosions afflicting the joint.

5.3.4 Histopathological analyses

Histopathology of the ankle joints was done according to a slightly modified method of Mythilypriya (2007) using a light microscope. The ankle joint of the hind paw of the rats were removed and separated from the surrounding tissues and weighed. The joints fixed in 10 % formalin were decalcified, sectioned and finally stained with haematoxylin and eosin to examine the histopathological changes during the experimental period in all the above groups under light microscope.

5.3.5 Determination of MPO levels

The myeloperoxidase levels in the rat paw homogenates were determined using a slightly modified method of Bradley *et al.,* (1982) as discussed below.

Reagents

5 g Hexadecyltrimethyl Ammonium Bromide in 1L potassium phosphate buffer 50 mM, pH 6.0

Phosphate buffer (180 μl) containing 1 mM *O*-dianisidine dyhydrochloride and 0.001% hydrogen peroxide

Procedure

Rats from different groups were sacrificed with ether anaesthesia, and the right hind paws were weighed, cut and homogenated in 1 ml of hexadecyltrimethylammonium bromide (HTAB) buffer containing 5 g HTAB in 1L potassium phosphate buffer 50 mM, pH 6.0. (Bradley *et al.*, 1982) using a Polytron homogenizer (two cycles of 10 s at maximum speed). After centrifugation at 10,000 r.p.m. for 2 min, supernatant fractions were assayed for MPO activity, as an index of cellular migration, using the method described by Bradley *et al.* (1982). Samples (20 μl) were mixed with phosphate buffer (180 μl) containing 1 mM *O*-dianisidine dyhydrochloride and 0.001% hydrogen peroxide in a microtiter plate. Absorbance was measured at 450 nm, taking three readings at 30-s intervals. Calculation of units of MPO was realized considering that 1 U MPO=1 μmol H_2O_2 split and 1 μmol H_2O_2 gives a change in absorbance of 1.13×10^{-2} (change in absorbance=nm min^{-1}).

5.3.6 Determination of NO_x exudate levels

The nitric oxide levels (measured as the sum total of nitrites and nitrates) were estimated using the methods employed by Thomsen *et al.*, (1990) and Misko *et al.*, (1993).

Reagents

30% zinc sulphate solution in distilled water

Procedure

Rats from different groups were sacrificed, inflamed paws were cut and centrifuged at 4000 r.p.m. for 30 min. Exudates (supernatants) were collected with 100 μl of saline (0.9% w/v) and were used for NO_x (nitrite plus nitrate) quantification. To determine NO_x levels, exudates were deproteinized with $ZnSO_4$ 30% for 15 min (Thomsen *et al.*, 1990). Supernatants and a standard curve of sodium nitrate were incubated in a microplate with cadmium (50 mg $well^{-1}$) for 1 h to convert NO_3^- to NO_2^- (Thomsen *et al.*, 1990). After centrifugation at 14,000 r.p.m. for 15 min, total nitrite (NO_x) content was determined fluorometrically in microtiter plates using a standard curve of sodium nitrite (Misko *et al.*, 1993). NO_x content was calculated by using the internal standard curve.

5.3.7 Determination of cyclooxygenase (COX) levels

The COX levels in CFA-induced rat paws were determined using Cayman's COX fluorescent activity assay kit. It provides a convenient fluorescent based method for determining COX I and COX II activities in both crude (tissue homogenates) and purified enzyme preparations. The assay utilises the peroxidise component of the COXs. The reaction between PGE-2 and ADHP (10-acetyl-3,7-dihydroxy phenoxazine) produces the highly fluorescent compound resorufin. Resorufin fluorescence can be easily analysed with an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm. The kit also includes isozyme-specific inhibitors for distinguishing COX II activity from COX I. The COX concentration is expressed as Fluorophore Units (FU) from the standard curve for resorufin. 1 FU is defined as the amount of enzyme that will cause the formation of 1 nm fluorophore per minute at 22°C.

5.3.8 Determination of counts of Red Blood Corpuscles (RBC) and White Blood Corpuscles (WBC)

Total RBC and WBC count was determined using haemocytometer as described in the textbook of Laboratory Methods in Microbiology (Srivastava and Singhal, 1997).

5.3.8.a) RBC

An aliquot of 0.02 ml blood was added to 3.98 ml of diluting fluid (1% formalin in 31.3 g/l trisodium citrate) in a test tube and mixed well. A small quantity of the diluted blood was put between the coverslip and the ruled platform of the counting chamber of Neubaur Haemocytometer. The chamber was not allowed to overflow and there was no air bubble in the chamber. The solution was allowed to settle for a couple of minutes and then the counting was done under the high power microscope. Count of the cells in 16 small squares in five different small squares was noted down. The total no of RBC per 1 cu. mm of blood was calculated as follows:

RBC per 1 cu. mm blood

= No. of RBC present in each small square X Volume of blood present in each small square (1/4000 cu.mm.) X Dilution factor

5.3.8.b) WBC

An aliquot of 0.02 ml blood was added to 0.38 ml of diluting fluid (1.5 ml glacial acetic acid+1 ml of 1% aqueous solution of gentian violet + 98 ml distilled water) in a test tube and mixed well. A small quantity of the diluted blood was transferred to the counting chamber of haemocytometer. The chamber was not allowed to overflow and there was no air bubble in the chamber. The solution was allowed to settle for 2 minutes and then the counting was done under the high power microscope. The number of cells in the four larger corner squares was noted down. The total no of WBC per 1 cu. mm of blood was calculated as follows:

WBC per 1 cu. mm blood = No. of cells counted X 50

5.3.9 Estimation of blood creatinine

Creatinine in blood was estimated by the method of Slot (1965).

Reagents

a. Picric acid : 1.2g of picric acid was dissolved in one litre of distilled water.

b. Sodium hydroxide: 30g dissolved in 1l water.
c. Alkaline picrate reagent: Equal volumes of solutions (1) and (2) were mixed just before use.
d. Sodium tungstate solution : 50g / l of water
e. Sulphuric acid: 0.33 M
f. Creatinine standard: 100 mg of creatinine was dissolved in l00ml of 0. 1M HCl. Before use, this stock standard was diluted to 10 fold with water.
g. Glacial acetic acid.
h. Trichloro acetic acid (TCA): 10%

Procedure

0.1 ml of blood was mixed with 3.9 ml of 10% TCA to precipitate protein. To 3.0 ml of deproteinised supernatant added 2.0ml of alkaline picrate solution. Blank containing 3.0ml of water and aliquots of standard in 3.0 ml of water were also treated in a similar manner. After 30 minutes the colour was measured at 520nm against the reagent blank. The values were expressed as mg/dl blood.

5.3.10 Estimation of blood urea

Urea was determined by the method of Natelson *et al.*(1951) using diacetylmonoxime.

Reagents

1. Diacetylmonoxmine reagent : 2.0 g of diacetylmonoxime was dissolved in 100 ml of 2.0% acetic acid.
2. Sulfuric acid-phosphoric acid mixture: 25 ml of concentrated sulfuric acid, 75 ml of 85% O-phosphoric acid and 70 ml of distilled water were mixed.
3. Sodium tungstate solution : 10%
4. Sulfuric acid : 0.67 N
5. Standard urea solution: 20 mg of urea dissolved in 100 ml of distilled water.

Procedure

To 0.1 ml of sample was added 3.3 ml of water and mixed with 0.3 ml of 10% sodium tungstate and 0.3 ml of 0.67 N sulfuric acid reagent. The suspensions were centrifuged and to 1.0 ml of the supernatant, 1.0 ml of water 0.4 ml of diacetylmonoxime and 2.6 ml of sulfuric acid-phosphoric acid reagent were added in that order. Aliquots of standard urea were also treated in a similar manner and

heated in a boiling water bath for 30 minutes, cooled and the colour developed was measured at 480 nm in a Shimadzu-UV spectrophotometer. The value was expressed as mg/dl blood.

5.3.11 Diagnostic Marker enzymes

The assay of alanine aminotransferase (ALT) Mohur and Cook (1957), aspartate aminotransferase (AST) Mohur and Cook (1957) and lactate dehydrogenase (LDH) King (1965a) has been mentioned in the previous chapter (Chapter 4).

5.3.12 Estimation of protein, free amino acids

5.3.12.a) Estimation of protein

The protein content in the sample was estimated by the method of Lowry *et al.* (1951) as mentioned in the previous chapter.

5.3.12.b) Estimation of albumin/globulin ratio

The albumin and globulin content of the plasma was estimated by the method of Varley (1980)

Reagents

a. Sodium sulphite solution: 25 g of anhydrous sodium sulphite was dissolved in 100 ml of distilled water.
b. Diethyl ether
c. Biuret reagent : 1.5 g copper sulphate and 6g sodium potassium tartarate were dissolved in 50 ml distilled water, 300 ml 10% sodium hydroxide was added. Solution was made up to 1l. One g potassium iodide was added and stored in plastic containers.

Procedure

To 0.04 ml of plasma, 4 ml of sodium sulphite solution and 4 ml of ether were added and centrifuged. After centrifugation 2.0 ml of lower layer was taken and 2.0 ml of freshly prepared biuret reagent was added to it. The blank contained 2.0 ml of sodium sulphite solution. All the tubes were left at room temperature for 15 minutes. The colour developed was read at 540 nm in a Shimadzu UV Visible spectrophotometer. The albumin content was determined from the absorbance and

deducted from total protein to get the globulin content. From the values of albumin and globulin, albumin / globulin ratio was calculated.

5.3.13 Electrophoretic separation and densitometric analysis of tissue proteins

Proteins were separated by SDS-PAGE technique as described by Laemmli (1970). It is based on the principle that, in the presence of 10% SDS and 2-mercaptoethanol, proteins dissociate into their subunits and bind large quantities of the detergent which mask the charge of the proteins, thus giving a constant charge to mass ratio. As a result the proteins move according to their molecular weight in an electric field. In this discontinuous buffer system, the separating (resolving gels) and stacking gels are made up in the electrode buffer, Tris-glycine. During electrophoresis, the leading ion is chloride while the trailing ion is glycine. In this experiment, 7.5% gel concentration is used for the effective separation. Densitometric analysis was carried out using the software Quantity One (Biorad, USA)

Reagents

1. 0.5M Tris-HCl, pH 6.8
2. 1.5M Tris-HCl, pH 8.8
3. 10% SDS
4. Acrylamide/Bis (30%T, 2.67 %C)
5. 10% Ammonium per sulphate (APS)
6. Sample buffer
    1. Distilled water: 3.8ml
    2. Tris-HCl 0.5M,pH 6.8: 1ml
    3. Glycerol: 0.8ml
    4. 10%SDS: 1.6ml
    5. 2-mercapto ethanol: 0.4ml.
    6. 1% Bromophenol blue: 0.4ml.
7. Stock electrode buffer
    1. Tris base: 9g
    2. Glycine: 43.2g
    3. SDS: 3g

These chemicals were dissolved in 600ml of distilled water. Working solution was prepared by diluting 100ml stock electrode buffer to 500ml with distilled water.

8. 7.5% Separating gel
    1. Distilled water: 4.85ml
    2. Tris-HCl 1.5M: 2.5ml
    3. 10%SDS: 100µl
    4. Acrylamide: 2.5ml
    5. APS 10%: 50µl
    6. TEMED: 5µl
9. 4% Stacking Gel
    1. Distilled water: 6.1ml
    2. Tris-HCl 0.5M: 2.5ml
    3. 10% SDS: 100µl
    4. Acrylamide: 1.33ml
    5. APS 10%: 50µl
    6. TEMED: 10µl

Procedure

Taken 0.1ml of the suitably diluted sample into a micro centrifuge tube and added 0.1ml of the sample buffer, heated in a boiling water bath for 4min, cooled and kept at 4°C in a refrigerator.

The separating gel was prepared without TEMED and APS. Evacuated for 15min to remove air bubbles. Added TEMED and APS with intermittent shaking after each addition immediately transferred the solution to the apparatus. Added a little water on the top of the gel to level it. Kept for 45min. The stacking gel was prepared in the same way. Kept the comb over the apparatus, tilted it to 45^0, poured the gel slowly, and pressed the comb slowly and evenly. Kept for 45min. Marked the wells. After removing the comb, the whole apparatus was transferred to the sandwich clamp assembly into the inner cooling core. Rinsed the apparatus and wells with electrode buffer and filled the inner chamber of the apparatus completely and the outer chamber to the optimum level. Added 10µl of the sample into the wells by using a syringe. The electrode lid was placed at proper position and connections were established. The power of 200V was supplied. Electrophoresis was carried out for 45min approximately until the dye reaches the bottom. Subsequently, the gel was removed and is placed in a big petri-dish containing the stain, Coomassive blue.

Kept for 30min, and transferred the gel into 7% acetic acid for destaining. 7% acetic acid was changed intermittently till the gel got completely destained.

5.3.14 Estimation of free amino acids

Free amino acid in the liver tissue was determined as per the procedure of Ishida *et al.* (1981).

Reagents

1. Ethanol
2. 0.05M HCl
3. Buffer A: Dissolved 32.7g trisodium citrate in 500ml of double distilled water, added 140ml distilled alcohol (ethanol of 99.5%) and 16.6ml perchloric acid. The pH was adjusted to 3.2 by adding 60% perchloric acid and made up to two litre using double distilled water.
4. Buffer B: Dissolved 117.6g trisodiumcitrate and 24.8g boric acid in 500ml double distilled water, added 45ml 4N NaOH. Adjusted the pH to 10 by adding 4N NaOH, and made up the volume to 2 litres using double distilled water.
5. Ortho-phthaldehyde (OPA) Buffer: Dissolved 81.4g of Na_2CO_3, 27.1g of H_3BO_3 (Boric acid) and 37.6g of K_2SO_4 in 500ml double distilled water and made up the solution to 2 litre using double distilled water.
6. Ortho-phthaldehyde solution (OPA): Dissolved 80mg OPA in 1.4ml-distilled alcohol (ethanol). Added 0.25ml mercaptoethanol and 0.15ml of 30% w/v Brij. Mixed well and made up the volume to 100ml by using OPA buffer.
7. Sodium hypochlorite solution: 4% w/v Sodium hypochlorite in OPA buffer i.e. 0.3ml Sodium hypochlorite in100ml OPA buffer.

Procedure

Taken 100mg sample accurately and extracted with 2.5ml ethanol by grinding in mortar, process repeated for two times. Transferred this into a centrifuge tube and centrifuged at 3000 rpm for 10min. Supernatant was transferred to another test tube and evaporated off the ethanol. Content made up to 1ml with 0.05M HCl. Samples were filtered through a membrane filter of 0.45µm and injected 20µl of this to an amino acid analyzer (HPLC- LC 10 AS) equipped with cation exchange column packed with a strongly acidic cation exchange resin i.e. styrene di vinyl benzene co

polymer with sulphonic group. The column used was Na type i.e. ISC- 07/S 1504 Na having a length of 19cm and diameter 5mm.

The instrument was equipped with Shimadzu FL 6A fluorescence detector and Shimadzu CR 6A Chrompac recorder. The mobile phase of the system consisted of two buffers, Buffer A and buffer B. Gradient system was followed for the effective separation of amino acids. The oven temperature was maintained at 60^0C. The total run was programmed for 62 min. The amino acid analysis was done with non-switching flow method and fluorescence detection after post-column derivatization with o-phthaldehyde. In the case of proline and hydroxyl proline, imino group is converted to amino group with hypochlorite. Amino acid standard (Sigma chemical Co., St. Louis, USA) was also run to calculate the concentration of amino acids in the sample. The amount of each amino acid was expressed as µmol/g tissue

5.3.15 Estimation of glycoprotein components

5.3.15.a) Estimation of hexose

Hexose was estimated by the method of Niebes (1972).

Reagents

1. (3:2, v/v) Sulphuric acid: water mixture
2. 800mg of orcinol dissolved in 50ml of 1N H_2SO_4.
3. Orcinol-sulphuric acid mixture: 1.0ml of reagent (2) was mixed with 7.5ml of reagent (1). This mixture was prepared fresh at the time of assay.
4. Standard hexose: Equal quantities of galactose and mannose were dissolved in water to give a concentration of 100µg/ml.

Procedure

An aliquot of the delipidised sample was treated with 1.0ml of 0.1N NaOH. Blank contained 1.0ml of 0.1N NaOH .The tubes were cooled by placing in an ice-bath and 8.5ml of Orcinol-sulphuric acid mixture was added slowly and mixed well. The tubes were stoppered, incubated at 80°C for 15min in a water bath. Cooled and the color was allowed to develop in the dark for 25min. The intensity was measured at 540nm. Standard solutions containing 0.025 to 0.1mg were treated similarly and hexose concentration was estimated.The concentration of hexose was expressed as mg/dl in plasma and as mg/g in liver tissue.

5.3.15.b) Estimation of hexosamine

Hexosamine was estimated by the method of Wagner (1979).

Reagents

1. Acetyl acetone reagent: 3.5% acetyl acetone in 1N trisodium phosphate containing 0.5N potassium tetra borate (98: 2 V/V).
2. Ehrlich's reagent: 3.2g of P-dimethyl aminobenzaldehyde was dissolved in 30ml of 1N HCl and diluted to 210ml with isopropanol.
3. Standard Hexosamine: Galactosamine hydrochloride solution containing 10mg/100ml was prepared.

Procedure

An aliquot of the delipidised sample was hydrolyzed with 3N HCl in a boiling water bath for 4h and neutralized. 0.8ml of the neutral hydrolysate was mixed with 0.6ml of acetyl acetone reagent. The mixture was heated in a boiling water bath for 30min, cooled and 2.0ml of Ehrlich's reagent was added. The contents of the tubes were mixed and the absorbance was measured at 535nm. Standard hexosamine solution containing 20μg to 80μg was used for the preparation of standard curve.

Hexosamine was expressed as mg/dl (plasma); mg/g (liver).

5.3.15.c) Estimation of sialic acid

Sialic acid was estimated by the method of Warren (1959).

Reagents

1. Sodium meta arsenite : A 10% solution was prepared in 0.5 M sodium sulphate in 0.1 N H_2SO_4.
2. Sodium meta periodate : 0.2 solution in 9 phosphoric acid.
3. Thiobarbituric acid reagent : 0.6% solution was prepared in 0.5 M sodium sulphate.
4. Adidified butanol : 5 ml of concentration hydrochloric acid in 95 ml of n-butanol.
5. Standard sialic acid: 10 mg of N-acetyl neuraminic acid was dissolved in 100 ml of water.

Procedure

An aliquot of the delipidised sample was hydrolysed with 0.1 N H_2SO_4 at 80^0C for 1 hour. 0.2 ml of the hydrolysate was mixed with 0.1 ml of metaperiodate and the

solution was kept at room temperature for 20 min. 1.0 ml of sodium meta arsenite was added and shaken well so that the yellow brown colour disappeared. 3.0 ml of thiobarbituric acid reagent was added and heated in a boiling water bath for 15 min. After cooling, 4.3 ml of acidified butanol was added, shaken well and the colour was extracted into butanol phase. The butanol phase was transferred to another set of tubes and the colour intensity was transferred to another set of tubes and the colour intensity was measured at 530 nm. The blank containing 0.2 ml of 0.1 N H_2SO_4 and standard sialic acid solutions were treated similarly. Sialic acid was expressed as mg/dl (plasma); mg/g (liver).

5.3.16 Carbohydrate components

5.3.16.a) Estimation of blood glucose

Blood glucose was estimated by the method of Sasaki *et al.* (1972), using o-toluidine reagent.

Reagents

1. Trichloro acetic acid (TCA) : 10%
2. O-Toluidine reagent : 12.5 g of thiourea and 12.0 g of boric acid were dissolved in 50 ml of distilled water by heating over a mild flame. Exactly 75 ml of redistilled o-toluidine and 375 ml of acetic acid were mixed with thiourea-boric acid mixture and the total volume was made upto 500 ml with distilled water. The reagent was left in a refrigerator overnight and filtered.
3. Standard glucose solution: 10 mg of pure glucose was dissolved in 100 ml of 0.2% boric acid in water.

Procedure

Mix 0.1 ml of sample with 1.9 ml of TCA solution to precipitate protein and then centrifuge. 1.0 ml of the supernatant was mixed with 4.0 ml of o-toluidine reagent and was kept in a boiling water bath for 15 minutes. The green colour developed was read at 600 nm in a Shimadzu-UV spectrophotometer. A set of standard glucose solutions were also treated similarly. Blood sugar levels were arrived at by comparison with the standard curve.

The value was expressed as mg/dl in blood.

5.3.16.b) Estimation of hexokinase activity (EC. 2.7.1.1)

Hexokinase was assayed by the method of Brankstrup *et al.*, (1957).

Reagents

1. 0.005 M glucose solution
2. 0.72 M ATP solution
3. 0.05 M magnesium chloride solution
4. 0.0125 M dipotassium hydrogen phosphate solution
5. 0.1 M potassium chloride solution
6. 0.5 M sodium fluoride solution
7. 0.01 M Tris-HCl buffer (pH 8.0)

Procedure

The reaction mixture in a total volume of 5.0 ml contained the following : 1.0 ml of glucose solution, 0.5 ml of ATP solution, 0.1 ml of magnesium chloride, 0.4 ml of dipotassium hydrogen phosphate solution, 0.4 ml of potassium chloride, 0.1 ml of sodium fluoride solution and 2.5 ml of Tris-HCl buffer (pH 8.0). The mixture was pre-incubated at 37^0C for 5 minutes. The reaction was initiated by the addition of 2.0 ml of tissue homogenate. 1.0 ml aliquot of the reaction mixture was taken immediately (zero time) to tubes containing 1.0 ml of 10% TCA. A second aliquot was removed after 30 minutes of incubation at 37^0C. The precipitated protein was removed by centrifugation and the residual glucose in the supernatant was estimated by the O-toluidine method of Sasaki *et al.* (1972) as described previously. A reagent blank was run with each test. The difference between the two values gave the amount of glucose phosphorylated.

The enzyme activity is expressed as nmoles of glucose-6-phosphate formed/min/mg protein.

5.3.16.c) Estimation of aldolase activity (EC. 4.1.2.13)

The enzyme was assayed by the method King (1965c).

Reagents

1. Tris HCl buffer : 0.1 M, pH 8.6
2. Substrate : Fructose-1, 6-diphosphate, 0.05 M

3. Hydrazine Sulphate : 0.56 N
4. TCA : 10%
5. Colour reagent: 10% solution of 2, 4-dinitrophenyl – hydrazine in 2N hydrochloric acid.
6. Sodium hydroxide : 0.75N
7. Standard: 12.3 mg of DL-glyceraldehyde was dissolved in 100 ml of distilled water and kept at room temperature for 4 days for depolymerisation.

Procedure

The incubation mixture contains 0.25 ml of substrate, 0.25 ml hydrazine sulphate, 1 ml Tris-HCl buffer and 0.1 ml of tissue homogenate. It was incubated at 37^0C for 15 minutes. The reaction was terminated by the addition of 1 ml of 10% TCA and the tubes were centrifuged. An aliquot of the supernatant was transferred to tubes containing 1 ml of 0.75 N sodium hydroxide. The tubes were left at room temperature for 10 minutes. Then 1 ml of 2, 4-dinitrophenyl hydrazine reagent was added and incubated at 37^0C for 60 minutes. The colour developed after the addition of 7 ml of 0.85 N sodium hydroxide was read at 540 nm using a Shimadzu UV spectrophotometer. Standard DL-glyceraldehyde solution was also treated in a similar manner.

The enzyme activity was expressed as nmoles of glyceraldehyde-3-phosphate formed/min/mg protein.

5.3.16.d) Estimation of glucose-6-phosphatase activity (EC. 3.1.3.9)

Glucose-6-phosphatase was assayed according to the method of King (1965b).

Reagents

1. Citrate Buffer: 0.1 M, pH 6.5
2. Substrate : Glucose-6-phosphate, 0.10 M
3. Ammonium molybdate :
4. Amino naphthol sulphonic acid (ANSA): 0.5g of ANSA was dissolved in 195ml of 15% sodium metabisulphite and 5 ml of 20% sodium sulphite was added for complete solubilization. The solution was filtered and stored in a brown bottle
5. TCA : 10%

Procedure

The incubation mixture in a total volume of 1 ml contained 0.3 ml of buffer, 0.5 ml of substrate and 0.2 ml of the sample. Incubation was carried out at 37^0C for 60 minutes. The reaction was arrested by the addition of 1 ml of TCA and centrifuged. The phosphorus content of the supernatant was estimated by the method of Fiske and Subbarow (1925).

The enzyme activity was expressed as nmoles of Pi liberated/min/mg protein.

5.3.16.e) Estimation of fructose-1,6-diphosphatase (EC 3.1.3.11)

Fructose 1,6 diphosphatase was assayed by the method of Gancedo (1971).

Reagents

1. Tris-HCl buffer: 0.1M, pH 7.0
2. Substrate, Fructose-1, 6-diphosphate: 0.05 M
3. $MgCl_2$: 0.1 M
4. KCl: 0.1 M
5. EDTA: 0.001 M
6. TCA: 10%
7. Ammonium molybdate
8. ANSA

Procedure

The assay medium in a final volume of 2 ml contained 1.2 ml of buffer 0.1 ml of substrate, 0.25 ml of magnesium chloride, 0.1 ml of potassium chloride, 0.25 ml of EDTA and 0.1 ml of tissue homogenate. The incubation was carried out at 37^0C for 15 minutes. The reaction was terminated by the addition of 1 ml of 10% TCA. The suspension was centrifuged and the phosphorous content of the supernatant was estimated according to the method of Fiske and Subbarow (1925).

The enzyme activity is expressed as nmoles of Pi liberated/min/mg protein.

5.3.17 Estimation of inorganic phosphorous

Inorganic phosphorus was estimated by the method of Fiske and Subbarow (1925). The method is based on the formation of phosphomolybdic acid by the reaction between a phosphate and molybdic acid and its subsequent reduction to a dark blue

phosphomolybdic acid, the intensity of which is proportional to the phosphate ion concentration.

Reagents

1. Ammonium molybdate reagent : 2.5 g of ammonium molybdates was dissolved in 100 ml of 3N sulphuric acid.
2. Amino naphthol sulphonic acid (ANSA)
3. Standard Phosphorus: 35.1 mg of potassium dihydrogen phosphate was accurately weighed, dissolved and made upto 100 ml with distilled water. One ml contained 80 µg phosphorus.

Procedure

To suitable aliquots of the supernatant, 1.0 ml of ammonium molybdate reagent was added 0.4 ml of ANSA was added after 10 minutes incubation at room temperature, standards and blank were also treated in the above manner. The blue colour developed was read after 20 minutes at 640 nm in a Shimadzu-UV-spectrophotomer.

The values were expressed as µg per mg protein.

5.3.18 Estimation of tissue glycogen content

Tissue glycogen was estimated by the method of Morales *et al.* (1973).

Reagents

1. Potassium hydroxide solution : 30%
2. Absolute ethanol
3. Anthrone reagent : 0.2% solution in concentrated sulphuric acid, prepared just prior to use.
4. Standard glucose solution: 100 mg of glucose was dissolved in 100 ml of distilled water.

Procedure

A weighed amount of dried defatted tissue was subjected to alkali digestion by heating with 30% potassium hydroxide solution in a water bath for 20 minutes. The tubes were cooled and to this was added 5.0 ml of absolute ethanol to precipitate glycogen. The tubes were placed in a freezer overnight. The precipitated glycogen was collected by centrifugation at 2000g for 20 minutes. They glycogen was

injecting different concentrations of standard of ATP to HPLC and plotting the linearity curve. The ATP content was expressed as nmol of ATP/g tissue.

5.3.20 Lipids

5.3.20.a) Extraction of fat

The total lipid content of the tissue was extracted by the method of Folch *et al.* (1957).

Reagents

1. Chloroform-methanol mixture (2:1 v/v)
2. Chloroform

Procedure

A weighed amount of the tissue was subjected to lipid extraction using chloroform-methanol mixture (2:1). The extraction was repeated twice with fresh aliquot of chloroform-methanol mixture. The lipid extracts were transferred to a separating funnel and added 20% of water into it and left overnight. Next day the lipid extracts were drained through filter paper containing anhydrous sodium sulphate and was collected in round bottom flask and was evaporated to dryness in a flash evaporator. The lipid in the round bottom flask was made up to 10ml by using chloroform. From this 1.0ml was taken into a pre-weighed vial and allowed to dry in warm temperature to constant weight and total lipid content were calculated from the difference in weight. Sample made up to 10ml was used for the estimation of various lipid components viz., cholesterol, triglycerides, free fatty acids and phospholipids after evaporating the solvent in air at room temperature.

5.3.20.b) Estimation of total cholesterol

The total cholesterol present in plasma and liver was estimated according to method of Parekh and Jung (1970) with slight modifications.

Reagents

1. Standard cholesterol solution (stock): 1mg /ml in chloroform
   Working standard: 1.0ml of the stock was diluted to 10ml with chloroform.
2. $FeCl_3$ stock solution: 10g $FeCl_3$ in 100ml acetic acid.
3. $FeCl_3$ - H_2SO_4 reagent: 2.0ml of $FeCl_3$ stock solution was diluted to 200ml with conc. H_2SO_4.

4. 33% KOH (w / v): 10g of KOH was dissolved in 20ml distilled water.
5. Alcoholic KOH solution: 6.0ml of 33% KOH was made up to 100ml with distilled ethanol. This solution was prepared fresh just before use.

Procedure

0.1ml of the lipid sample was taken into a 25ml glass stoppered tube and evaporated off the chloroform. Added 5ml of freshly prepared alcoholic KOH solution. The tubes were shaken well and incubated in a water bath at 37^0C for 55min. After cooling to room temperature, added 10ml of petroleum ether and inverted the tubes once to mix the contents. Then added 5.0ml of distilled water and shaken the tubes vigorously for 1min. Took 0.5-2ml aliquots from the supernatant (petroleum ether) into test tubes. Evaporated the petroleum ether extract under nitrogen. To each of the sample as well as the standard tubes including the blank, added 3.0ml of glacial acetic acid followed by 0.1ml -distilled water. Mixed the tubes thoroughly and added 2ml of the $FeCl_3$ - H_2SO_4 reagent through the sides of the test tubes. A brown ring was formed at the interface; tap the bottom of the tubes well to effect mixing and a light colour appeared which changed to an immense purple colour, which was measured in a Shimadzu-UV spectrophotometer at 560nm.

The amount of total cholesterol was expressed as mg/dl (plasma); mg/g (liver).

5.3.20.c) Estimation of triglycerides

The level of triglycerides in plasma and liver were determined by the method of Rice (1970) with slight modifications.

Reagents

1. Activated silicic acid.
2. Saponification reagent: 5.0g of potassium hydroxide was dissolved in 60ml distilled water and 40ml isopropanol.
3. Sodium metaperiodate reagent: To 77g of anhydrous ammonium acetate in 700 ml distilled water, added 60ml glacial acetic acid and 650mg of sodium metaperiodate and was dissolved and diluted to 1litre with distilled water.
4. Acetyl acetone reagent: To 0.75ml of acetyl acetone, 20ml of isopropanol was added and mixed well.
5. Stock solution: 400mg of triolein was dissolved in 100ml chloroform.
6. Working standard: 1.0ml of the stock solution was diluted to 10ml.

Procedure

0.2ml of the lipid sample was taken into a test tube and evaporated off the chloroform, added 4.0ml isopropanol. It was mixed well and added 0.4g of activated silicic acid. It was shaken in a vortex mixer for 15min and centrifuged at 4000 rpm for 5min. To 2.0ml of the supernatant and standards ranging from 20-100 mg made up 2.0 ml with isopropanol, 0.6 ml of saponifying reagent was added and incubated at 60-70^{0}C for 15min. After cooling, 1.0ml sodium metaperiodate solution was added and mixed. To this, 5ml acetyl acetone was added, mixed and incubated at 50^{0}C for 30min. After cooling, the colour was estimated by measuring OD at 405 nm in a Shimadzu-UV spectrophotometer.

The value of triglyceride in plasma was expressed as mg/dl and in liver as mg/g tissue.

5.3.20.d) Estimation of free fatty acids

Free fatty acids in plasma and liver were estimated by the modified method of Horn and Menahan (1981) with colour reagent of Itaya (1977).

Reagents

1. Activated silicic acid
2. Chloroform, heptane, methanol (CHM) solvent mixture: It was prepared by mixing chloroform, heptane and methanol in the ratio of 200:150:7(v/v)
3. Copper-triethanolamine solution: 50ml of 0.1M copper nitrate and 50ml of 2M triethanolamine were mixed with 33g of sodium chloride. The pH of the solution was adjusted exactly to 8.1.
4. Diethyldithiocarbomate (DDC) solution: 0.1% DDC in butanol was prepared.
5. Standard Stock: A solution containing 2mg/ml of palmitic acid was prepared in CHM solvent. For working standard, the stock was diluted 1:10 in CHM to give a concentration of 200μg/ml.

Procedure

To 1.0ml of the lipid sample, 6.0ml of CHM solvent and 200mg of silicic acid were added. The mixture was shaken well, centrifuged at 4000 rpm for 5min and 3.0ml of the supernatant taken. Standard solution in the range of 25-100μg were taken and made up to 3.0ml with CHM solvent. The blank contained 3.0ml of CHM solvent. To

all these samples, 2.0ml of copper triethanolamine solution was added and then mixed on a mechanical shaker for 10min. The tubes were centrifuged at 4000 rpm for 5min. To the 2.0ml of the supernatant taken, 1.0ml of DDC solution was added and shaken well. The colour intensity was read immediately at 430nm in a Shimadzu UV-spectrophotometer.

Values are expressed as mg/dl plasma and mg/g wet tissue.

5.3.21 Lipid peroxidation and tissue antioxidant status

5.3.21.a) Estimation of lipid peroxides (LPO)

Lipid peroxides content was determined by thiobarbaturic acid reaction as described by Ohkawa *et al.* (1979) in liver tissue.

Reagents

1. Acetic acid 20%: 20ml of glacial acetic acid dissolved in 100ml distilled water.
2. 8% Thiobarbaturic acid (TBA) in 20% acetic acid.
3. 8% Sodium dodecyl sulphate (SDS).
4. Standard: 41.66mg of tetraethoxy propane (TEP) dissolved in 100ml distilled water. 1.0ml of above was made up to 10ml with distilled water.

Procedure

To 0.2ml of sample, 1.5ml of 20% acetic acid, 0.2ml of SDS and 1.5ml of TBA were added. The mixture was made upto 4.0ml with distilled water and heated in a boiling water bath for one hour. After cooling the mixture was centrifuged at 3000g for 10min. Supernatant was taken and absorbance was read at 532nm in a Shimadzu-UV spectrophotometer.

The lipid peroxides content was expressed as nmol of malondialdehyde/g tissue.

5.3.22 Antioxidant defense system

5.3.22.I) Non-enzymatic antioxidants

5.3.22.a) Determination of total reduced glutathione (GSH)

The total reduced glutathione was determined by the method of Ellman (1959). The method is based on the reaction of reduced glutathione with 5, 5'-dithiosbis (2-nitrobenzoic acid) (DTNB) to give a compound that has absorbance at 412nm.

Reagents

1. DTNB: 0.6mM in 0.2M Phosphate buffer pH 8.0
2. 0.2M Phosphate buffer, pH 8.0.
3. 5% Trichloroacetic acid
4. Standard: 61.4mg of reduced glutathione was dissolved in 100ml 0.02M EDTA, 0.1ml of this is made up to 10ml with 0.02M EDTA.
5. Working standard: 2.0ml of the above was made up to 10ml.

Procedure

0.5ml of liver homogenate was precipitated with 5% of TCA. The contents were mixed well for complete precipitation of proteins and centrifuged at 4000 rpm for 5 min. To an aliquot of clear supernatant, 2.0ml of DTNB reagent and 0.2M phosphate buffer were added to make a final volume of 4.0ml. The absorbance was read at 412nm against a blank containing TCA instead of sample, series of standards treated in a similar way were also run to determine the reduced glutathione content. The amount of glutathione was expressed as nmol/mg protein.

5.3.22.b) Ascorbic acid (Vitamin C)

Ascorbic acid (Vitamin C) was determined in the liver tissue by the method of Roe and Kuether (1943).

Reagents

1. DTC reagent: Take 0.4g thio urea, 0.05g $CuSO_4$, 3g DNPH and dissolved in 100ml of 9N H_2SO_4.
2. 10% TCA
3. 65% H_2SO_4
4. Standard: Stock: 25mg ascorbic acid dissolved in 50ml of 5% TCA. Working standard: 5mg /ml in 5% TCA

Procedure

100mg of liver sample was homegenated with 2.0ml of Tris Hcl buffer pH 7, centrifuged for 10min at 3000rpm. 1.0ml of the supernatant was taken into test tube, added 1.0ml 10% ice cold TCA and centrifuged for 20min. 0.5ml of the supernatant was taken into another test tube, added 2.0ml 5% TCA and 0.1ml DTC reagent, incubated for 3h at 37^{O}C. Cooled to room temperature and added 0.75ml ice cold 65% H_2SO_4, kept it for 30min and read OD at 520nm. A blank was carried out using 1.0ml of distilled water. The standards were also treated similarly.

The content of vitamin C was expressed as mg/g tissue

5.3.22.c) Vitamin E (α-Tochopherol)

α-Tochopherol (Vitamin E) was determined in liver tissue by the method of Baker *et al.* (1980).

Reagents

1. 2% pyrogallol (or epinephrine) in ethanol
2. Saturated KOH in ethanol
3. Hexane
4. Ethanol
5. 0.2% Bathophenanthroline in ethanol
6. 0.001M $FeCl_3$ in ethanol
7. 0.001M H_3PO_4 in ethanol
8. Standard: 1mg/ml in ethanol.

Procedure

100mg heart sample was homegented with 2.0ml of ethyl alcohol, centrifuged and took 1.0ml of the supernatant into a test tube. To this added 1ml of 2% epinephrine and incubate at 70^{O}C for 2min. Added 0.3ml of of saturated KOH and kept in a water bath at 70^{O}C for 2min. Cooled in ice bath and added 1ml H_2O and 4.0ml hexane. Centrifuged and took 3ml of the upper hexane layer and evaporated to dry. To the residues and standards (0.1-0.5ml) added 3.0ml ethyl alcohol, 0.2ml bathophenanthroline, 0.2ml $FeCl_3$ and 0.2ml H_3PO_4. Mixed well and read OD at 550nm.

The content of vitamin E was expressed as mg/g tissue

5.3.22.II) Antioxidant enzymes

5.3.22.d) Assay of glutathione-S-transferase (EC 2.5.1.18)

Glutathione-S-tranferase (GST) activity was determined by the method of Habig *et al.* (1974).

Reagents

1. 0.3M Phosphate buffer, pH 6.5
2. 30mM 1-chloro-2, 4-dinitrobenzene (CDNB).
3. 30mM reduced glutathione (GSH).

Procedure

The reaction mixture containing 1.0ml of buffer, 0.1ml of CDNB and 0.1ml of tissue homogenate was made up to 2.5ml with water. The reaction mixture was pre-incubated at 37°C for 5min. 0.1ml of GSH was added and the change in the absorbance was measured at 340nm for 3min at 30sec intervals.

The enzyme activity was expressed as μmol of CDNB-GSH conjugate formed/min/mg protein.

5.3.22.e) Estimation of glutathione peroxidase (EC 1.11.1.9)

The method of Paglia and Valentine (1967) was adopted for assay of glutathione peroxidase (GPx).

Reagents

1. 0.4 M Phosphate buffer, pH 7.
2. 0.4M Tris buffer, pH 8.9
3. 0.4mM EDTA
4. 2mM GSH
5. 10mM NaN_3
6. 10% TCA
7. DTNB: 99mg in 25ml of methanol.
8. H_2O_2: 1mM was prepared freshly from commercial 30%solution.
9. GSH standard: 61.4mg GSH was dissolved in 100ml distilled water. 1.0ml of this solution was made up to 10ml with distilled water.

Working standard: 2ml of the stock was made up to 10ml with distilled water.

Procedure

0.2ml of tissue homogenate was added to a mixture containing 0.2ml of buffer, 0.2ml of EDTA and 0.1ml of sodium azide. Mixed well and added 0.1ml reduced glutathione and 0.1ml of hydrogen peroxide. Incubated in a water bath at 37°C for 10min. At the end of incubation period, 0.5ml of 10%TCA was added and centrifuged at 10000 rpm for 5min. 1.0ml of the supernatant was taken into a separate test tube and added 2.0ml Tris buffer and 50μl DTNB. Immediately read the OD at 412nm.

The enzyme activity was expressed as nmol of glutathione oxidized/min/mg protein.

5.3.22.f) Assay of glutathione reductase (EC 1.6.4.2)

Glutathione reductase activity was assayed spectrophotometrically by the method adopted by Dulber and Anderson (1981).

Reagents

1. Sodium phosphate buffer: 50 mM, pH, 7.5
2. EDTA: 1.0mM
3. Glutathione (oxidised) : 0.67 M
4. Nicotinamide adenine dinucleotide phosphate (reduced) (NADPH): 0.1 mM

Procedure

The reaction mixture containing 50mM phosphate buffer, pH 7.5, 1.0 mM EDTA, 0.67 mM GSSG and 0.1 mM NADPH was made upto 3.0 ml with distilled water. After the addition of suitable aliquot of the tissue homogenate the change in optical density at 340 nm was monitored at 27^0C for 2 min at 30 sec. intervals.

The enzyme activity was expressed as nmol of NADPH oxidized/min/mg protein.

5.3.22.g) Assay of superoxide dismutase (EC 1.15.1.1)

The superoxide dismutase was assayed according to the method of Misra and Fridovich (1972) based on the oxidation of epinephrine-adrenochrome transition by the enzyme.

Reagents

1. 0.1M Carbonate-bicarbonate buffer, pH 10.2 containing 57mg/dl EDTA.
2. 3mM Epinephrine

Procedure

50µl of sample was taken into the cuvette and added 1.5ml buffer and 0.5ml epinephrine. Mixed well and immediately read the change in optical density at 480nm for 2min in a Shimadzu-UV spectrophotometer.

One unit of SOD activity was the amount of protein required to give 50% inhibition of epinephrine auto oxidation.

5.3.22.f) Assay of catalase (EC 1.11.1.6)

Catalase (CAT) was assayed according to the method of Takahara *et al.* (1960).

Reagents

1. 50mM Phosphate buffer, pH 7.0
2. 30mM Hydrogen peroxide solution in the above buffer.

Procedure

To 2.45ml of the phosphate buffer, 50µl of the liver homogenate was added and the reaction was started by the addition of 1.0ml of H_2O_2 solution. The decrease in absorbance was measured at 240nm at 30sec intervals for 2min. The enzyme blank was run simultaneously with 1.0ml of distilled water instead of hydrogen peroxide.

The enzyme activity was expressed as nmol of H_2O_2 decomposed/min/mg protein.

5.3.23 Membrane-bound phosphatases

5.3.23.a) Assay of Na+/K+-dependent ATPase

Na^+/K^+-dependent ATPase activity was measured from the amount of Pi released according to the method of Bonting (1970).

Reagents

1. 184mM Tris buffer, pH 7.
2. 50mM Magnesium Sulphate.
3. 50mM Potassium Chloride.
4. 600mM Sodium Chloride.
5. 1.0mM EDTA.
6. 40mM adenosine triphosphate (ATP).
7. 10% trichloroacetic acid (TCA).

Procedure

One ml of Tris-buffer and 0.2ml each of the above assay reagents were mixed together. So the assay medium, in the final volume of 2.0ml contained, 92mM tris-buffer, 5mM magnesium sulphate, 60mM sodium chloride, 5mM potassium chloride, 0.1mM EDTA and 4.0mM ATP. After 10min equilibration at 37°C in an incubator, reaction was started by the addition of 0.2ml of the tissue homogenate. The assay medium was incubated for 30 min and at the end of the incubation period. The reaction was stopped by the addition of 2 volumes of ice cold 10% TCA. The phosphorus (Pi) liberated was estimated by the method of Fiske and Subbarow (1925).

The enzyme activity was expressed as µmol of Pi liberated/min/mg protein.

5.3.23.b) Assay of Ca 2+-dependent ATPase

Ca 2+-dependent ATPase was assayed by the method of Hjerten and Pan (1983).

Reagents

1. 0.125M Tris-HCl buffer, pH 8.0
2. 0.05M Calcium chloride.
3. 0.01M ATP.
4. 10% TCA.

Procedure

Tris-HCl buffer 0.1ml, Calcium 0.1ml, ATP solution 0.1ml and distilled water 0.1ml were taken in test tubes. The reaction was started by the addition of 0.1ml of tissue homogenate. Tubes were incubated at 37°C for 15min. The reaction was arrested by the addition of 10% TCA to the incubation mixture. 0.1ml enzyme source was added to the control tubes. The contents were centrifuged at 4000 rpm for 5 min. The supernatant was used for the estimation of inorganic phosphorous. The phosphorus (Pi) liberated was estimated by the method of Fiske and Subbarow (1925).

The enzyme activity was expressed as μmol of Pi liberated/min/mg protein.

5.3.23.c) Assay of Mg2+-dependent ATPase.

Mg^{2+}-ATPase was assayed according to the method described by Ohinishi *et al.* (1982)

Reagents

1. 0.375M Tris- HCl buffer, pH 7.6
2. 0.205M Magnesium chloride
3. 0.01M ATP

Procedure

Reaction mixture contained 0.1ml buffer, 0.1ml of ATP, 0.1ml magnesium chloride and distilled water. The reaction was started by the addition of 0.1ml of enzyme preparation. The tubes were then incubated at 37°C for 15min. The reaction was stopped by the addition of 1.0ml of 10% TCA. 0.1ml of enzyme was added to the control tubes. The phosphorus (Pi) liberated was estimated by the method of Fiske and Subbarow (1925)

The enzyme activity was expressed as μmol of Pi liberated/min/mg protein.

5.3.24 Estimation of minerals using atomic absorption spectrophotometer

Minerals were estimated according to the method of the AOAC (1980).

Reagents

1. Nitric acid :Perchloric acid mixture 9:4
2. Stock solution of sodium, potassium, calcium were prepared by diluting concentrated solution of 1000 mg/L (Merck).

Procedure

Samples size of 0.5 g of liver tissue was used for the experiment. To the sample containing flask, 7ml of nitric acid and perchloric acid (9:4) mixture was added, covered with a watch glass and left at room temperature over night. The sample was then digested using a microwave digester (Milestone ETHOS PLUS Closed Vessel Microwave Digestion System). The completely digested samples were allowed to cool at room temperature, filtered (by using glass wool) carefully transferred into a clean 50ml volumetric standard flask and then diluted to the mark with ultra pure water (Milli Q, Millipore). The digested samples were analyzed using Varian model 220 atomic absorption spectrophotometer equipped with a deuterium back ground corrector, for the determination of minerals viz sodium, potassium, calcium.

5.3.25 Lysosomal marker enzymes

5.3.25.a) Assay of β-glucosidase (EC 3.2.1.21)

β- glucosidase was assayed according to the method of Conchie *et al.* (1967) based on the principle that β- glucosidase acts on P-nitrophenyl-β-D-glucopyranoside and liberate P-nitrophenol, which was measured at 410 nm in alkaline pH.

Reagents

1. Substrate: 10 mM, p-nitrophenyl β-D-gluco pyranoside in buffer
2. Phosphate buffer : 0.1 M Citrate-0.2 M phosphate, pH 4.5
3. Glycine-NaOH buffer : 0. 4 M pH-10.4
4. Standard : 6 mM, p- nitrophenol
5. Working standard : 1 ml diluted to 10 ml using distilled water.

Procedure

To 0.5 ml of substrate and 0.3 ml of citrate buffer in a test tube, 0.2 ml of the enzyme solution was added, shaken gently and incubated at 37°C for 1h. 3 ml of glycine–NaOH buffer was added for reaction termination. Mixed and read at 410 nm using shimadzu UV-1601 spectrophotometer.

The activity of β-glucosidase was expressed as µmol p-nitro phenol liberated/mg protein.

5.3.25.b) Assay of β-galactosidase (EC 3.2.1.23)

β-galactosidase was assayed according to the method of Conchie *et al.* (1967) based on the principle that β-galactosidase acts on p-nitrophenyl- β-D-glucopyranoside and liberate P-nitrophenol, which was measured at 410 nm in alkaline pH.

Reagents

1. Substrate : 2 mM, p-nitro phenyl β-D-galactopyranoside in buffer
2. Phosphate buffer : 0.1M Citrate-0.2 M phosphate, pH 4.5
3. Glycine-NaOH buffer : 0.4 M pH-10.4
4. Standard : 6 mM, p- nitrophenol
5. Working standard : 1 ml diluted to 10 ml using distilled water

Procedure

To 0.5 ml of substrate and 0.3 ml of citrate buffer in a test tube, 0.2 ml of the enzyme solution was added, shaken gently and incubated at 37°C for 1h. 3 ml of glycine–NaOH buffer was added for reaction termination. Mixed and read at 410 nm.

The activity of β-galactosidase was expressed as µmol p-nitrophenol liberated/ mg protein.

5.3.25.c) Assay of β-glucuronidase

B-glucuronidase activity was measured by the method of Kawai and Anno (1971) using p-nitrophenol- β-glucuronide as the substrate.

Reagents

1. Substrate : 0.01M , p-nitro phenyl β-D-glucuronide in buffer
2. 0.1M Sodium acetate buffer, pH 4.5
3. 0.4M Glycine-NaOH buffer, pH-10.4
4. Standard: 6mM, p-nitrophenol

Working standard: 1.0 ml diluted to 10 ml using distilled water

Procedure

To 0.5 ml of substrate and 0.3 ml of Sodium acetate buffer in a test tube, 0.2 ml of the enzyme solution was added, shaken gently and incubated at 37°C for one hour. 3.0 ml

of glycine-NaOH buffer was added for reaction termination. Mixed and read at 410 nm.

The activity of β-glucuronidase was expressed as µmol of p-nitrophenol liberated/h/ 100 mg protein.

5.3.25.d) Assay of acid proteinase (Cathepsin-D)

Acid proteinase (Cathepsin-D) was assayed by the method of Sapolsky *et al.*, (1973) using Haemoglobin as substrate.

Reagents

1. Substrate : 1.5% haemoglobin in buffer
2. 0.2M Sodium formate buffer, pH 3.5
3. 10% TCA solution
4. 4% Sodium carbonate solution in 0.1M NaOH
5. Folins reagent: Commercial diluted in 1:2
6. Standard: 100mg/ml Tyrosine in dilute HCl

Procedure

Reaction mixture contained a final volume of 2ml consisting 0.8ml buffer, 1.0ml substrate, 0.2ml enzyme homogenate. Incubated at 37^0C for 2h. Enzyme reaction was arrested by adding 2ml of 10% TCA. Control received enzyme after arresting the reaction. After 30min tubes are centrifuged at 1000×g for 15min. Took 2.0ml of the supernatant and added 2.5ml of Sodium carbonate. Mixed well and added 0.5ml folins reagent. Blue colour developed was read at 670nm.

The activity of acid proteinase was expressed as µmol of tyrosine/h/100mg protein.

5.3.25.e) Assay of acid phosphatase (EC 3.1.3.2)

Acid phosphatase was assayed by the method of King (1965^b) using disodium phenyl phosphate as the substrate.

Reagents

0.1M Citrate buffer, pH 4.9: 2.1014 g of citric acid and 2.941 g of sodium citrate were dissolved in 100 ml of distilled water

Substrate: 0.01M Disodium phenyl phosphate solution.

Folin's phenol reagent: This was diluted 1:2 with double distilled water before use.

15% Sodium carbonate

Standard phenol solution:Stock: 50 mg phenol dissolved in 100 ml distilled water which is of a conc of 500 µg /ml

Working std: 1 ml of stock diluted to 10 ml using distilled water, which has a conc of 50 µg /ml.

Procedure

The incubation mixture contained the following components in a final volume of 3.0 ml. 1.5 ml of citrate buffer, 1.0 ml of substrate, 0.3 ml of distilled water and requisite amount of the enzyme source (0.2 ml serum). The reaction mixture was incubated at 37^0C for 15 min. The reaction was terminated by the addition of 1.0 ml of Folin's phenol reagent. If turbidity appeared, the tubes were centrifuged. Controls without enzyme sources were also incubated and the enzyme source was added after the addition of Folin's phenol reagent. 1.0 ml of 15% sodium carbonate solution was added and incubated for a further 10min at 37^0C. The blue colour developed was read at 640 nm against a blank. The standards were also treated similarly.

The activity of the enzyme was expressed as µmol of phenol liberated/h/l (plasma); µmol of phenol liberated/ mg protein (tissue).

5.3.25.f) Assay of alkaline phosphatase (EC 3.1.3.1)

Alkaline phosphatase was assayed by the method of King (1965b) using disodium phenyl phosphate as the substrate.

Reagents

1. 0.1M Carbonate-bicarbonate buffer, pH 10.0
   Solution A: 0.1M Na_2CO_3: 1.0599g Na_2CO_3 dissolved in 100ml distilled water.
   Solution B: 0.1M $NaHCO_3$: 0.8401g $NaHCO_3$ dissolved in 100ml distilled water.
   27.5ml of Solution A was mixed with 22.5ml of Solution B and the mixture was made up to 200ml with distilled water to get the required buffer
2. Substrate: 0.01M disodium phenyl phosphate solution.
3. Folin's phenol reagent: This was diluted 1:2 with double distilled water before use.
4. 15% Sodium carbonate
5. 0.1M Magnesium chloride
6. Standard phenol solution:

Stock standard: 50mg phenol dissolved in 100ml distilled water which was of a concentration of 500μg /ml

Working standard: 1ml of stock diluted to 10ml using distilled water, which was having a concentration of 50μg /ml

Procedure

The incubation mixture contained the following components in a final volume of 3.0ml with 1.5ml of carbonate-bicarbonate buffer, 1.0ml of substrate and 0.1ml of magnesium chloride and 0.2 ml of sample. The reaction mixture was incubated at 37°C for 15min. The reaction was terminated by the addition of 1.0ml of Folin's phenol reagent. If turbidity appeared, the tubes were centrifuged. Controls without samples were also incubated and the sample was added after the addition of Folin's phenol reagent. The 1.0ml of 15% sodium carbonate solution was added and incubated for a further 10min at 37°C. The blue colour developed was read at 640nm against a blank. The standards were also treated similarly.

The activity of the enzyme was expressed as U/dl (plasma); nmol of phenol liberated/min/ mg protein (liver tissue).

5.3.26 Statistical analysis

All data were expressed as mean ± SD and analyzed statistically by one-way analysis of variance using Duncan's multiple comparison test with a level of significance set at $P<0.05$. The statistical software, SPSS for Windows version 16 (SPSS Inc., Chicago, IL), was employed for the analyses.

5.4 RESULTS AND DISCUSSION

Rheumatoid arthritis is a condition that causes pain and inflammation and stiffness in many joints, especially in the hands and feet. It could start at any age, but most commonly between 40 and 50 years old. Three times as many women as men are affected. Rheumatoid arthritis is a common condition, but it is only rare complications of the disorder and its treatment that could be fatal. It is one of the most potential debilitating inflammatory disorders that affect more than 2.5 million Americans and over 7 million Indians every year. Rheumatoid arthritis caused 0.04% of all deaths worldwide in 2002, an average of 4 deaths per million people per year. Though not highly significant in terms of death toll the overall number of patients that live with this disorder is large.

During this inflammatory disorder, periods of active inflammation and tissue damage marked by worsening of symptoms (flares) are interspersed with periods of little or no activity, in which symptoms get better or go away altogether (remission). The duration of these cycles varies widely among individuals. Approximately 40% of people have some degree of disability 10 years after their diagnosis. For most, rheumatoid arthritis is a chronic progressive illness, but about 5%-10% of people experience remission without treatment. Rheumatoid arthritis is not fatal, but complications of the disease shorten life span by a few years in some individuals. Although generally rheumatoid arthritis cannot be cured, the disease gradually becomes less aggressive and symptoms may even improve. However, any damage to joints and ligaments and any deformities that have occurred are permanent. Rheumatoid arthritis can affect parts of the body other than the joints. The early use of DMARDs and biologic response modifiers in rheumatoid arthritis have resulted in patients experiencing more profound relief of symptoms and less joint damage and less disability over time. DMARDs can actually stop or slow the progression of rheumatoid arthritis. They can also suppress the ability of the immune system to fight infections.

Since prolonged consumption of modern medications is linked up with negative side effects the need for natural remedies for treatment of rheumatoid arthritis is fast emerging. Epidemiological studies have shown lower incidence of inflammatory disorders among the population who consume fish oils in their diet (Parkinson *et al.*, 1994; Mannisto *et al.*, 1999; Augustsson *et al.*, 2001), Evidence on the role of fish

oils of deep sea elasmobranchs on various metabolisms associated with rheumatoid arthritis is scarce. In the present study, we decided to investigate the effect of feeding liver oil of the deep sea shark *C.scalpratus* and compare its efficiency with gingerol concentrate (a traditional and natural anti-inflammatory agent) , on the progression of CFA-induced inflammation and thereby analyse some of the important metabolisms undergoing within blood and liver during arthritis.

5.4.1 Effect of *C.scalpratus* liver oil in CFA-induced arthritis in male Albino rats

To the best of our knowledge the present study is the first one of its kind to evaluate the anti-arthritic potential of *C.scalpratus* liver oil against CFA-induced arthritis in male Albino rats and thereafter compare its efficacy with yet another natural anti-inflammatory substance, gingerol. Adjuvant arthritis is a rat model of autoimmune erosive arthritis widely used to evaluate etiopathogenetic mechanisms in rheumatoid arthritis as well as for testing the potency of anti-inflammatory drugs (Joe, 1999). Although animal models can only represent partial aspects of the complex pathology of human disease, they allow the development of therapeutic approaches for rheumatoid arthritis. The development of rat arthritis induced by adjuvant is accompanied by the induction of cyclooxygenase-2 (COX-2) and iNOS, and high levels of derived metabolites can be measured in the serum and synovium of arthritic rats (Connor *et al.*,1995; Fletcher *et al.*,1999)

5.4.2 Paw sizes and inflammatory score

The effect of CFA-induced inflammations on paw sizes was analysed (Fig. 5.1). Maximum thicknesses for paw sizes were observed on the 14^{th} day of the experiment in groups II, III and IV. However, there were no significant differences in paw sizes for either of the Control groups I and V. When Groups III and IV were treated with CS oil and gingerol significant ($p<0.05$) reductions in paw sizes were noted from the 15^{th} day of the experiment. The paw sizes of gingerol treated rats achieved faster reductions in edema and they resembled those of the control animals from the 27^{th} day of the experiment. Fish oil treated group III animals attained normal paw structure on the 36^{th} day. However there was no significant reductions in paw sizes for group II animals induced with inflammation/edema.

Similarly the arthritic index was also monitored and maximum edema was observed for the groups II, III and IV on the 14th day where a maximum score of 3 was recorded (Fig. 5.2). Three days after treatment with CS oil for group III animals, there was a sudden decrease in inflammatory score from 3 to 2.5 to less than 0.5 over a time span of 20 days. However the gingerol treated group attained a score of 2 to 1.0 to less than 0.5 on the 27th day of the experiment.

Inflammatory joint tissue in RA is characterized by infiltration and accumulation of mononuclear phagocytes, lymphocytes, and plasma cells; proliferation of synovial cells and expression of proinflammatory cytokines. The sequence of events which occur during an inflammatory response can vary, depending on the type or cause of injury (i.e., bacteria, cold, heat, trauma), the site of the injury, and the state of the body. In a localized infection damage causes the release of chemicals called immune mediators from the injured cells. These cause dilation of blood vessels producing heat and redness which then leak lymph into the damaged area (swelling). The chemicals also stimulate nerve fibres (pain) to draw the animal's attention to the injury and to encourage it to stop doing whatever caused the injury. Depending on the area involved, there may also be stimulation of secretion of mucus. Blood clotting factors are also stimulated in case there has been sufficient damage to cause haemorrhage. White blood cells are attracted to clean up any infection resulting in pus. As a consequence maximum edema was recorded for all the CFA-induced groups II, III, IV on the 14th day.

It is interesting to note that the dietary supplementation of CS oil and gingerol significantly reduced edema in group III and IV compared to group II rats (Fig 5.1, 5.2). Reports by Ramos *et al.* (2004) showed that the dietary intake of n-3 fatty acids was effective in attenuating edema in inflammation-induced rats. Studies carried out by Cleland et al (2003) on arthritis patients taking fish oil medication showed decreased joint pain, morning stiffness less swelling of the affected areas. Investigations carried out by Mythilypriya etal, (2007) in adjuvant-induced rats showed reductions in the paw volume in arthritis induced rats upon feeding Kalpaamruthaa, a siddha drug. The present finding confirmed the same pattern and showed lesser edema volume in groups III and IV rats compared to that of group II rats, indicating the anti-inflammatory effect of dietary CS oil and gingerol concentrate

supplementation on CFA-induced inflammatory conditions. Though it is evident from the present finding that both CS oil and gingerol concentrate can reduce inflammation the exact mechanism involved is not known. Kim *et al.*, (2010) proposed that the formation of cytostatic and cytotoxic compounds after peroxidation of long chain PUFA was responsible for the beneficial effect against arthritis. The other proposed mechanisms include the modulation of antioxidant system (Fernandes *et al.*, 1996), alteration in prostaglandin synthesis, change in gene/oncogene expression (Das, 1999) and control on cell cycle phases (Lai *et al.*, 1996; Chen and Istfan, 2001).

5.4.3 Effect on body weight

Cachexia is a complex metabolic problem and commonly manifested as weight loss with the depletion of muscle protein and fat (Tisdale, 1996). Loss of host body weight and raise in level of inflammatory mediators has been primarily used as an early tool to assess severity of arthritis in animals (McPhee *et al.*, 2010). In the present study, group-II, III and IV rats demonstrated a significant decrease in the body weight upto the 14^{th} day of experiment. However treatment with CS oil and gingerol extracts significantly improved appetite and bodyweights of both Groups III and IV from the 15^{th} day while Group II rats showed an overall loss of body weight at the end of the experiment (Fig 5.3) which is supported by earlier report by Gomaa *et al.* (2009).

Anorexia is an eating disorder associated with reduced rate of food intake and loss of weight in normal persons. Although anorexia is often associated with cachexia, in the present study loss of body weight can't be explained by reduced feed intake alone, as the onset of anorexia appeared after 14^{th} day of experiment for group II rats where as cachexia noticed in the 1^{st} week itself. Earlier studies by Roubenoff (2009) reported that gain in lean tissue mass in cachectic patient with inflammatory disorders was difficult to be explained by increased food intake until the metabolic abnormalities involved in induction of cachexia were corrected. Cytokines such as TNF-α, IL – 1, IL- 6, Interferon γ and other factors such as proteolysis inducing factor (PIF) derived from inflammation, act in a co-ordinated manner to induce cachexia (Hultgren *et al.*, 2004).

Figure 5.1 Effect of *C.scalpratus* liver oil in comparison to gingerol on paw-sizes in CFA-induced arthritis in rats

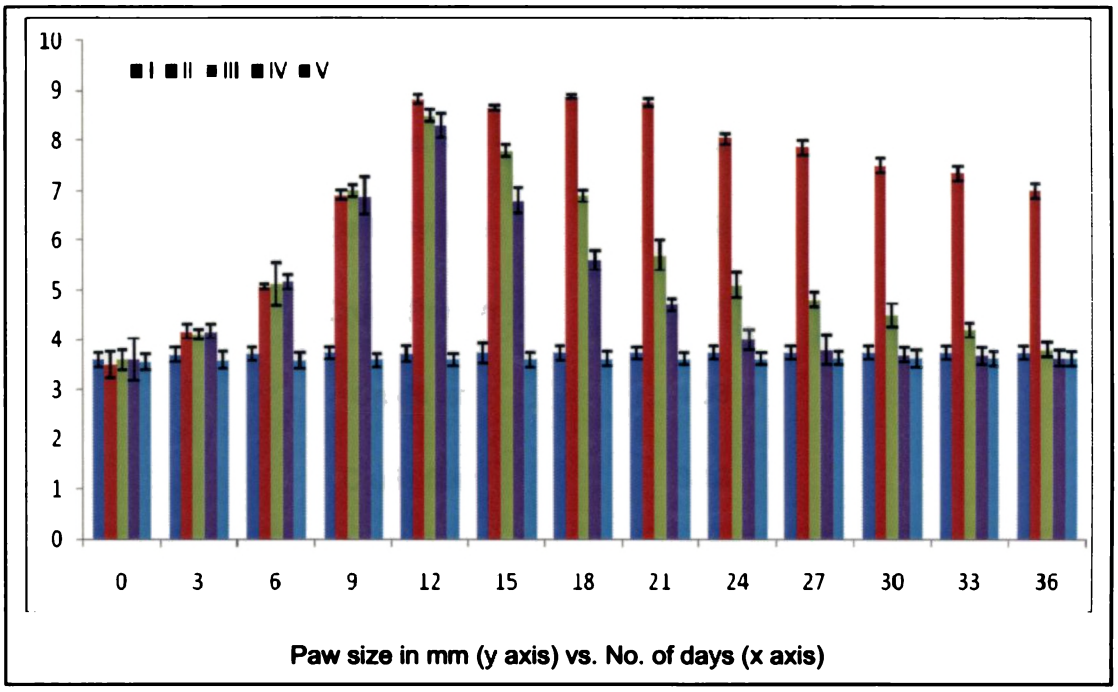
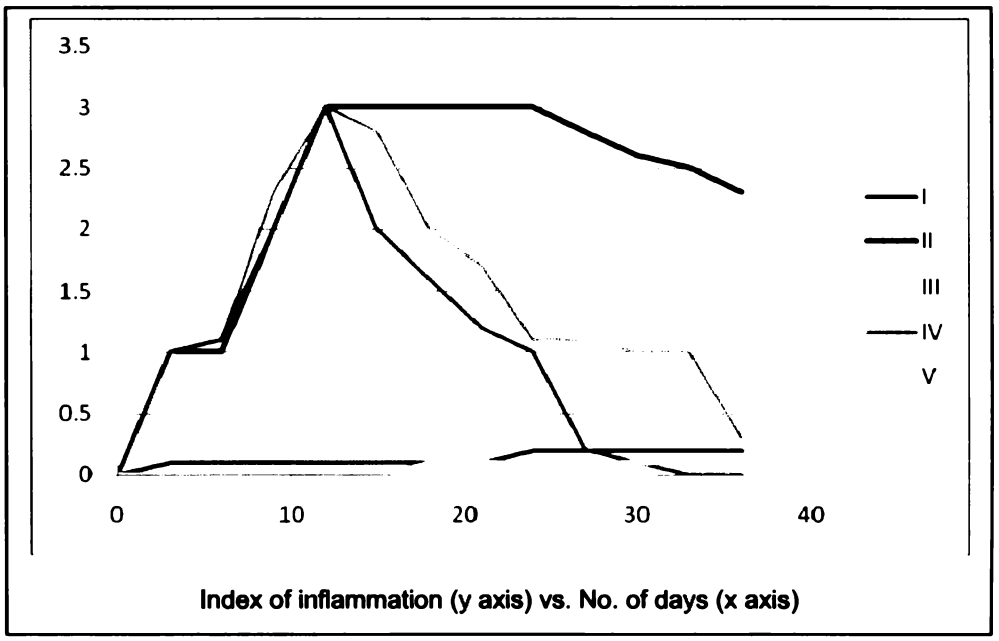


Figure 5.2 Effect of *C.scalpratus* liver oil in comparison to gingerol on the inflammatory score in CFA-induced arthritis in rats

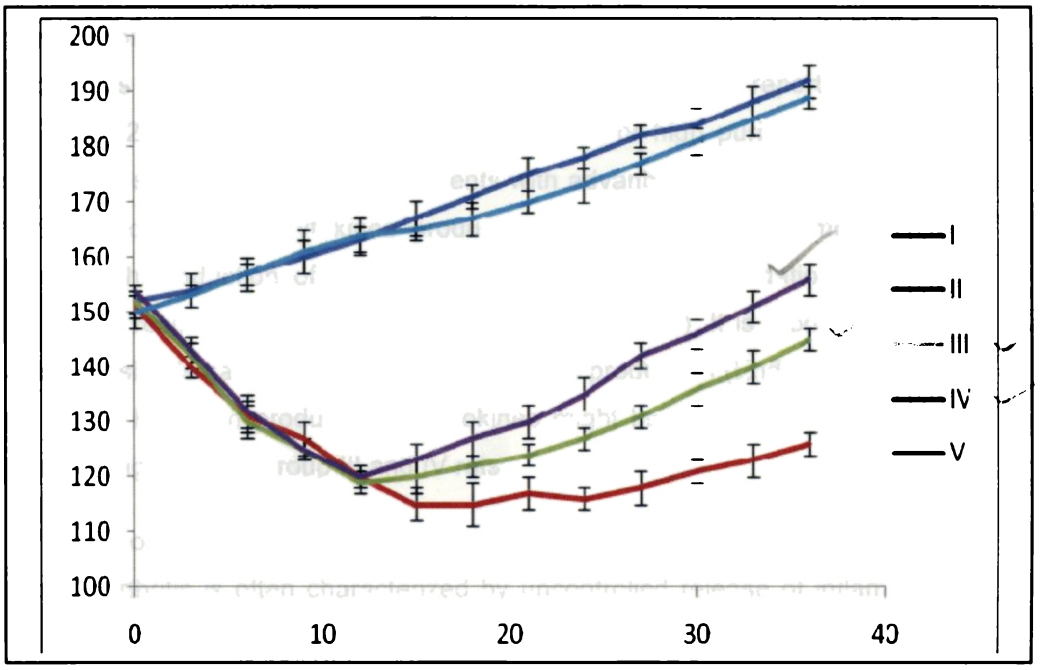


Score values: 0 – no inflammation/edema 2 – moderate inflammation/edema
1 – low inflammation/edema 3 – high inflammation/edema

Table 5a. Composition of the basal diet

Specifications	(% by weight)
Moisture	10.2
Carbohydrate	54.2
Crude Protein	22.1
Crude fat	4.03
Crude fiber	3.12
Minerals	5.10
Sand silica	1.11

Figure 5.3 Changes in body weight in CFA-induced inflammations in Albino rats



Body weight (y axis) vs. No. of days (x axis)

Results are mean ± SD for n=6 animals.

Group I – normal control (provided with normal basal diet alone)

Group II – CFA induced arthritis (0.01 ml suspension in paraffin oil– 10mg/ml heat killed *Mycobacterium tuberculosae* injected into right hind paw) ; provided normal basal diet

Group III - CFA induced arthritis + *Centrophorus scalpratus* (CS) oil – oral feeding (1.0 g/kg bwt) from the 15th day onwards; provided normal basal diet

Group IV - CFA induced arthritis + gingerol – oral feeding (250 mg/kg bwt) from the 15th day onwards *(ref. std.)*; provided normal basal diet

Group V – normal control + CS oil fed from 15th day onwards; provided normal basal diet.

Dietary supplementation of fish oil and gingerol concentrate reduced the loss of body weight and improved the gain in body weight of groups III and IV rats in comparison to group II rats (Fig 5.3), which is in agreement with the earlier reported study by Ramos *et al.*, 2004. Administrations of n-3 fatty acid or high purity EPA capsules have shown to stabilize the weight in patients with advanced arthritis (Kremer *et al.*, 1990). Elevated levels of cytokines produced during an acute inflammation are suspected in the induction of cachexia and anorexia and degradation of muscle protein and fat reserves in cachectic patients (Tisdale, 2000). It is reported that n-3 fatty acids in fish oils can down regulate the cytokine production (Endres *et al.*, 1989; Calder, 2002). Reduced production of cytokines might be another possible reason for reduced weight loss in group III and IV rats.

5.4.4 Effect of on liver weight

Rheumatoid arthritis is often characterized by uncontrolled release of inflammatory mediators and increase in acute phase proteins (Germolec, 2010) at the inflammatory site. In the present study, growth of liver tissue as a whole was used as an index to assess the severity of inflammation. Liver weight in group II rats was significantly higher ($p< 0.05$) compared with group I control rats (Fig 5.8) which is in line with earlier observation by Castillero *et al.* (2009). Morton *et al.* (1970) reported that the relative liver weight was significantly higher in animals induced with inflammaiton and treatment of these animals with extracts of gingerol reversed the liver weight to that of control animals. The faster multiplication of hepatoma cells might be the probable reason for increase in liver mass noticed in group II rats.

In the present study it was interesting to see that dietary supplementation of CS oil and gingerol extracts significantly decreased ($p<0.05$) the liver weight in groups III and IV rats in comparison to group II rats (Fig. 5.8). The present result is in line with the earlier report by Funk (2009). Supplementation of n-3 PUFA either in cell culture (Ruggiero *et al.*, 2009) or in animal studies Wooki *et al.*, 2010) showed to inhibit the cell proliferation by producing lipid peroxides. In the present instance similar kind of effect of n-3 PUFA in proliferation of hepatoma could be the factor that reduced the enlargement of liver size reflecting lesser weight of liver in group III and IV rats compared to group II rats.

Figure 5.7 Effect of CFA-induced inflammations on RBC & WBC counts in CFA-induced inflammations in rats

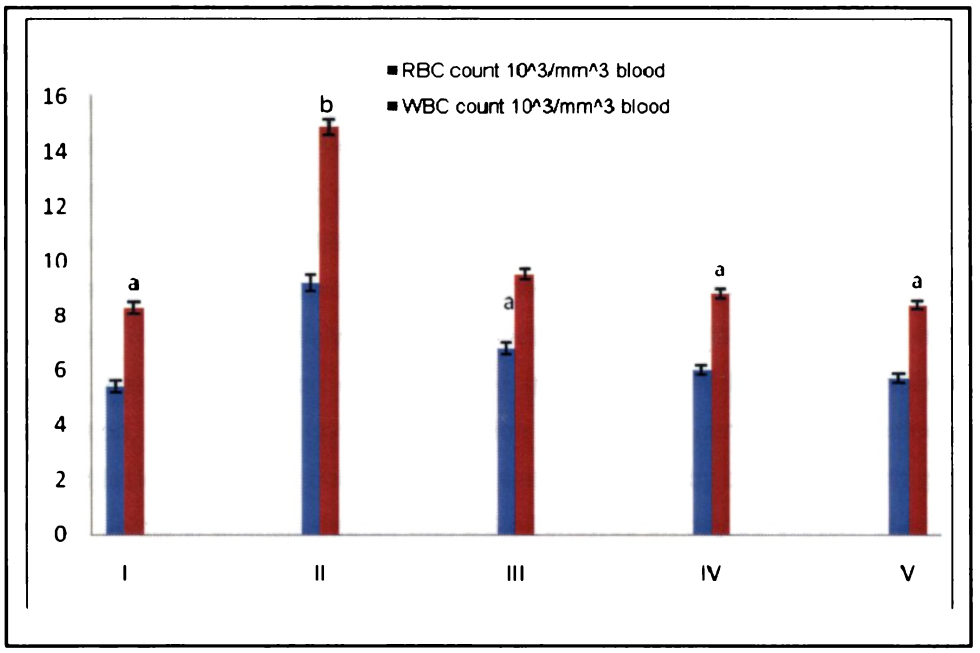
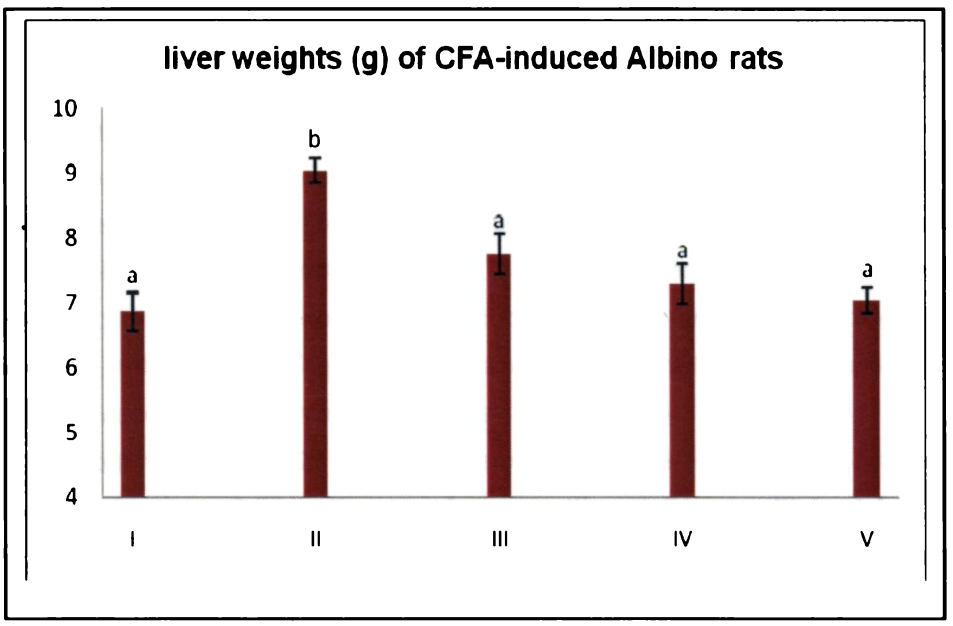


Figure 5.8 Effect of CFA-induced inflammations on the liver weights of rats



Values are mean±SD for n=6, groups with different letters[a,b,c,d,e] are significantly different ($p<0.05$)

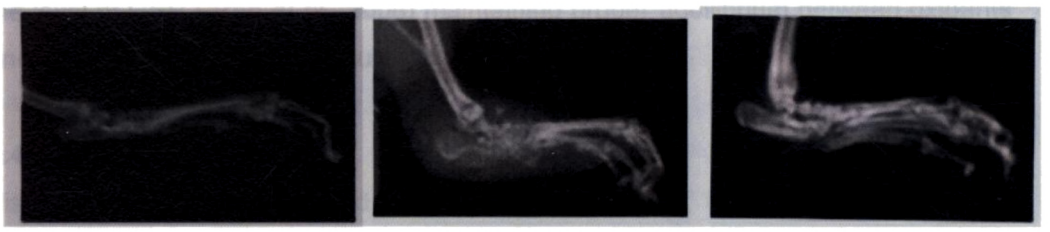
5.4.5 X ray and bone histopathology

Arthritic changes were evident in radiographs of hind paws and were confirmed by histological analysis (Plate V.1). Evidence of chronic inflammation was apparent in joints from arthritic rats on day 36. The arthritic control group showed a characteristic periarticular inflammation with edema and infiltration of polymorphonuclear neutrophils and lymphocytic cells, synovitis and synovial hyperplasia as well as a severe loss of articular cartilage. Periostitis and bone resorption were associated with a marked osteoclastic activity, new bone formation and ankylosis (Devessa *et al.,* 2007) . Histological analysis of joint sections of animals of groups III and IV, indicated that administration of CS oil and gingerol inhibited leukocyte infiltration, inflammation, hyperplastic synovitis, erosion of articular cartilage and osteolysis. Stabilized lesions and new bone formation were observed by day 36 for both groups III and IV. Joints of animals treated with CS oil showed on day 25 erosion of articular cartilage with pannus accompanied by a regenerative process including fibrosis and new bone formation. However by day 36, there was a general increase in inflammatory parameters in the joints of CFA-induced and untreated group II animals, with leukocyte infiltration, erosion of cartilage with pannus as well as osteolysis.

Bone tissue sections from groups I and V rats showed normal architecture and a regular arrangement of cells with small granulated cytoplasm around the central vein (Plate V.2). The revealed the loss of architecture and fragmentation of bone tissue of group II rats. Enlarged view of section of bone from CFA-induced and untreated rats also showed presence of adjuvant filled globules, which is a common indication of cellular inflammation (Plate II) The marked changes in the architecture of bone sections could be explained by the loss of equilibrium between oxidant and antioxidant status and shifting of redox status towards oxidants in bone tissues of animals induced with inflammations (Rahman *et al.,* 2008).

Supplementation of CS oil and gingerol concentrate by and large restored the normal architecture of bone tissues of groups III and IV rats (Plate 4.1.5). The histological section also showed reductions in adjuvant filled globules and better integrity of cells. This suggested that usage of CS oil and gingerol concentrate in the present study was non toxic and effective against inflammations induced by CFA. This could be possibly explained by the capability of CS oil to induce activation of

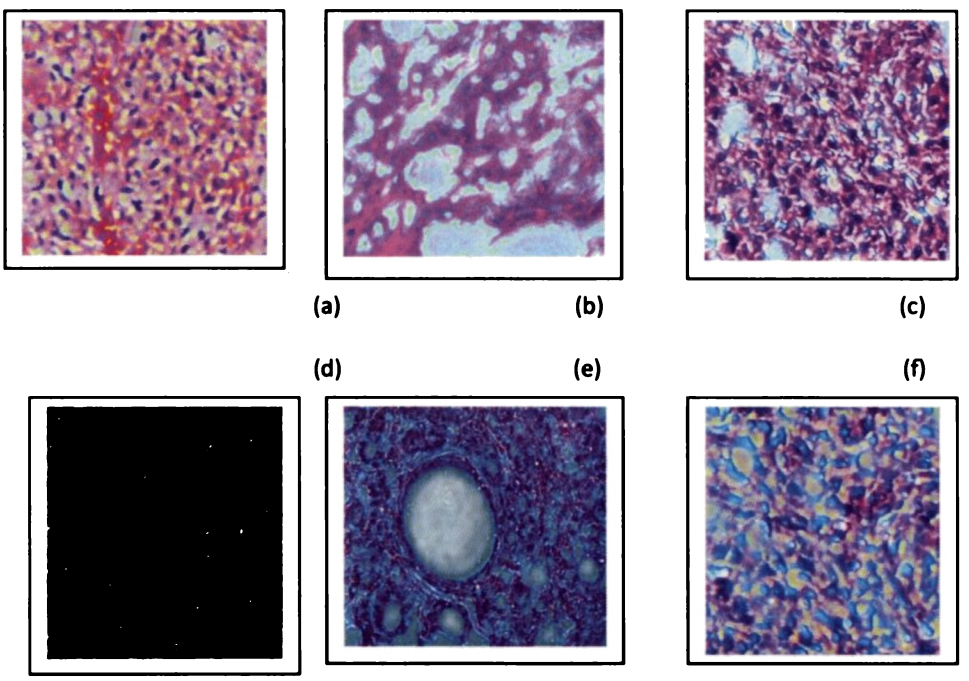
Plate V.1. Bone Radiographs



(a) (b) (c)

(a)normal bone architecture of rat paw(b) bone radiograph of rat paw with CFA-induced arthritis with eroded cartilage and swollen joints (c) bone structure of rat paw with induced arthritis and treated with CS oil/gingerol extract with lower inflammations and better healing of the eroded tissue

Plate V.2. Histopathology of bone tissues



(a) (b) (c)

(d) (e) (f)

(a,d) Normal architecture of rat bone tissue.
(b,e) CFA induced tissues with periarticular inflammation, edema and infiltration of polymorphonuclear neutrophils and lymphocytic cells, synovitis and synovial hyperplasia as well as a severe loss of cartilage and bone. Presence of more adjuvant filled globules observed.
(c,f) Administration of CS oil/gingerol extracts reduced leukocyte infiltration, inflammation, hyperplastic synovitis, erosion of articular cartilage and osteolysis. Stabilized lesions observed.

(Magnifications in both 20X (a,b,c) and 40X (d,e,f) using CKX-41 microscope.

antioxidant enzymes (Fernandes *et al.*, 1996; Hardman *et al.*, 2001) and enhance the level of GSH (Arab *et al.*, 2006) maintaining proper equilibrium between oxidants and antioxidant systems in tissue.

5.4.6 MPO levels

Tissue MPO levels increased with the progression of inflammation. Significant increase ($p<0.05$) in MPO levels were observed in CFA-induced and untreated group II animals and a corresponding decline in their levels were observed in both treated groups III and IV at the end of the experimental period. Similar results have been reported by Vázquez *et al.*, (2004) (Fig. 5.4). However, there were no siginificant differences in MPO levels in either of the control groups I and V or between the treated groups III and IV. Both CS oil and gingerol were able to maintain the cellular integrity of the organelles under the inflammatory conditions and hence no differences in their levels were observed. Though CFA-induced inflammation results in the infiltration of neutrophils in the paw tissues of Group II animals, administration of CS oil and gingerol reduced adhesion molecule expressions and inflammatory-related mediators at the site of injury, as observed in group III and IV animals. These results are in close agreements with previously reported works. Chiu et al (2009) reported that fish oil administration reduced MPO levels during hypercholesterolemia and sepsis, both of which are inflammatory disorders. Studies by Yuen Yin Lee confirmed (2009) the increase in plasma MPO activity in RA patients compared to healthy controls and is associated with abnormal HDL anti-inflammatory function and increased disease activity. Oxidative modification of HDL by MPO may provide another mechanism by which active RA increases cardiovascular morbidity and mortality.

5.4.7 COX activity in CFA induced arthritis

There was a significant increase in COX activity in groups II, III and IV animals induced with arthritis (Fig. 5.6). However there was no increase in COX levels in both the control groups I and V. The activities of both COX I and COX II were individually monitored in all the groups with the help of enzyme specific inhibitors SC-560 and DUP-697 which are selective COX I and COX II inhibitors (Laine *et al.*, 2008) respectively. For the CFA-induced and untreated group II animals there was

significant ($p<0.05$) inhibition of COX I activity (82%) but significantly less inhibition of COX II activity (17%) compared to the CFA-induced and treated groups III and IV animals (Table 5b). There was significant increase in COX I activity in both the treated groups III and IV and corresponding decrease in COX II levels in the same groups of animals. Similar studies on the protective effects of fish oils against inflammation have been reported (Maroon *et al.*, 2006; Thomas *et al.*, 2007). However the gingerol treated group IV animals showed maximum inhibition of COX II activity (79%). CS oil and gingerol (Lantz *et al.*, 2007) were able to selectively inhibit COX-2 are thus highly desirable since inflamed tissues could be targeted without disturbing the homeostatic functions of prostaglandins in noninflamed organs. Theoretically, then, selective COX-2 inhibition should preserve the anti-inflammatory efficacy without causing the associated toxicities of NSAIDs (Rao *et al.*, 2008). The control groups I and V showed no or negligible COX II activities thus confirming the fact that COX II is an inducible form of COX released only during conditions of acute inflammations.

Pharmacological inhibition of COX can provide relief from the symptoms of inflammation and pain. Non-steroidal anti-inflammatory drugs, such as aspirin and ibuprofen, exert their effects through inhibition of COX. However these NSAIDS inhibit the activities of both COXs (COX I and COX II). COX I is involved in the normal homeostasis mechanisms in the body and its inhibition can lead to the development of dyspepsia, erosions, gastric ulcers and renal dysfunction (Laine L, 2002).

5.4.8 Nitric oxide levels in CFA-induced arthritis

Nitric oxide is an important mediator in animal models of arthritis that mimic human RA. This simple gas NO has multiple functions in the body which include roles in host resistance to tumors and microbes, regulation of blood pressure and vascular tone, neurotransmission, learning, and neurotoxicity, carcinogenesis, and control of cellular growth and differentiation (Magrinat *et al.*,1992; Punjabi *et al.*,1992). In the presence of oxygen, NO rapidly (seconds) is converted to nitrite and nitrate, substances that are generally not bioactive. In the present study there was a significant increase in the nitric oxide levels (measured as the sum total of nitrites and nitrates) in the CFA-induced and untreated group II animals in the paw exudates

Figure 5.4 Effect of CFA-induced inflammations on MPO levels in Albino rats

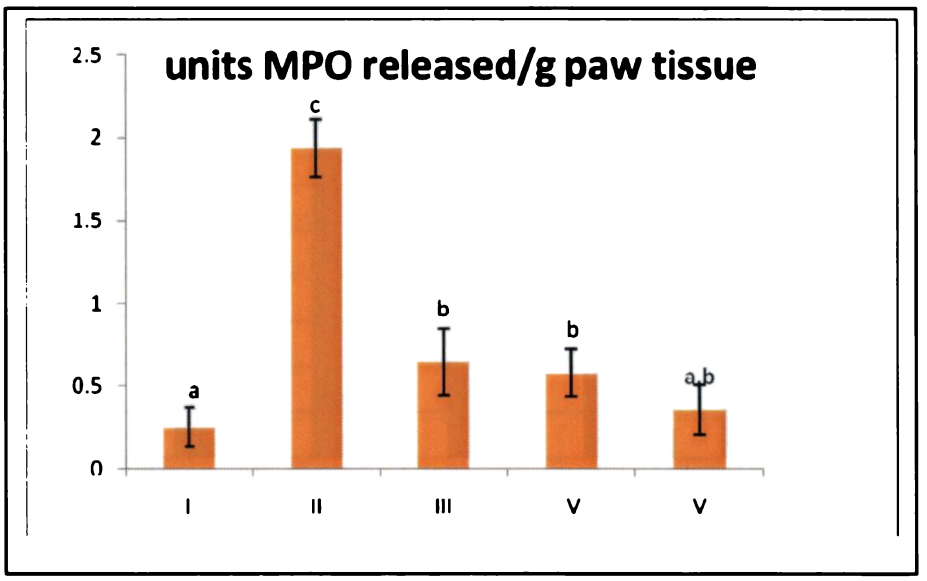
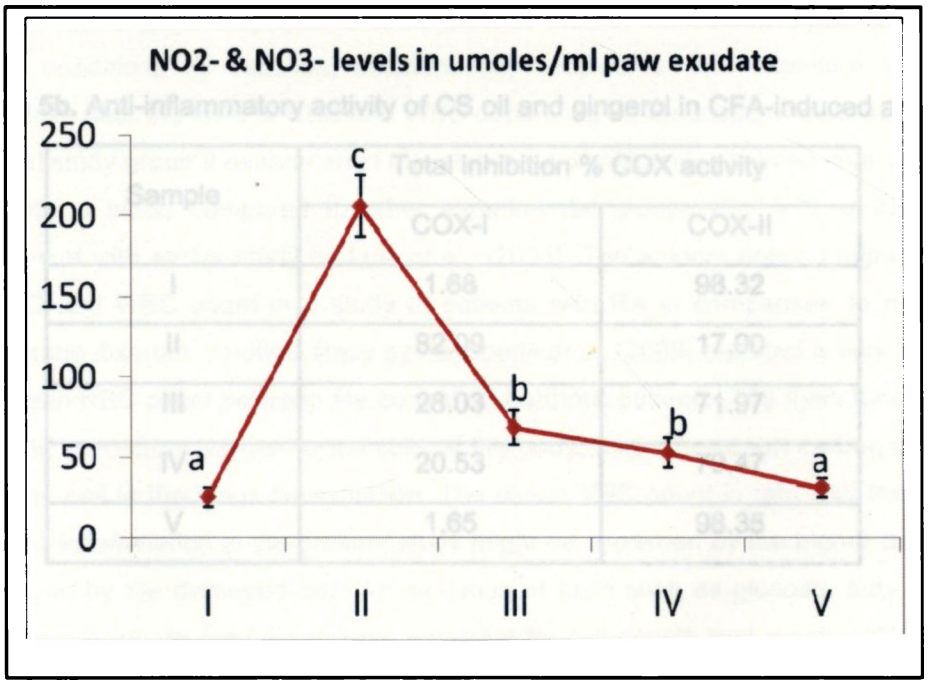
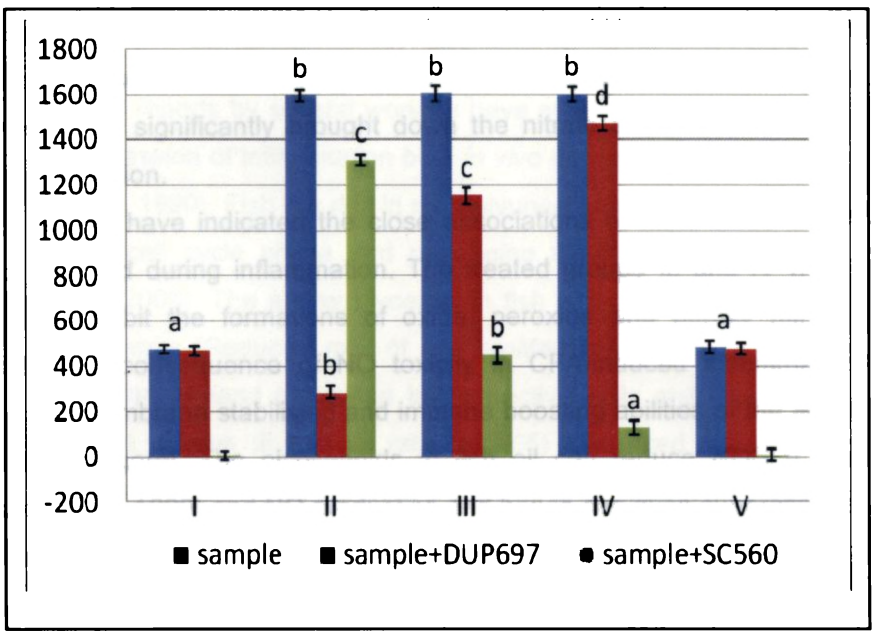


Figure 5.5 Effect of CFA-induced inflammations on nitrite/nitrate levels in Albino rats



Values are mean±SD for n=6, groups with different letters[a,b,c,d,e] are significantly different (p<0.05)

Figure 5.6 COX activity in CFA-induced arthritis in rat paw homogenates



Values are mean±SD for n=6, groups with different letters[a,b,c,d,e] are significantly different (p<0.05); DUP-697: selective COX II inhibitor, SC-560: selective COX I inhibitor

Table 5b. Anti-inflammatory activity of CS oil and gingerol in CFA-induced arthritis

Sample	Total inhibition % COX activity	
	COX-I	COX-II
I	1.68	98.32
II	82.09	17.90
III	28.03	71.97
IV	20.53	79.47
V	1.65	98.35

compared to the CFA-induced and treated groups III and IV (Fig. 5.5). There was no rise in the nitric oxide levels in the control groups I and V and were not significantly different. Likewise the CS oil and gingerol (Zhou *et al.*, 2006) treated groups III and IV, respectively, significantly brought down the nitrate and nitrite levels, released during inflammation.

Several studies have indicated the close associations of nitric oxide with the free radicals released during inflammation. The treated groups III and IV were able to significantly inhibit the formations of oxide, peroxide and peroxy nitrite radicals released as a consequence of NO toxicity in CFA-induced inflammations, thus showing the membrane stabilising and immune boosting abilities of the administered CS oil and gingerol. The eicosanoids in fish oil can reduce NOS II expression (Jeyarajah *et al.*, 1999) and NO production and hence can even suppress the activity of COX II.

5.4.9 Effect on RBC and WBC count

RBC together with other components of blood is used as a tool to evaluate status of disease where specific symptoms of the disease are not manifested. Deterioration of health conditions by infection, inflammation, neoplasms, and exposure to toxic substance alter the level of RBC and WBC depending on the stage of disease. In the present study group II experimental rats showed a significantly elevated level of RBC ($p<0.05$) in blood compared to other experimental groups (Fig 5.7) which is in agreement with earlier study by Luis *et al.*, (2005). The authors noticed higher level of RBC and WBC count in a study of patients with RA in comparison to patients without the disorder. Another study by Pasupathi *et al.* (2009) reported a very narrow change in RBC count between the control and arthritic patients. The main function of the RBC is to carry oxygen to the cells of the body, and to transport carbon dioxide from the cell to the lungs for excretion. The rise in RBC count in rats with the CFA-induced inflammation in the present study might be explained by the higher demand of oxygen by the damaged cells for oxidation of fuels such as glucose, fatty acids, amino acids etc. to produce energy, essential for cell growth and repair. WBCs are the main components involved in inflammatory immune responses. Elevated level of WBC count commonly noticed during illness is used as an index to measure the status of the disease (Daniel *et al.*, 2007). In the present study, higher level of WBC

count in group II rats ($p<0.05$) indicated the severity of inflammation compared with group I and V controls (Fig 5.7).

Previous reports by several workers have showed the inhibitory effect of fish oil on the progression of inflammation both *in vivo* and *in vitro* (Germain *et al.*, 1998; Deschner *et al.*, 1990). Fish oils rich in n-3 polyunsaturated fatty acids were reported to control the cell cycle phase and prolonging G2/M phase of cell proliferation (Aurelia *et al.*, 2006). The alkoxy glycerols in fish oils have a major role in the cell signalling pathways. Reduced rate of cell proliferation during inflammation might explain the lower demand of oxygen by respective cells and lower count of RBC observed in the groups III and IV rats ($p<0.05$) compared to group II rats (Fig 4.2.4.1). In contrast to the present study Hardman *et al.* (2001) had showed that fish oil fed mice produced higher RBC count than corn oil fed mice bearing breast cancer xenografts. The authors suggested that incorporation of fish oil concentrate could enhance the bone marrow cellularity (Atkinson *et al.*, 1997) that might result in higher RBC count. The major function of WBC is to defend the body against microbes, toxins and other foreign agents. Dietary supplementation of CS oil and gingerol boosted the defence system of rats with induced inflammations (groups III and IV) and simultaneously lowered the WBC and lymphocytes in arthritic animals. Anti-oxidant effect of CS oil components (n-3 PUFA, glycerol ethers, squalene, vitamin E) and of gingerol on arthritic progression might attenuate the inflammatory burden in the host by boosting their immunity status and thereby decrease the production of WBC in the system.

5.4.10 Effect of CFA-induced arthritis on blood and liver proteins

Significant increase ($p<0.05$) in plasma and liver proteins was observed for the CFA-induced and untreated group II animals compared to groups I, II, III and IV (Fig. 5.9). However there was no significant difference in liver proteins of groups III, IV and V animals. Many workers have reported an increase in serum proteins during an inflammation or infection (Saso *et al.*,2000; Sutton *et al.*,2009). The significant increase in plasma and liver proteins observed in group II rats may be due to the release of acute phase proteins in inflammatory disorders. Saso et al. (2000) reported a marked rise of two prominent acute phase proteins, α-2 macroglobulin and hemopexin in streptozotocin-induced diabetic rats. Glycoproteins (GP) are one

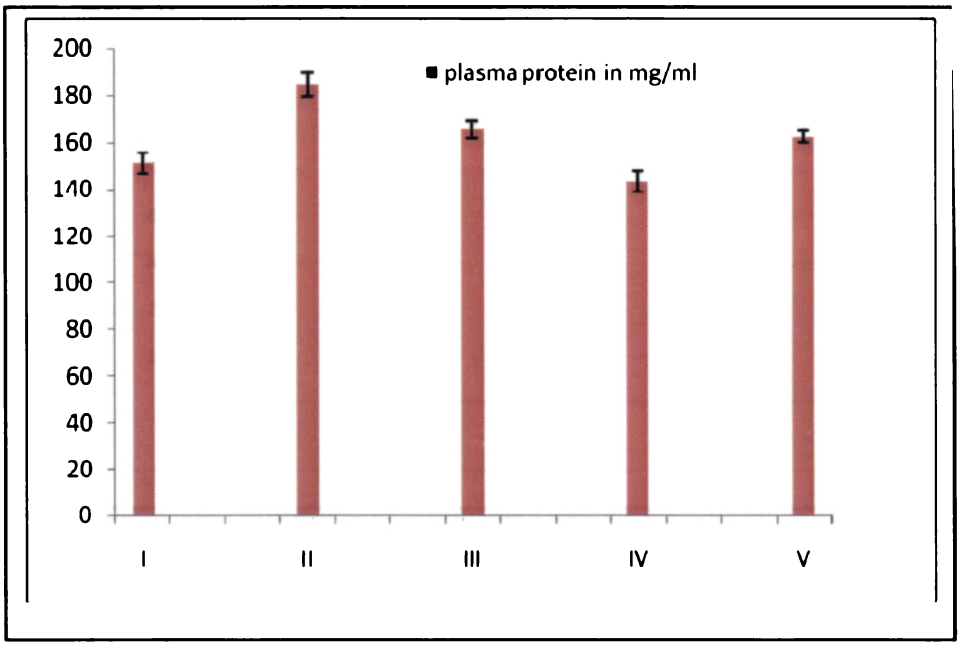
of the many acute phase proteins (APP) present in serum. The presence and levels of G-reactive protein, transferrin, ceruloplasmin, albumin, a1-antitrypsin etc. have been used to diagnose the extent of inflammation in patients with arthritis.

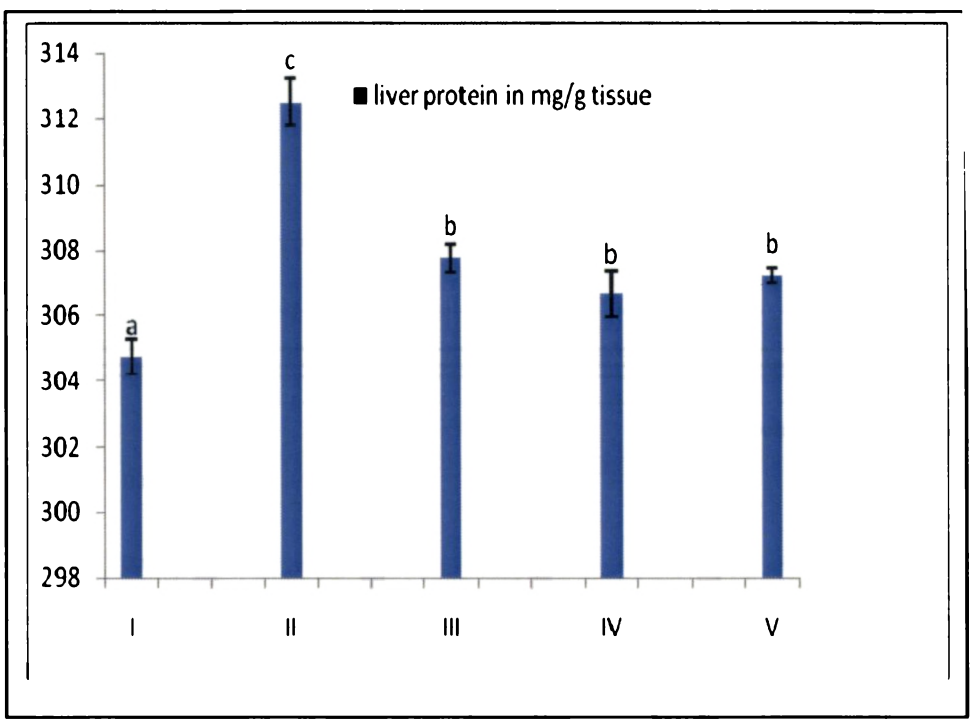
In arthritis, proteins (Sutton *et al.,* 2009), hormones, cytokines and growth factors are produced at faster rate. Cytokines, growth factors and hormones act as messengers of cell signalling pathways essential for repair of damaged cells. Higher rate of synthesis of various hormones, proteins, enzymes, growth factors, cytokines and immunoglobulins etc in liver and its release to circulatory system (Predeteanu *et al.*, 2009) could enhance the levels of proteins in tissue and plasma and this may be the reason for the higher protein levels observed in group II rats.

In the present study there was a significant ($p<0.05$) decrease in albumin and a significant increase in globulin proteins in blood plasma in the CFA-induced and untreated group II animals (Fig. 5.12). Treatment with CS oil significantly ($p<0.05$) raised the levels of albumin and decreased the levels of globulin in the blood of the CFA-induced and treated group III animals. Treatment with gingerol had also recorded similar effects however there was a significant decrease in the globulin levels in the CFA-induced and gingerol treated group IV animals. The albumin/globulin ratio was significantly ($p<0.05$) low in the untreated and CFA-induced group II animals. Similar reductions in albumin contents were observed in turpentine-induced inflammations in rats which was associated with a reduced concentration of albumin mRNA in the liver.

Albumin and globulin are the major blood proteins and they play a significant role in the immune response. Albumin, abundantly present in circulatory system, is synthesized in liver. Any damage to liver affects the plasma concentration of albumin and hence blood plasma albumin is clinically used as a marker for liver disease (Waitzberg *et al.,* 1989). The decreased level of albumin in plasma of group II rats ($p<0.05$) indicated that the hepatic tissue of group II rats was damaged by CFA (Fig 5.12). Electrophoretic pattern of liver proteins confirmed the lower level of albumin in group II rats in comparison to control rats (Plate-V.3). The densitometric analysis of liver protein showed decrease in the albumin band intensity and increase in positive acute phase proteins for group II rats compared to group I rats. This finding is in agreement with a previous study by Schett *et al.*, (2008) in acute inflammatory conditions. The authors noticed a reduced rate of synthesis of albumin in liver of

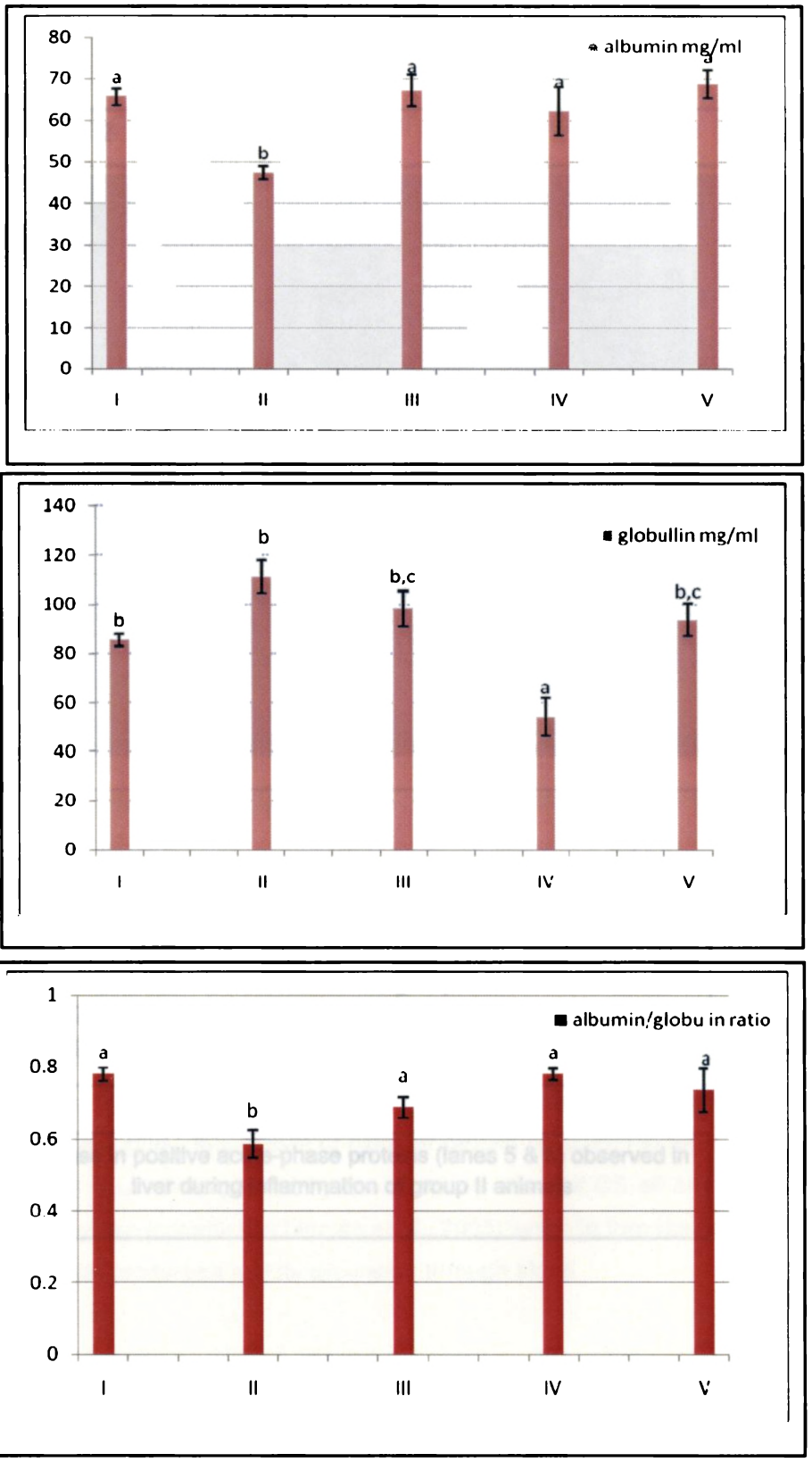
Figure 5.9 Effect of CFA induced arthritis on blood and liver proteins of Albino r





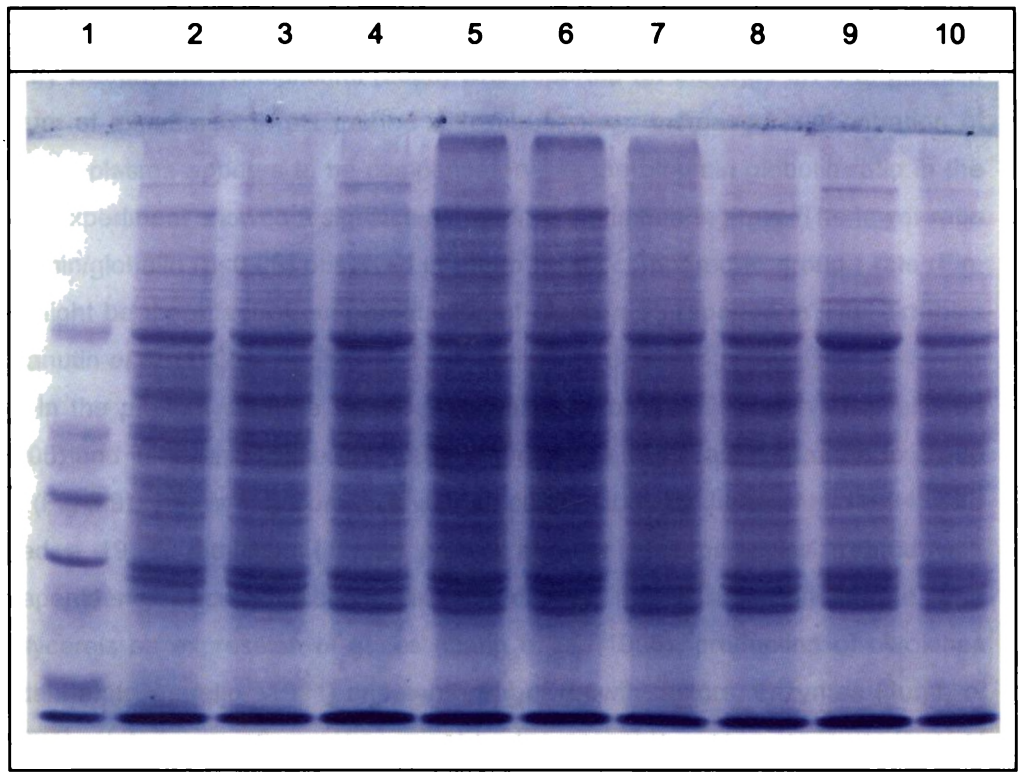
Values are mean±SD for n=6, groups with different letters[a,b,c,d,e] are significantly different (p<0.05)

Figure 5.12 Effect of CFA-induced arthritis on albumin and globulin contents in blood



Values are mean±SD for n=6, groups with different letters[a,b,c,d,e] are significantly different (p<0.05)

Plate V.3. Effect of CFA-induced arthritis on acute phase proteins in liver tissues of rats (SDS-PAGE photograph)



Lane 1 – low molecular weight protein marker
Lane 2 – control, group I
Lane 3,4 – control & CS oil treated, group 5
Lanes 5,6 – CFA induced arthritis, group 2
Lane 7,8 – CFA induced arthritis & CS oil treated, group 3
Lane 9,10 – CFA induced arthritis & gingerol treated, group 4

Increase in positive acute-phase proteins (lanes 5 & 6) observed in liver during inflammation of group II animals

animals with inflammatory disorders. Increased rate of catabolism of proteins and amino acids might be another factor inducing hypoalbuminemia in arthritis bearing animals (Watkins and Swannell, 1985).

The findings of the present study are in agreement with an earlier reported study by Deepa and Varalakshmi (2005) who reported an elevated level of globulin in serum of experimental rats treated with nitrosamine. Increased concentration of globulin in plasma appears to be compensatory, as the albumin globulin ratio in the present experiment showed a significant drop in arthritis bearing rats. The lower ratio of albumin/globulin ($p<0.05$) observed in group II rats compared to group I rats (Fig. 5.12) might be the effect of over production of globulin as reported in liver cirrhosis by Chanutin *et al.* (1938).

In the present study, a significant lower level of protein observed in plasma ($p< 0.05$) and in hepatic tissue ($p<0.001$) of group III and IV rats compared to group II rats (Fig 5.9). This is in accordance with an earlier study by Jump *et al.* (1994) who showed a 19 % reduction in total protein content of hepatocytes treated with eicosapentaenoic acid. Inhibitory role of dietary n-3 PUFA concentrate and alkylglycerols on expression of genes (Jiang *et al.*, 1996), production of cytokines (Meydani and Dinarello., 1991) and synthesis of growth factors, enzymes (Jump *et al.*, 1994) related with inflammation might be the probable reason for lower level of protein observed in plasma and liver of group III and IV rats compared to group II rats. Immunoglobulins are important globular proteins, generally increases in blood during inflammation (Mairesse *et al.*, 1988) and liver diseases (Weiss *et al.*, 2006). During inflammation and auto-immuno disease T-lymphocytes proliferate and produce cytokines that act directly on B-lymphocytes, promoting polyclonal activation and subsequent synthesis of immunoglobulins such as IgG, IgE, IgM, and IgA (Delrieu *et al.*, 1972). Unsaturated fatty acids derived from vegetable oils were reported to inhibit IgG and IgM production in spleen or lymphocytes (Lantz et *al.*, 2007. In the present study, lower level of globular protein ($p<0.001$) in group III and IV rats (Fig 5.12) could be explained by the inhibitory effect of CS oil and gingerol components on cytokine production (James *et al.*, 2003) which in turn may suppress the immunoglobulin production and its circulation through blood.

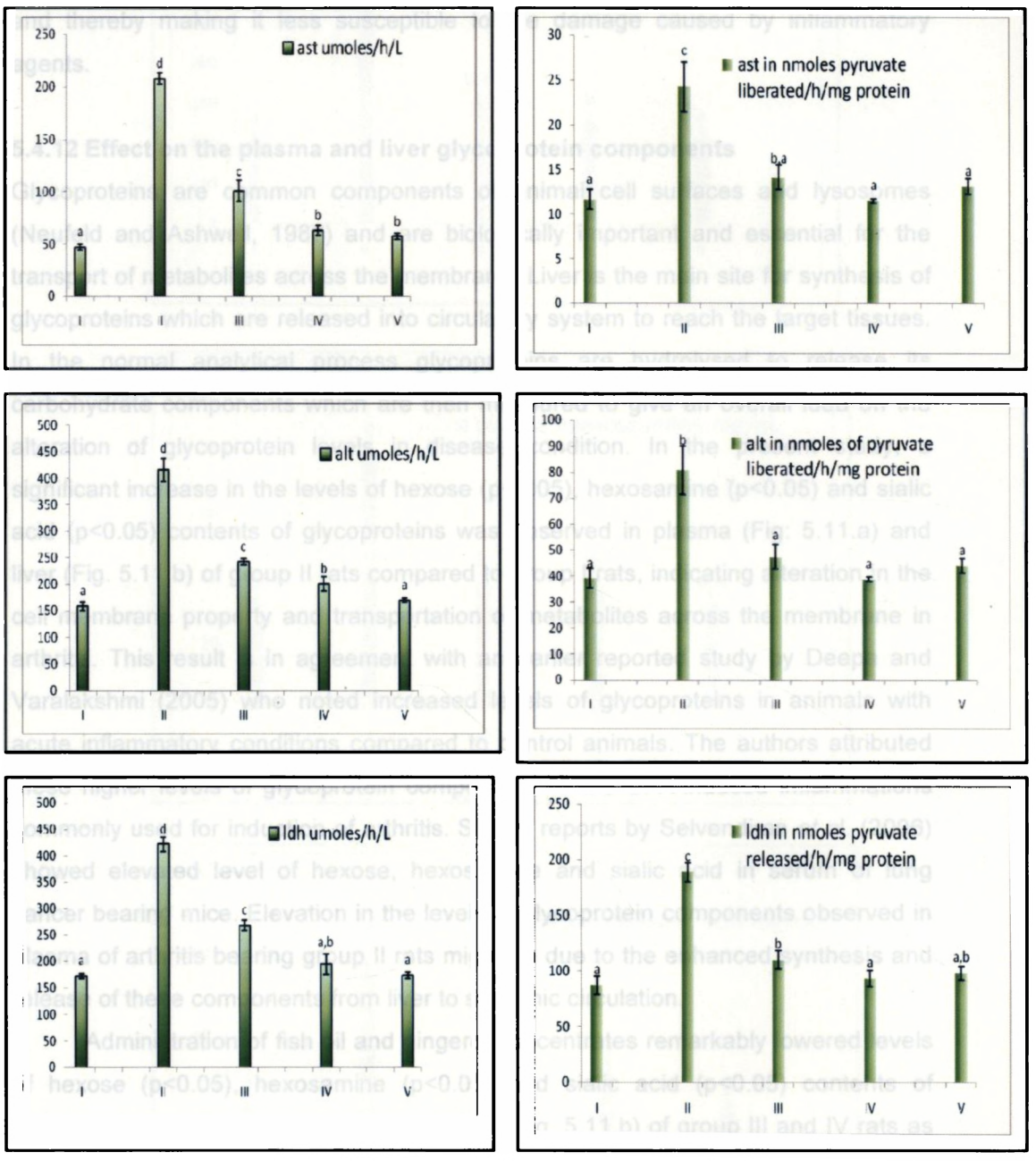
5.4.11 Effect on diagnostic marker enzymes

A significant ($p<0.05$) rise in the activities of alanine amino transaminase (ALT), aspartate amino transaminase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), and acid phosphatase (ACP) were noticed in plasma (Fig. 5.10) and liver tissues of group II rats as compared to group I rats. The present observations are in line with earlier reported studies by Mythilypriya *et al.* (2008) and Latha *et al.* (1998) in experiments of adjuvant-induced inflammations. Diagnostic marker enzymes such as AST, ALT and LDH in plasma are used as tools to assess hepatic damages caused by hepatotoxic agents. Clinical evidence for liver disease in rheumatoid arthritis is limited. Liver scintigraphy has been used to assess liver size when 22% out of 32 RA patients were diagnosed with hepatomegaly. CFA-induced inflammations might have caused damages to hepatic tissue resulting in an elevated level of activities of AST, ALT, LDH, ACP and ALP in plasma and hepatic tissues of group III mice. Similar kind of observations has been reported by Narendhirakannan *et al.* (2007) in serum of experimental rats treated with adjuvants. Parikh *et al.* (2005) observed that the activity of diagnostic marker enzymes was raised significantly in serum samples from patients with acute sepsis.

Being highly susceptible to oxidation, long chain n3 PUFAs in CS oils might enhance the lipid mediated damage to the tissues and cells and thereby increase in levels of diagnostic marker enzymes was expected in plasma and liver of group III rats. But contrary to this expectation, a significant decrease in level of ALT, AST, LDH, ALP, and ACP in plasma (Fig: 5.10) and liver tissue was observed in CS oil fed rats in the present study. The presence of antioxidant vitamins, hydrocarbons and alkoxy glycerols in fish oil would have quenched the free radicals and hydroperoxides formed during inflammatory conditions. Moreover the presence of flavonoids, triterpenes and antioxidants in gingerol would have boosted the antioxidant defense status in the blood and liver tissues of group IV rats and hence a reduction in the level of marker enzymes in their system (compared to the arthritic untreated group II rats) was observed. Several authors have reported decreased levels of serum transaminases in experimental rats fed with docosahexaenoic acid and then treated with carbon tetrachloride to induce liver injury. Clinical diagnostic reports have also shown reduction in the activities of serum alkaline phosphatase and gamma-glutamyl transaminase in cancer patients administered a diet supplemented with unsaturated fatty acids (Booyens *et al.*, 1984). The exact mechanism of action by which fish oil

Figure 5.10 Effect of CFA-induced arthritis on diagnostic marker enzymes (DME) of blood and liver of Albino rats

DME in blood plasma **DME in liver**



Values are mean±SD for n=6, groups with different letters[a,b,c,d,e] are significantly different (p<0.05)

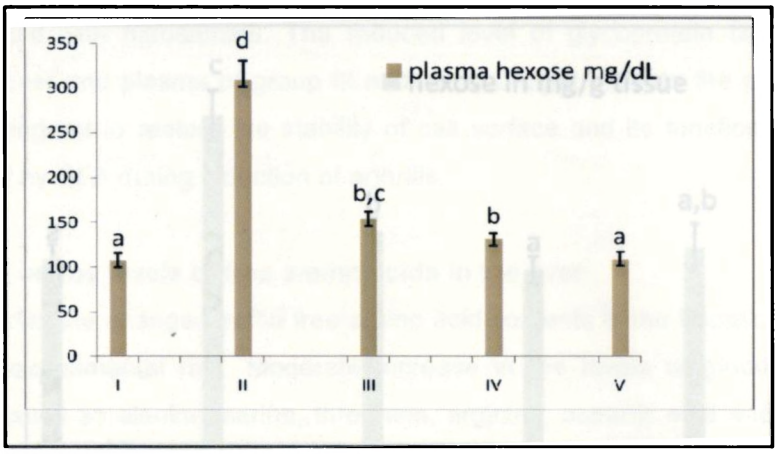
can regulate the level of plasma and tissue diagnostic marker enzymes in arthritic condition is not known. It is believed that the dietary n-3 PUFA and squalene may stabilize the cell membranes by modulating the lipid environment (Kim *et al.*, 2000) and thereby making it less susceptible to the damage caused by inflammatory agents.

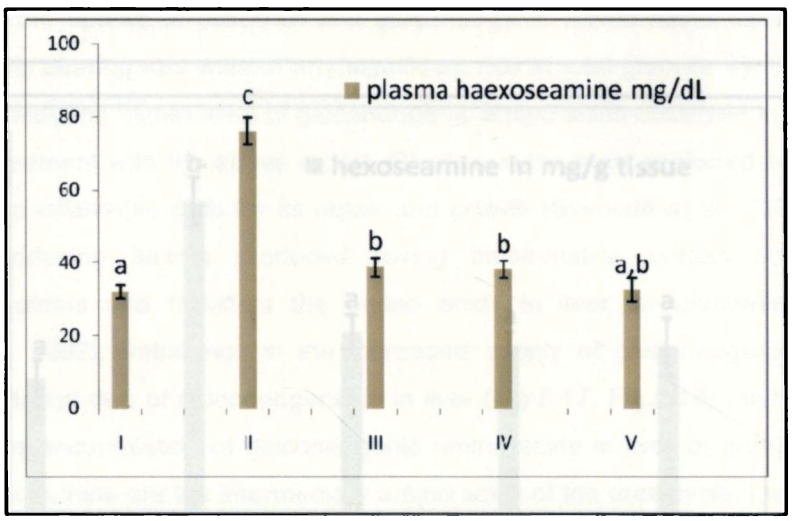
5.4.12 Effect on the plasma and liver glycoprotein components

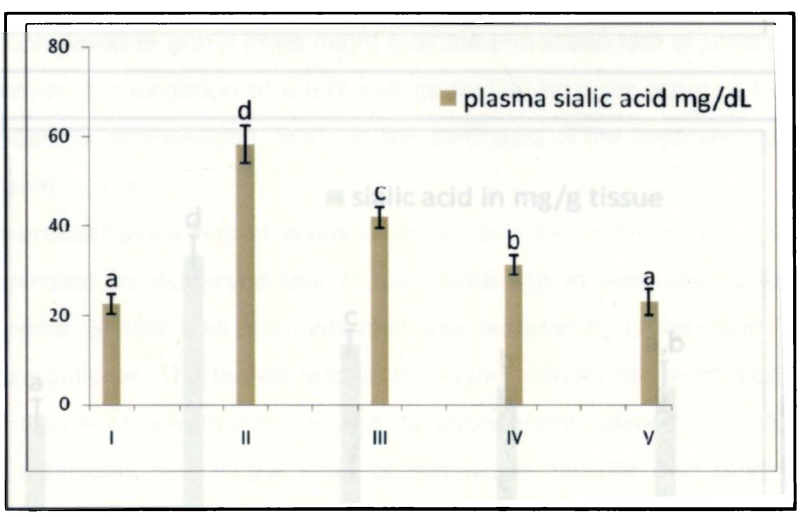
Glycoproteins are common components of animal cell surfaces and lysosomes (Neufeld and Ashwell, 1987) and are biologically important and essential for the transport of metabolites across the membrane. Liver is the main site for synthesis of glycoproteins which are released into circulatory system to reach the target tissues. In the normal analytical process glycoproteins are hydrolysed to release its carbohydrate components which are then measured to give an overall idea on the alteration of glycoprotein levels in disease condition. In the present study, a significant increase in the levels of hexose ($p<0.05$), hexosamine ($p<0.05$) and sialic acid ($p<0.05$) contents of glycoproteins was observed in plasma (Fig: 5.11.a) and liver (Fig. 5.11.b) of group II rats compared to group I rats, indicating alteration in the cell membrane property and transportation of metabolites across the membrane in arthritis. This result is in agreement with an earlier reported study by Deepa and Varalakshmi (2005) who noted increased levels of glycoproteins in animals with acute inflammatory conditions compared to control animals. The authors attributed these higher levels of glycoprotein components to the CFA-induced inflammations commonly used for induction of arthritis. Similar reports by Selvendiran *et al.* (2006) showed elevated level of hexose, hexosamine and sialic acid in serum of lung cancer bearing mice. Elevation in the levels of glycoprotein components observed in plasma of arthritis bearing group II rats might be due to the enhanced synthesis and release of these components from liver to systemic circulation.

Administration of fish oil and gingerol concentrates remarkably lowered levels of hexose ($p<0.05$), hexosamine ($p<0.05$) and sialic acid ($p<0.05$) contents of glycoprotein in plasma (Fig. 5.11.a) and liver (Fig. 5.11.b) of group III and IV rats as compared to group II rats. The mechanism of action of CS oil and gingerol on alteration in glycoprotein levels during inflammation is not clear. Altered levels of glycoprotein conjugates are well documented in inflammatory disorders (Shetlar *et*

Figure 5.11.a Effect of CFA-induced arthritis on glycoprotein components of blood

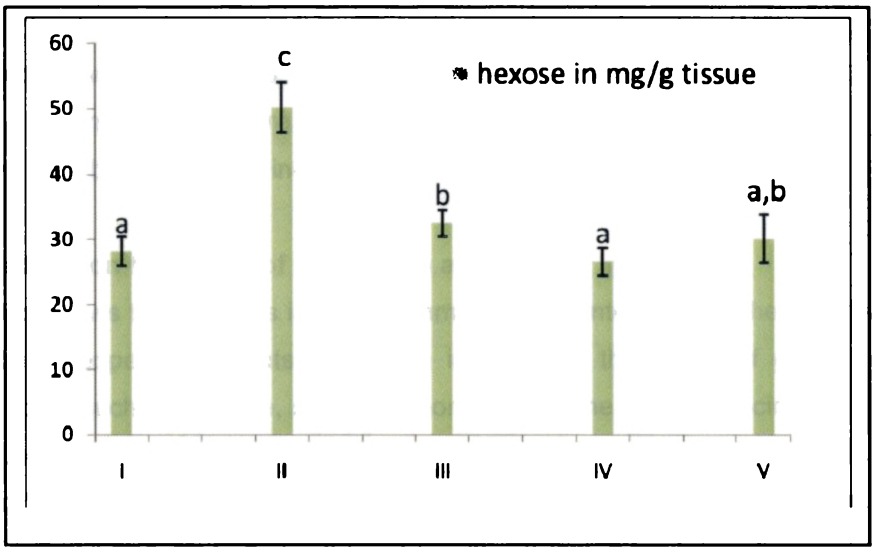


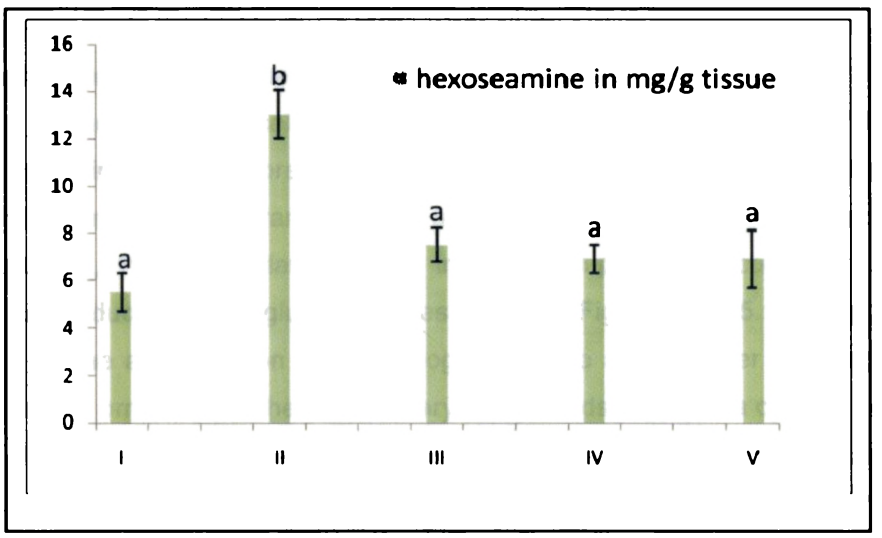


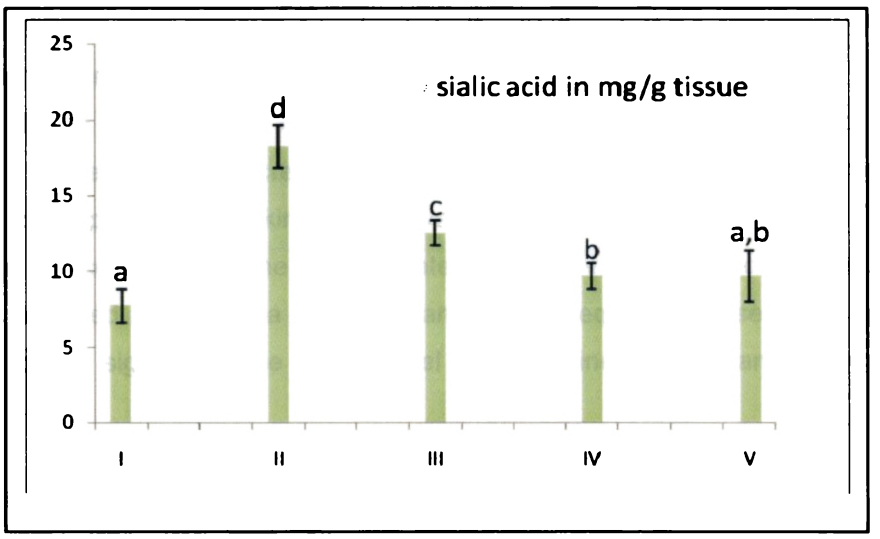


Values are mean±SD for n=6, groups with different letters[a,b,c,d,e] are significantly different (p<0.05)

Figure 5.11.b Effect of CFA-induced arthritis on glycoprotein components in liver







Values are mean±SD for n=6, groups with different letters[a,b,c,d,e] are significantly different ($p<0.05$)

al., 1950). Rachesky *et al.*, (1983) reported an increased level of glycoprotein in animals treated with nitrosamine. The reduced level of glycoprotein components observed in liver and plasma of group III and IV rats might indicate the potential of CS oil and gingerol to restore the stability of cell surface and its functioning which was impaired by CFA during induction of arthritis.

5.4.13 Effect on the levels of free amino acids in the liver

Table 5c. shows the changes in the free amino acid contents in the hepatic tissue of normal and experimental rats. Moderate increase in the levels of gluconeogenic amino acids such as alanine, serine, threonine, arginine, aspartic acid and tyrosine was observed in group II rats in comparison to group I rats. Sorkin *et al.* (1992) showed that the uptake of essential and gluconeogenic amino acids increased in liver of arthritis bearing rats without any significant rise in total glucose synthesis. In the present study the higher level of gluconeogenic amino acids observed in group II rats is in agreement with the above report. Glucose is the most preferred source of energy for the inflammed cells for its repair and growth (Svenson *et al.*, 1987). The proteolysis inducing factors produced during inflammation breaks down the peripheral proteins and transfers the amino acids to liver for gluconeogenesis (Christensen, 1982). Imbalance in the increased supply of gluconeogenic amino acids and reduced rate of gluconeogenesis in liver (Fig.5.17, Fig.5.18) might be the reason for the accumulation of gluconeogenic amino acids in liver of group II rats. Arginine and ornithine are the intermediary amino acids of the urea cycle. Liver is the main site for ureagenesis. The increase in arginine observed in hepatic tissues of group II rats compared to group I rats might indicate enhanced rate of urea synthesis in liver. Moreover, the oxidation of a terminal guanidino nitrogen atom of L-arginine, which is induced by inflammation, leads to the synthesis of the cytotoxic nitric oxide products in group II rats.

In the present study higher levels of urea observed in blood of group II rats (Fig. 5.13) revealed an increased rate of urea synthesis in liver and its release to blood for disposal. Similar kind of observation was reported by Lattermann (2003) in bladder cancer patients. The author related the higher concentration of plasma urea to an elevated rate of urea synthesis and its subsequent release into plasma for excretion. A significant rise in the level of isoleucine, leucine and phenylalanine

Table 5c. Effect of CFA-induced arthritis on free amino acids in liver

% increase in free amino acids in liver w.r.t. normal control rats			
Amino acid	Group II	Group III	Group IV
Asp	37.12	12.04	17.88
Thr	34.49	11.59	14.97
Ser	36.14	33.43	31.40
Glu	51.86	31.83	27.61
Pro	21.02	6.84	4.35
Gly	70.32	57.21	55.73
Ala	76.07	50.63	57.03
Cys	26.42	12.81	11.94
Val	20.78	10.65	15.40
Met	18.51	9.39	5.92
Ile	16.34	8.21	11.97
Leu	39.37	21.83	31.47
Tyr	9.87	13.78	4.11
Phe	23.79	13.17	18.64
His	5.77	3.06	1.69
Lys	26.58	18.45	14.29
Arg	33.04	15.74	14.31

indicated their possible role in hormonal secretion, action and intracellular signalling (Nair and Short, 2005) during the progression of inflammation. The increase in sulphur-containing amino acids Cysteine and Methionine in group II animals might be interpreted as a sign of an enhanced glutathione (GSH) catabolism during arthritis (Hernanz *et al*, 1999).

Oral supplementations of CS oil and gingerol concentrate restored the free amino acids, in hepatic tissues of groups III and IV rats. Progression of an inflammatory disorder is associated with elevated rate of catabolism of protein in skeletal muscle (Watkins and Swannell, 1972). Amino acids released due to degradation of proteins are transported to the inflamed tissue either for energy or biosynthesis. Polyunsaturated fatty acids were reported to suppress the synthesis of prostaglandin PGE_2 and activity of arthritis derived proteolysis inducing factor associated with degradation of muscle protein and reduce the supply amino acids to arthritic cells for its growth and division (Jean *et al.*, 2008; Lawand *et al.*, 2000). Free amino acids play an important role as precursors in different metabolisms like urea, polyamine, hormone and protein metabolisms, associated with arthritis. As it is not clear how fish oils/gingerol extracts control these metabolic processes, it would be difficult to interpret the effect of fish oil components on the level of these free amino acids in group III and IV rats. But the observations of present study have shown that dietary supplementation of CS oil and gingerol decreased the rate of synthesis of protein, urea, production of ATP via glycolysis and oxidative stress in arthritic condition. Inhibition of these processes might cumulatively reduce the demand for their precursor amino acids in hepatic tissue of group III and IV rats compared to group II rats.

5.4.14 CFA-induced arthritis and metabolism of carbohydrates

Since, a definite correlation exists between progression of inflammation and the activities of glycolytic and gluconeogenic enzymes (Rzymowska and Dyrda, 1993), these enzymes have been used as markers of inflammatory arthritis. In the present study, hexokinase and aldolase activities were monitored to assess the rate of glycolysis in arthritic cells and fructose-1,6-diphosphatase and glucose 6 phosphatase activities were assayed, as a measure for gluconeogenesis. Blood glucose and tissue glycogen contents were assayed to know the net effect of

glycolysis on liver glycogen storage and glucose transport by systemic circulation. Hepatic ATP content was estimated to find out the effect of glycolysis on ATP availability in inflamed tissues.

5.4.15 Effect on blood glucose

Blood sugar is checked as a part of routine clinical test in arthritic patients to assess the alteration in glucose metabolism during acute inflammations. Inflammation places rigorous metabolic demands on the host and exerts a stress on the host to maintain normal blood glucose level (Argiles and Azcon-Bieto, 1988). In the present study a sharp drop in blood glucose level was observed in group II rats ($p<0.05$) in comparison to group I controls (Fig. 5.14). This finding is in agreement with a similar study by Foster *et al.* (2001) who noticed a decrease in blood glucose concentration in Ehrlich Ascite mice with induced tumour. Glucose-6-phosphatase located at the terminal steps in the gluconeogenic and glycogenolytic pathways plays a key role in the maintenance of blood glucose homeostasis (Kelmer-Bracht *et al,* 2003). Inhibited activities of glucose 6 phosphatase observed in the present study (Fig. 4.2.12.4) might be the reason for lower blood glucose level in CFA-induced and untreated group II rats.

Animals treated with fish oil and gingerol (groups III and IV respectively) showed a significant increase in ($p<0.05$) blood glucose level compared to group II rats (Fig. 5.14) however the blood glucose levels of groups III and IV were comparable to the control groups I and V. Study on the maintenance of plasma glucose homeostasis by dietary unsaturated fatty acids in arthritic condition is scarce. Many studies have been reported on the beneficial effects of fish oils on insulin resistance (Berry, 1997) and glucose homeostasis in diabetic condition (Malasanos and Stacopoole, 1991; Suresh and Das, 2003). The normal levels of blood glucose observed in CFA-induced and treated groups III and IV compared to group II rats indicates the ability of CS oil and gingerol to restore the homeostasis of glucose in blood which was found disturbed in group II rats .

5.4.16 Effect on glycolysis

The activities of glycolytic enzymes were significantly high in inflammatory conditions as evident from the observed low levels of glucose in the blood. Hexokinase, the first

enzyme of the glycolytic pathway, plays a pivotal role in glucose metabolism in transformed cells (Naughton, 2003). Figure 5.15 showed that the activity of hexokinase enzyme in group II rats was higher ($p<0.001$) in comparison with group I control rats. This finding is in agreement with several past studies which reported enhanced activity of hexokinase in various inflammatory disorders (Shull *et al.*, 1956, Sevenson *et al.*, 1987). Studies on human breast cancers showed a 13-fold increase in hexokinase activity in comparison to normal mammary tissue (Hennipman *et al.*, 1987).

Aldolase acts at the middle position of glycolytic pathway and catalyses the conversion of fructose 1,6 diphosphate to glyceraldehydes 3 phosphate and dihydroxy acetone. The maintenance of aldolase activity indicates an uninterrupted production of energy from catabolism of glucose via glycolysis. In the present study a marked increase in the level of aldolase activities ($p<0.05$) was observed in group II rats in comparison with group I controls (Fig 5.16). Similar finding has been reported by Meyer and Kunin (1969) who observed higher activities of aldolase in inflammatory condition. Naughton, (2003) reported an enhanced activity of aldolase enzyme in liver of rats bearing arthritis.

Glucose is the most preferred source of fuel to inflammatory cells and about 60% of energy in terms of ATP is derived by these cells via glycolysis (Nakashima *et al.*, 1986). This might be the reason for higher level of activities of hexokinase and aldolase enzymes observed in the present study (Fig. 5.15, Fig. 5.16). Glucose 6 phosphate, which is rapidly produced by action of hexokinase in the first step of glycolytic pathway, not only serve as source for ATP but also is utilised as a precursor for biosynthesis of lipid and nucleic acid which help in faster cell repair, maintenance and growth. The increased level of both hexokinase and aldolase activity noticed in the present study indicated increased rate of glycolysis in acute cases of inflammation. Enhanced activities of glycolytic enzymes (Fig. 5.15; Fig. 5.16) and reduced supply of glucose through circulatory system might lead to depletion of glucose level in inflammatory cells. Glucose deprivation in arthritic tissues was reported to cause metabolic oxidative stress imposed by super oxides and hydrogen peroxides (Sevenson *et al.*, 1987). In the present study higher levels of oxidative stress noticed in group II experimental rats (Fig 5.29) could be partly related to the altered glucose metabolism in CFA-induced arthritis.

Figure 5.14 Effect of CFA-induced arthritis on blood glucose

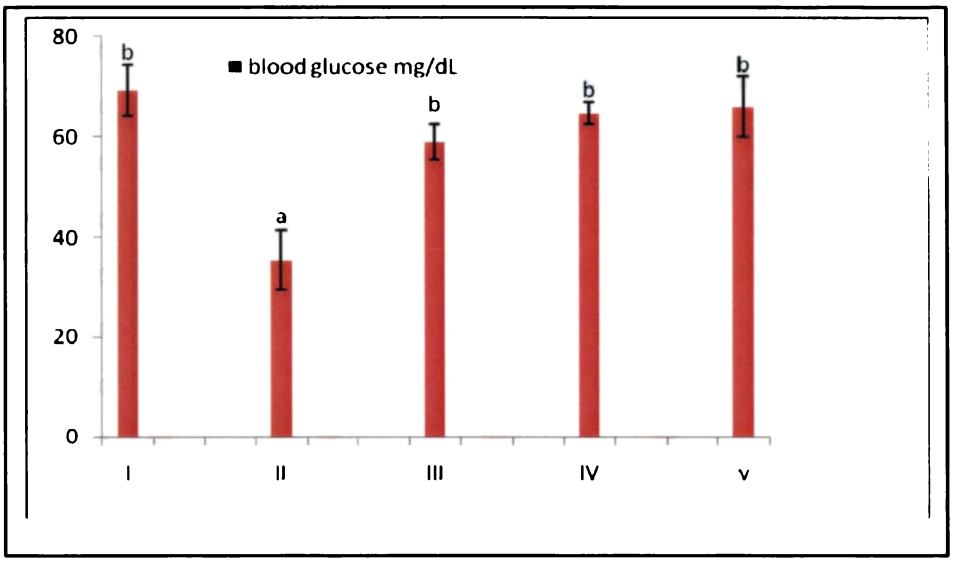
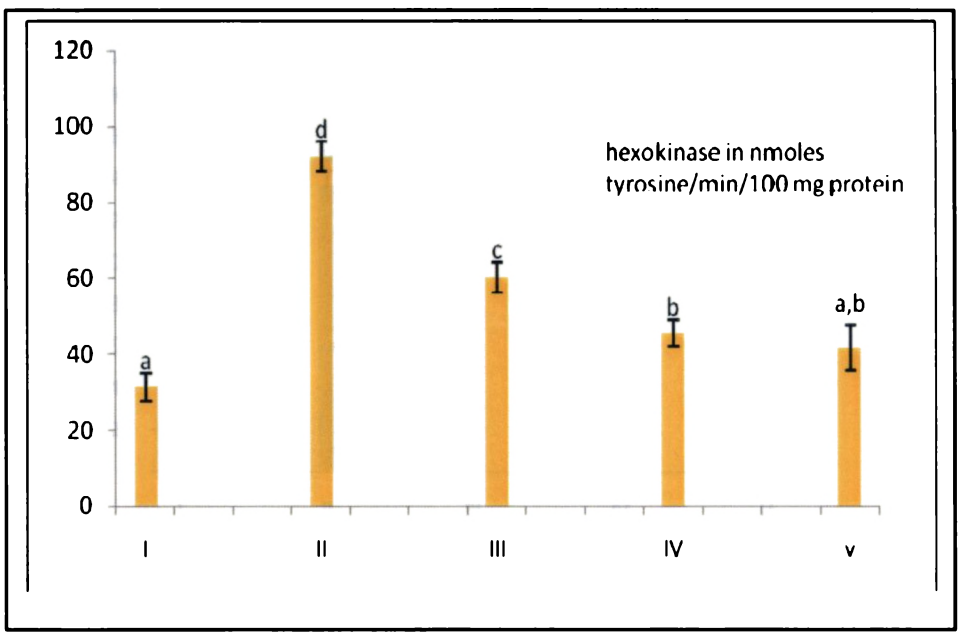


Figure 5.15 Effect of CFA-induced arthritis on the activity of hexokinase in liver



Values are mean±SD for n=6, groups with different letters[a,b,c,d,e] are significantly different (p<0.05)

Figure 5.16 Effect of CFA-induced arthritis on activity of aldolase in liver

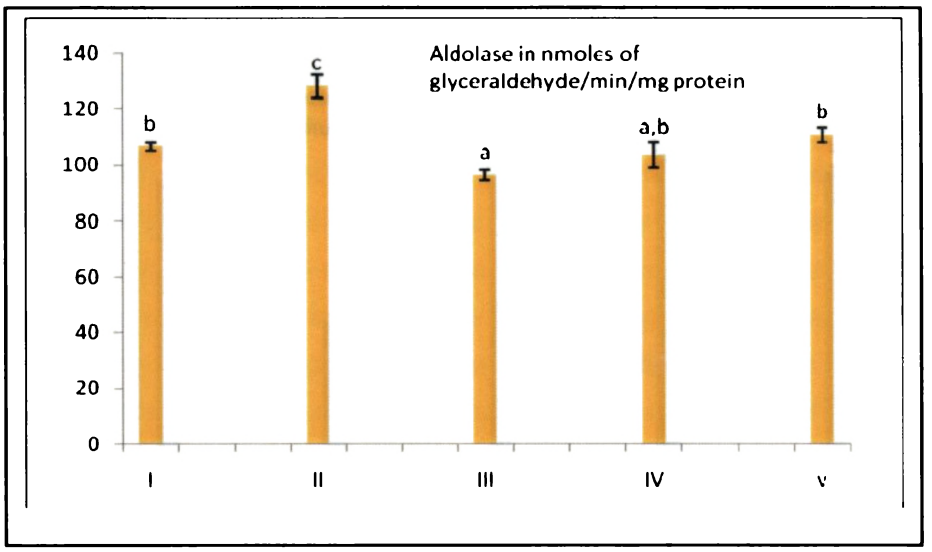
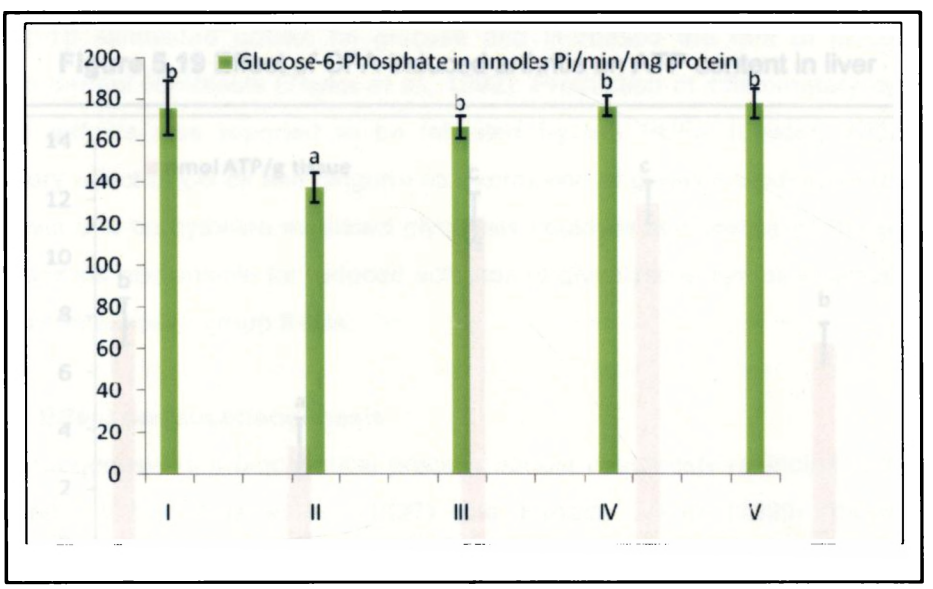


Figure 5.17 Effect of CFA-induced arthritis on activity of glucose-6-phosphate in liver



Values are mean±SD for n=6, groups with different letters[a,b,c,d,e] are significantly different (p<0.05)

Figure 5.18 Effect of CFA-induced arthritis on the activity of fructose-1,6-diphosphatase in liver

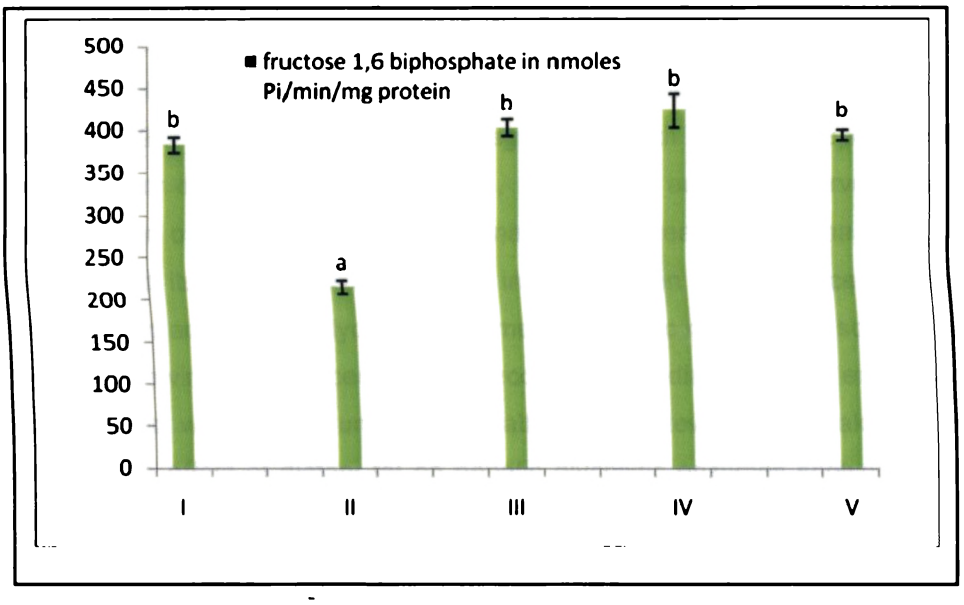
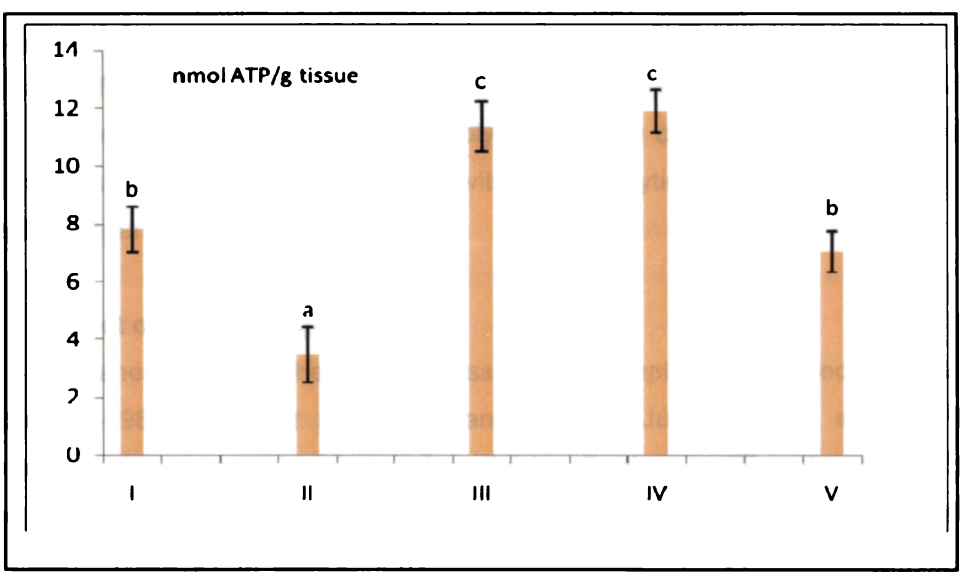


Figure 5.19 Effect of CFA-induced arthritis on ATP content in liver



Values are mean±SD for n=6, groups with different letters[a,b,c,d,e] are significantly different (p<0.05)

Inflammatory cells exhibit higher catabolism of glucose for energy (Pedersen, 1978). A significant drop in the activity of hexokinase and aldolase enzymes ($p<0.05$) observed in group III and IV rats compared to group II rats, indicated the ability of CS oil and gingerol concentrate to limit the supply of ATP to inflammatory cells through glycolysis (5.19). The present finding is in agreement with an earlier reported study on inflammatory diseases (Miyasaka *et al.*, 1996). The authors observed that the rate of inhibition of glycolysis was proportional to the increase in the chain length of fatty acids and its concentration. Though information on the influence of fish oil components on activities of glycolytic enzymes in arthritic condition is scanty, there are reports showing the reduced rate of glycolysis in cardiac tissue of experimental animals treated with dietary unsaturated fatty acids (Stewart and Blakely, 2000). Studies on the effect of dietary fat on hepatic glycolytic enzymes showed reduced activity of glucokinase in rats fed with unsaturated fatty acids (Clarke *et al.*, 1977). Polyunsaturated fatty acids can inhibit the genes responsible for expression of glycolytic enzymes (Jump *et al.*, 1994). Proinflammatory cytokines such as TNF-α and IL-1β stimulated uptake on glucose and increased the rate of glycolysis in human dermal fibroblasts (Taylor *et al.*, 1992). Production of inflammatory cytokines during arthritis was reported to be inhibited by n-3 PUFA (Calder, 2002). The inhibitory effect of CS oil and gingerol on expression of genes encoding for glycolytic enzymes and on cytokine mediated glycolysis could be considered as the probable mechanisms responsible for reduced activities of glycolytic enzymes in group III and IV rats compared to group II rats.

5.4.17 Effect on gluconeogenesis

Gluconeogenesis is a biochemical process almost completely restricted to the liver (Quistorff, 1985). Katz *et al.* (1997) and Fedatto Júnior (1999) showed that gluconeogenic enzymes have a preferential localization in different zones of the hepatic lobules and hence the severity of diseases affecting liver can be estimated by assaying activity of certain enzymes of this pathway. In the present study activities of glucose-6-phosphatase and fructose 1, 6 diphosphatase were measured to find out the extent of gluconeogenesis in liver tissue of CFA-induced experimental animals. The activities of these two enzymes showed lower values for group II rats than that of group I (Fig. 5.17; Fig. 5.18). The present finding is in line with an earlier

reported study by Kelmer-Bracht *et al.* (2003); Kelmer-Bracht *et al.*, (2006). The authors found lower level of glucose-6-phosphatase and fructose 1,6 diphosphatase in hepatic tissues of rats with arthritis.

Higher activities of ALT, AST and LDH noticed in group II rats than in group III and IV rats (Fig 5.10) had been discussed earlier in the present study. In addition to their role as diagnostic markers, these enzymes play role in gluconeogenic pathway which produces glucose from non-carbohydrate sources. Lactate dehydrogenase converts lactate to pyruvate where as AST and ALT convert glutamate and alanine to their keto acids respectively. Elevated activities of enzymes like LDH, AST, ALT which provide precursors for generation of pyruvate at the beginning of gluconeogenesis and lower activities of glucose-6-phosphatase and fructose 1, 6 diphosphatase in later part of gluconeogenesis, indicate the difficulty of inflamed cells to convert pyruvate to glucose due to lack of energy. In other words, the pyruvate synthesized from lactate and amino acids might be metabolized to acetyl coA or oxaloacetate and enters to Krebs cycle for biosynthesis of fatty acids, nucleic acid, proteins etc. Enhanced level of fatty acid synthase and acetyl coA carboxylase in various inflammatory disorders have been reviewed by Dabhi *et al.*, (2008). Significant increase in the activities of glucose-6-phosphatase ($p<0.05$) and fructose 1,6 diphosphatase enzymes ($p<0.05$) in groups III and IV rats compared to group II rats showed that CS oil and gingerol concentrate might stimulate gluoconeogenesis in CFA-induced arthritis. (Fig. 5.17, Fig. 5.18). Though studies on effect of CS oil and gingerol on gluconeogenesis in arthritic conditions are limited, one study showed that use of fish oil as dietary source of lipid induced gluconeogenesis in mammalian liver tissues (Yilmaz *et al.*, 2004).

5.4.18 Effect on liver glycogen content

The glycogen content in hepatic tissues of control and experimental animals is shown in Fig 5.20. Significant decrease in the level of glycogen was noticed in CFA-induced group II rats ($p <0.05$) in comparison to control groups I and V. The present finding is in agreement with earlier study by Shutle *et al.* (1977) who noted a lower level of glycogen in liver and muscle tissue of rats induced with arthritis. The authors claimed that intraperitoneal inoculation of the tumor was more aggressive than that of subcutaneous inoculation. Results of the present study showed an elevated

activity of hexokinase (Fig. 5.15) and reduced activity of glucose-6 phosphatase (Fig 5.17) in arthritic animals indicating faster utilization of glucose from the hepatic glycogen storage than its conversion to glycogen. This might be the reason for depleted level of glycogen in liver tissue of group II experimental rats.

Supplementation of fish oil and gingerol concentrate significantly ($p<0.05$) improved the levels of glycogen in group III and IV rats ($p<0.05$) in comparison to group II rats. Rosell *et al.* (2009) reported that supplementation of fish oil increased the level of glycogen in arthritic rats compared to control rats fed with coconut oil. This finding is in agreement with the present result. Fedatto-Júnior *et al.* (2004) reported that glycogen synthesis increased when the rate of glycolysis was inhibited to a greater extent than the rate of glucose uptake and phosphorylation. The results of present study only showed reduced rate of glycolysis in arthritic animals treated with CS oil and gingerol and lack of data in respect of the glucose uptake in the present study makes it inconclusive to comment on the glycogen synthesis. However, there are reports showing that pretreatment with fish oil rich in n-3 fatty acids significantly improves muscular uptake of glucose without altering hepatic glucose production in rats (Sierra *et al.,* 1995). Effect of dietary n-3 PUFA on improved glucose uptake and inhibition of glycolysis might be one of the possible mechanisms to explain the higher level of glycogen observed in group III rats in the present study.

5.4.19 Effect on liver ATP content

Adenosine 5'-Triphosphate (ATP) is a multifunctional nucleotide and serves as one of the important sources of energy for various metabolisms. Inflammatory cells demand high-energy (Dunn, 1997) for synthesis of protein, nucleic acid, phospholipids and other molecules, essential for growth. This might be the reason for the faster utilisation of ATP observed in hepatic tissues of group II rats ($p<0.05$) as compared to groups I and V rats in the present study (Fig. 5.19). In arthritis, decreased expression of ectonucleotidases that break ATP, ADP, AMP (Gergely *et al.*, 2002) and increased rate of glycolysis that produces ATP (Mikirova *et al.,* 2004), might lead to the faster consumption of ATP in inflamed tissues. Wink *et al.* (2003) hypothesized that alteration in ATP metabolism could facilitate high proliferation and low death of cells.

Supplementation of CS oil and gingerol concentrate showed a significantly higher level of ATP in the hepatic tissue of groups III and IV rats ($p<0.05$) compared to group II rats. Inflammatory cells obtain upto 60% of their total ATP from glycolysis (Nakashima *et al.*,1986). Contrary to these findings it has been reported that Inhibition of ATP production in neoplastic cells has been believed to induce apoptotic cell death and has been proposed as therapeutic strategy for treatment of liver tumors (Geschwind *et al.*, 2002). CS oil and gingerol was able to bring back ATP production to levels slightly higher than normal levels, in experimental animals, indicating faster recovery and efficient repair of damaged tissues during arthritis.

5.4.20 Effect of CFA-induced arthritis on end-products of protein metabolism

Both urea and creatinine levels in the blood are increased during cases of acute inflammation in the body (Yassuda Filho *et al.*, 2003; Greisen *et al.*,1999). In the present study there was a significant ($p<0.05$) increase in the levels of both creatinine and urea in the blood plasma of CFA-induced and untreated group II animals (Fig. 5.13). However their levels were brought down to near normal levels in the inflammation-induced and treated groups III and IV. Even though there was a significant decrease in the creatinine and urea levels in groups III and IV compared to the group II animals, there was no significant ($p<0.05$) difference in their levels between the treated groups III and IV.

Creatinine in circulatory system originates either from breakdown of creatine phosphate or from the urea cycle. Creatinine is filtered from the blood by the kidneys and excreted into urine. Creatinine levels in the blood become elevated if kidney function is impaired. Several cases of renal failure have been reported in patients with severe erosive arthritis (Griffin Jr., 1984). Creatine phosphate serves as an alternative source of energy in muscle tissues. The rise in creatinine levels in Group II animals could be attributed to the enhanced breakdown of creatine phosphate during inflammatory disorder for the maintenance and repair of damaged tissues within the system. Earlier studies in arthritic conditions (Cersosimo *et al.*, 1996) and fibrosarcoma (Palani *et al.*, 1998) showed increased activities of creatine phosphokinase in experimental rats.

Both CS oil and gingerol extracts were able to significantly bring down the level of inflammation in vivo (Swaiman and Sandler, 1963) and in vitro (Berbert *et al.*, 2005) experiments. The anti-inflammatory effect of n-3 PUFA, squalene, alkyloxyglycerols and vitamin E contained in CS oil and of the triterpenes and flavonoids in the ginger extract could have arrested the elevations in level of inflammatory mediators, reduced the levels of arachidonic acid metabolites and lowered the formation of proinflammatory compounds, like prostaglandins and leukotrienes, by blocking their activity (Olivera *et al.* 2004) This might be one of the reasons for reduced breakdown of creatine phosphate, a corresponding decline in creatinine observed in blood ($p<0.05$) in the CFA-induced and treated groups III and IV in comparison to group II rats. Another possible mechanism which may explain the lower blood creatinine level in group III rats is due to activation of creatine phosphokinase by protein kinase C. Polyunsaturated fatty acids in CS oil can modulate the activity of protein kinase C (Fortin *et al.*, 1995; Kolahi *et al.*, 2010) which in turn affects the activation of creatine phosphokinase and reduce the break down of creatine phosphate in muscle and its releases to circulatory system for different activities (Chida *et al.*, 1990).

5.4.21 Effect on lipid metabolism

Various disease conditions affect lipid metabolism and hence the concentration of lipid components like triglycerides, free fatty acids and cholesterol etc are monitored for evaluating the progress of such diseases. Triglycerides and free fatty acids are important sources of energy in fasting condition or in abnormal glucose metabolism. Cholesterol is an important component of nervous system and also required for hormone synthesis. In the present study levels of cholesterol, triglycerides, free fatty acids and lipid peroxides were studied to find out the changes in lipid metabolism during CFA-induced arthritis.

5.4.21.a) Effect of CFA-induced arthritis on plasma and tissue total cholesterol content

Cholesterol is a neutral lipid that accumulates in liquid-ordered, detergent-resistant membrane domains called lipid rafts. Lipid rafts serve as membrane platform for signal transduction mechanisms that mediate cell growth, survival and a variety of

other processes during the progression of inflammation. A significant increase in the levels of cholesterol was observed in plasma ($p<0.05$) and hepatic tissue ($p<0.05$) of group II rats showing a positive relationship between cholesterol accumulation and arthritis (Fig. 5.21). Distribution of total cholesterol was reported to be altered during arthritis (Lakatos and Hárságyi., 1988).

Inflammation and dysregulated cholesterol metabolism are key components in the pathogenesis of inflammatory disorders. Although the cellular and molecular mechanisms underlying arthritis are not thoroughly understood, inflammation associated with the rheumatic disease state may promote the severity and lead to further complications. Increasing evidence indicates that the systemic inflammatory load in lupus disrupts cholesterol homeostasis, increasing vulnerability to cholesterol accumulation in cells of the artery wall, including macrophages and endothelium. The relationship between the inflammatory state and dyslipidemia in arthritis is complex, involving lipoproteins, cholesterol transporters, scavenger receptors, and oxysterols. (Steiner and Urowitz, 2009)

A number of studies demonstrated that cholesterol deposits in inflammatory states and that cholesterol homeostasis breaks down with the transition of the disorder to its malignant state (Lakatos and Hárságyi., 1988). Elevated levels of cholesterol and phospholipid were reported to modify the lipid fluidity of the inflammed cell membranes, thereby accelerating the disorder (Stamp et al., 2005). It was reported that the plasma HDL cholesterol was inversely related to the cholesterol levels in the inflamed tissues of RA patients (Steiner and Urowitz MB, 2009; Dessi et al., 1994). Cholesterol is an important lipid component involved in cell signalling process and synthesis of hormones. In the present study the higher content of cholesterol in plasma and liver of arthritic rats might indicate greater use and storage of cholesterol in the inflamed tissues which may modulate cell signalling pathway and hormone metabolism in favor of its growth.

Dietary supplementation of CS oil and gingerol has markedly reversed the plasma ($p<0.05$) and hepatic levels ($p<0.05$) of cholesterol to normal condition in group III and IV rats (Fig. 5.21). Fish oils have been reported to reduce the plasma cholesterol level in rats with induced inflammations (Straniero *et al.*, 2008). The observation of

the present study is in agreement with the above report. Low-density lipoproteins (VLDL and LDL) are the major cholesterol carrying lipoproteins in plasma and are the causative agents for several ailments. Fish oil, rich in n-3 PUFA, was stated to increase the removal rate of VLDL–cholesterol and LDL–cholesterol particles from plasma by improving hepatic microsomal membrane fluidity (McCarthy and Kenny 1992). Polyunsaturated fatty acids were reported to inhibit the activity of 3- hydroxy methylglutaryl coenzyme A (HMG CoA) reductase, which is a rate limiting enzyme in cholesterol biosynthesis, in rats with induced inflammations (Straniero *et al.*, 2008) through modulating the production of cytokines. Similarly the lower level of cholesterol observed in plasma and liver tissue of group IV rats could be attributed to inhibitory effect of gingerol on HMG CoA reductase activity and modulation of microsomal membrane fluidity associated with cholesterol synthesis and disposal respectively (Chrubasik, 2005; Bhandari *et al.*, 1998). Reduced level of total cholesterol observed in plasma and hepatic tissues of arthritic rats indicate that the hypocholesterolemic property, of CS oil and gingerol, is related to its anti-inflammatory property.

5.4.21.b) Effect on triglyceride levels

As shown in Fig 5.22, a significant increase in the levels of triglycerides was observed in the plasma ($p < 0.05$) and liver tissues ($p < 0.05$) of group II rats as compared to those of control mice. The present finding is in agreement with earlier reported studies showing higher level of triglycerides in patients with arthritis (Mythilypriya *et al.*, 2008) and experimental animals with induced inflammations Lopez-Soriano *et al.* (1996b) The authors explained the increase in plasma and hepatic triglyceride was associated with the elevated level of catecholamines and their actions via α-adrenergic receptor. Hypertriglyceridemia is frequently observed in various degrees in patients with rheumatoid arthritis in combination with increased very low-density lipoprotein and decreased high-density lipoprotein levels (Ghosh *et al.*, 2009). Inhibition of lipoprotein lipase activity was reported to reduce the uptake of triglycerides by the tissue (Williamson, 1991) and this is a common finding in arthritic animals. An inverse relationship has been reported to exist between serum triglyceride level and lipoprotein lipase activity in experimental animals (Vanheek and Zilversmit, 1990; Lottenberg *et al.*, 1992). The hypertriglyceridemic condition noticed

in group II rats could be explained by the lower activity of lipoprotein lipase which might reduce the utilization of triglycerides and thereby accumulate in higher concentration in plasma and tissue.

Dietary supplementation of CS oil and gingerol remarkably reduced the plasma ($p<0.05$) and hepatic ($p<0.05$) triglycerides content in group III and IV rats compared to group II rats (Fig 5.22). Liu *et al.*, (2001) reported that fish oils rich in polyunsaturated fatty acids, particularly EPA, reversed the hypertriglyceridemic condition associated with inflammatory disorders and the finding from the present investigation is in agreement with this. Though the exact mechanism of action associated with the hypertriglyceridemic effect of fish oil is not known, the ability of n-3 PUFA and of alkoxyglycerols to enhance the activity of lipoprotein lipase may facilitate the utilization of triglycerides and thus lower the triglyceride level. Levy *et al.*, (1990) found that rats fed with diet rich in n-3 PUFAs had a higher level of adipose tissue lipoprotein lipase activity with a concomitant decrease in the serum triglycerides. Polyunsaturated fatty acids, especially n-3 fatty acids and gingerol were reported to increase mitochondrial and peroxisomal oxidation of fatty acids and decreased synthesis and storage of triglyceride in liver (Ikeda *et al.*, 1998; Ide *et al.*, 2000). This might be another possible reason for the lower level of triglycerides observed in liver tissue of group IV rats in the present study.

5.4.22 Effect on level of free fatty acids in plasma and liver

The levels of free fatty acid (FFA) in plasma and liver tissue of experimental rats are presented in Fig.5.23. In both plasma ($p<0.05$) and liver ($p<0.05$), the fatty acids levels were significantly higher in group II rats in comparison to groups I and V control rats. Hypermetabolism, a common feature noticed during inflammatory disorders (Simkin *et al.*, 2005) showed an enhanced rate of lipolysis of adipose fat to mobilize fatty acids essential for the repair of damaged tissues (Thompson *et al.*, 1989). This might be the reason for elevated level of FFA that was noticed in the plasma of group II rats. The present finding is in line with earlier studies by Li *et al.* (1997), Fraser *et al.*,(1999) who reported that proportions of plasma saturated and monounsaturated fatty acids were significantly increased in the hepatocellular carcinoma patients compared to the control. Liver is a key organ for the metabolism

of free fatty acids (Cowen and Campbell, 1977) and in the present study higher concentration of FFA observed in liver, could serve as a source of substrate for triglycerides synthesis (Donnelly et al., 2005) leading to hypertriglyceridemia in group II rats with induced inflammation (Fig. 5.23).

Treatment with CS oil and gingerol reduced the levels of free fatty acid in plasma ($p<0.05$) and liver ($p<0.05$) of group II and IV rats compared to that of group II rats (Fig. 5.23). Dietary fish oils rich in vitamin E and polyunsaturated fatty acids were reported to inhibit the lipolysis of adipose fat (Tisdale, 1996) and regulate the release of free fatty acids into circulatory system. This might be the reason for lower level of plasma FFA observed in group III rats in the present study. In RA, inflammation manifests an elevation in the level of cytokines and other inflammatory mediators influencing mobilization of fatty acids from adipose fat. Lipolysis is also promoted through the intracellular mediator cyclic AMP, formed in response to the activation of adenylate cyclase by the binding of a hormone to its receptor. Polyunsaturated fatty acids were reported to inhibit the production of pro-inflammatory cytokines (Lawrence, 1990) and cyclic AMP (Tisdale, 1996), thereby reducing lipolysis of adipose fat during arthritic progression. The lipid lowering activity of fish oil has been associated with various hepatic mechanisms such as increased fatty acid oxidation (McCarthy *et al.*, 1992) and inhibition of de novo fatty acid synthesis secondary to decreased fatty acid synthase gene expression (Clarke *et al.*, 1990). Reduced supply of FFA to liver tissues due to inhibition of lipolysis of adipose fat together with increased FFA oxidation and decreased synthesis by n-3 fatty acids might explain the lower level of FFA that observed in liver of group III rats.

5.4.23 Effect on formation of lipid peroxides

Reactive oxygen species (ROS), constantly generated inside cells by several oxidase enzymes and dismutation of the super oxide anion formed by electron leakage during mitochondrial respiration (Fridovich, 1978), are capable of production of lipid peroxides in the absence of an effective antioxidant system. In normal condition, a sensitive balance between antioxidant and oxidant is maintained within cell. Shift of equilibrium in favor of the pro-oxidant was reported to be involved

directly or indirectly in various pathological processes leading to the development of inflammatory disorders (Gambhir *et al.*,, 1997) and other diseases.

The levels of lipid peroxides in the hepatic tissues of the control and experimental rats subjected to different treatments are presented in Fig. 5.24. Metabolic activation of cytochrome P 450 enzyme by CFA produces variety of radical and non-radical species, which are responsible for the onset of inflammation in different organs of experimental animals (Lijinsky, 1999). In the present study a significant increase ($p<0.05$) in lipid peroxide levels in hepatic tissues of group II rats can be explained by an enhanced activity of cytochrome P 450 enzyme in the inflammation induced animals . This finding is in agreement with an earlier report by Bansal *et al.* (2005). The authors noticed a higher level of lipid peroxides in the hepatic tissues of CFA-induced rats. Oxidative stress, commonly expressed in terms of ratio of GPX (scavanges free radicals) to SOD (produces free radicals), is used as a tool to measure inflammation (Yoshikawa *et al.*,, 1985). A decrease in GPX: SOD ratio indicates higher accumulation of free radicals than its removal, leading to an increased oxidative stress in tissues (Vijayakumar *et al.*, 2006). Surapneni and Chandrasada Gopan (2008) reported an uncompromised generation of free radicals in liver tissue of arthritic patients overwhelming the antioxidant status which in turn increases the oxidative stress in the system. In the present investigation, higher level of oxidative stress observed in CFA-induced and untreated group II rats (Fig. 5.24) is in agreement with the above report.

Supplementation of fish oil and gingerol significantly decreased the levels of lipid peroxides in plasma and liver tissues of group III and IV rats ($p<0.05$) in comparison with group II rats.(Fig 5.24) This finding is in agreement with previous studies carried out in CFA-induced inflammations in rats (Yoshikawa *et al.*,, 1985). The authors reported that the amount of lipid peroxides produced in these animals, was significantly higher in group with induced arthrtis than the fish oil and control groups. The presence of antioxidant vitamins and alkoxyglycerols in fish oils would have nulled the effect of oxidation of the double bonded PUFAs in fish oil and thus helped in recovering the celllar integrity of the damaged tissues during arthritic progression (Xi and Chen, 2000). In the present study the oxidative stress observed in group IV rats ($p<0.05$) was significantly lower as compared to group II rats (Fig 5.24)

indicating the beneficial effect of the gingerol (Kota *et al.*, 2008) concentrate against CFA-induced inflammations. Erdogan *et al.* (2004) reported that fish oil when used along with doxorubicin, reduced the oxidative stress in normal liver and increased the stress in arthritic tissues compared to corn oil fed mice.

The lipid peroxide levels in group V were similar to group I. This indicates dietary fish oil does not increase oxidative stress in normal condition but the modulation of oxidative stress depends on the physiological state of the tissue or cell. Contrary to the present finding, Lii *et al.* (2000) reported greater oxidative stress in rats induced with inflammation and fed with fish oil diet The authors found that the foci number was negatively correlated to live thiobarbituric acid reactive substance and 7-pentoxyresorufin-O-dealkylase acid reactive substance. Both beneficial and harmful effects observed in the above reported studies make it difficult to draw any conclusion on the role of lipid peroxide and oxidative stress on arthritis. Further studies are required to confirm the exact role lipid peroxides, antioxidant systems and the resultant oxidative stress on inflammatory growth and progression.

5.4.24 Effect on membrane bound ATPase activities

The activities of the membrane bound enzymes such as Na^{+}/K^{+} ATPase ($p<0.05$), Ca^{2+} ATPase ($p<0.05$) and Mg^{2+} ATPase ($p<0.05$), responsible for the transport of sodium, potassium, calcium and magnesium ions across the cell membranes at the expense of ATP (Kadir *et al.*, 2005), were found to be significantly inhibited in hepatic tissues of CFA-induced group II rats compared to controls (Fig. 5.25) The present observations are in agreement with an earlier study reported by Testa *et al.* (1987). The authors noted that the activities of ATPases in erythrocyte membrane, lung and liver tissues were inhibited in patients under inflammatory stress. In studies of patients with RA, lower level of activity of Na^{+} K^{+} ATPase enzyme was attributed to decreased expression of mRNAs of isomers of this enzyme during inflammation (Testa *et al.* 1987). Ohnishi et al. (1962) cited that the activity of Mg^{+2} ATPase in normal liver cell was 140% higher than that in inflammatory cells. This is in line with the present observation of lower activities of Mg^{2+} ATPase in liver tissues of group II rats compared to control group I rats (Fig. 5.25). Ca^{2+} ATPase is extremely sensitive to hydroperoxides (Jain and Shohet, 1981). Lipid peroxides can oxidize the -SH

groups of sulphur bearing amino acids and other disulphide linkages of ATPase (Patzelt-Wenczler et al., 1975) and thereby might alter the tertiary structures of these enzymes and reduce their activity. Impairment in the lipid architecture of cell membrane by lipid peroxides results in the loss of membrane integrity and reduce the activities of membrane bound enzymes (Kiziltunç *et al.*, 1998). CFA a potent inflammatory agent was reported to generate excess lipid peroxidation products which overpowers the antioxidant defense system shifting the equilibrium between pro-oxidants and antioxidants unfavorably towards oxidant side (Boitier et al., 1995). In the present study the reduced activity of membrane bound ATPase enzymes observed in CFA-induced and untreated animals can be linked to the higher production of lipid peroxide by the adjuvant which may alter the membrane integrity by attacking its lipid component and oxidise the functional groups of ATPase enzymes responsible for their catalytic activity.

However a significant increase in the activities of membrane bound enzymes was noticed in the CS oil fed group III rats and gingerol fed group IV rats compared to group II rats (Fig 5.25). Literature on effect of dietary fish oil directly on activities of ATPase enzymes in arthritic condition is scarce. Polyunsaturated fatty acids and squalene in the fish oil can activate the antioxidant enzymes and maintain the GSH pool of tissue by modulating eicosanoid pathway or regulating the activities of other enzymes directly or indirectly associated with antioxidant system (Darlington and Trevor, 2001; Drew *et al.*, 2005; Arab *et al.*, 2006). Polyunsaturated fatty acids used at lower dose reported to enhance the antioxidant status in liver tissues of experimental mice (Niranjan *et al.*, 2000). In the present study a significantly lower level of oxidative stress was observed in both groups III and IV ($P< 0.05$) compared to controls (groups I and V) (Fig 5.29). From the above reports and observations of the present study it can be suggested that dietary fish oil and gingerol concentrate can protect the cell membrane and its integrity by activating the antioxidant system and maintaining a delicate balance between lipid peroxide and antioxidant in tissue (Ali *et al.*, 2008). This might be one of the reasons for the restoration of membrane bound ATPases in hepatic tissues of group III and IV rats as compared to group II rats. Addition of polyunsaturated fatty acids to culture medium altered the fatty acid composition of cell membrane which in turn modified the activity of membrane bound

enzymes including ATPase (Vijaya Kumar *et al.*, 1992). Proinflammatory cytokines like TNF α and eicosanoid PGE_2 are produced in higher level during acute inflammation. TNF α has been reported to induce PGE_2 synthesis which reduces the expression of Na^+, K^+ ATPase enzyme and its activity (Kreydiyyeh *et al.*, 2007) during inflammatory disorders. Consumption of fish oil rich in n-3 fatty acids reported to decrease production of eicosanoids such as PGE_2 and LTB_4 (Chapkin *et al.*, 1990) and this might inhibit the activities of TNF-α and IL-1β in arthritis (Grimble *et al.*, 2002). Hence it could be postulated that n-3 PUFA might restore the activity of membrane bound Na^+, K^+ and Mg^{2+} ATPase by regulating the production of prostaglandins and cytokines during arthritic progression. Dietary supplementation of gingerol concentrate increased the activity of Ca^{2+} ATPase in group IV rats ($p<0.05$) as compared to group II rats (Fig. 4.2.17.2) The exact mechanism behind the higher activity of Ca^{2+} ATPase by gingerol in arthritis is not clear.

A possible mechanism by which fish oils might regulate the activity of membrane ATPases is through the phosphorylation and dephosphorylation of this enzyme by protein kinase C. Protein kinase C in active condition phosphorylates the ATPase enzymes to inactive state. Dietary n-3 PUFA is reported to inhibit the activity of protein kinase (Mirnikjoo *et al.*, 2001) responsible for phosphorylation of ATPases.

5.4.25 Effect on mineral content

Alteration of cell mineral metabolism has been proposed to be an important pathogenetic step in the development of arthritis (del Pozo *et al.*, 2009). In normal physiological condition the cytosol of animal cells contains a concentration of potassium ions (K^+) as much as 20 times higher than that in the extracellular fluid. Ca^{2+} ATPase located in the plasma membrane of all eukaryotic cells pumps Ca^{2+} out of the cell helping to maintain the ~10,000-fold concentration gradient of Ca^{2+} between the cytosol and the extra cellular fluid (ECF) In the present study significantly lower levels of sodium and potassium ($p<0.05$) and higher content of calcium ($p<0.05$) were noticed in hepatic tissue of group II rats (Fig 5.26). Similar observations have been reported by Premalatha and Sachdanandam (1998) in liver tissues of rats treated with aflatoxin B1. Higher intracellular calcium reported in arthritic conditions (del Pozo *et al.*, 2009) is essential for its pivotal role in cell signalling involved in cell proliferation (Waldron et al., 1994). Elevated level of

magnesium has been reported to accelerate cellular mitosis (Yamasaki *et al.*, 2001). In the present study inhibition of Ca^{2+}ATPase under conditions of inflammation would have resulted in calcium accumulation in group II CFA-induced rats. This would have lead to a disturbance in the equilibrium between the intracellular and extracellular calcium concentrations (Hämäläinen *et al.*, 2010). Moreover CFA administration would have induced lipolysis and production of reactive oxygen species that destabilized the hepatic membrane, resulting in the inactivation of Na^+, K^+-ATPase and the depletion of plasma potassium and rise in sodium concentration.

Active calcium transport and resultant low calcium concentrations are requirements for active Na^+/K^+ transport. Since calcium and sodium ions are competitive at a number of membrane sites, a high concentration of calcium ions in the inflammatory cells would compete with sodium specific sites at the inner surface of the membrane, leading to a decrease in hepatic sodium content as observed in group II rats.

Supplementation of fish oil and gingerol concentrate reduced the intracellular content of calcium ($p<0.05$) and increased the concentration of sodium ($p<0.05$) and potassium ($p<0.05$) in hepatic tissues of group III and IV rats in comparison to group II rats (Fig. 5.26). Dietary fat alters the membrane phospholipids fatty acids composition and this might modulate the activity of ion channels (Leaf *et al.*, 2002). Polyunsaturated fatty acids can regulate the membrane transport process by altering the lipid profile of the membrane (Coetzer *et al.*, 1994), controlling the steroid induced protein synthesis (Nunez, 1993), affecting the production of eicosanoids (Kogteva and Bezuglov, 1998), modulating the action of phospholipases A and C (Graber *et al.*, 1994) or activities of G proteins (Abramson *et al.*, 1991). All the above mechanisms might modulate the activity of membrane bound ATPases and restore the levels of minerals near to normal in group III rats in comparison to group II rats. Elevated level of glycolysis in arthrtitis is directly related to the level of calcium present in inflammatory cells. Polyunsaturated fatty acids was reported to inhibit the activity of glycolytic enzymes (Clarke *et al.*, 1977; Jump *et al.*, 1994) which in turn could reduce the flow and accumulation of calcium ions in arthritic cells.

5.4.26 Effect of CFA-induced arthritis on the hepatic antioxidant defence system

It has been well documented that oxidative stress defined by Blomhoff as a "condition that is characterized by the accumulation of non-enzymatic oxidative damage to molecules that threaten the normal function of a cell or the organism" is involved in the aetiology of a large number of human diseases (Blomhoff, *et al.*, 2006). The human body has several mechanisms to counteract damage by free radicals and other reactive oxygen species. These act on different oxidants as well as in different cellular compartments. One line of defense is via the enzymatic antioxidant system which includes enzymes such as catalase, superoxide desmutase, glutathion peroxidase, glutathion reductase etc. and the other line of defense is through the role played by the non-enzymatic antioxidants like glutathione, Vitamin E, Vitamin C etc. all of which are capable of scavenging out the free radicals that results in intracellular oxidative stress (Feng et al., 2001) and inhibit the progression of inflammation in animals and humans (Mythilypriya *et al.*, 2007).

5.4.27 Effect on non-enzymatic antioxidants

5.4.27.a) Effect on tissue glutathione levels

Glutathione is a tripeptide (γ-Glutamyl-cysteinyl-glycine) thiol present in virtually all animal cells. In normal condition both the reduced (GSH) and oxidized (GSSG) form of glutathione remain in a balanced state (Herebergs *et al.*, 1992). GSH acts as a free radical scavenger and neutralizes superoxides, peroxide radicals and singlet oxygen by donating hydrogen atoms (Meister, 1983). Antioxidant enzymes like glutathione S-transferase (GST) and glutathione peroxidase (GPX) utilise reduced form of GSH to carry out their scavenging and detoxification activities and at the end of the process oxidized form of GSSG comes out as final product (Meister, 1975). Conversion of oxidized glutathione (GSSG) to its reduced (GSH) form by glutathione reductase (GR) and NADPH, is one of the important factors that decides the level of GSH in cell. In the present study, high concentration of lipid peroxide, enhanced activities of GST and GPX (with a corresponding decline in their levels in Group II animals) and at the same time reduced rate of restoration of GSH by lower GR activity might be responsible for the lower level of GSH observed in hepatic tissue of group II rats ($p<0.05$) compared to group I controls (Fig 5.27).

Dietary supplementation of n-3 PUFA concentrate enhanced the level of reduced glutathione in group III and IV rats ($p<0.05$) compared to group II rats (Fig. 5.27). Similar to our findings, Kumar and Das (1995) reported a higher level of glutathione in rats with CFA-induced inflammations and treated with fish oil. Arab *et al.* (2006) reported that docosahexaenoic acid enhanced the cellular GSH level by elevating the activities of gamma-glutamyl-cysteinyl ligase and glutathione reductase enzymes Polyunsaturated fatty acids were reported to enhance the activity of glutathione reductase (Hsu *et al.*, 2001), and glucose-6-phosphate dehydrogenase enzymes (Yilmaz *et al.*, 2004) which in turn can restore GSH at faster rate. Higher level of GSH noticed in group IV rats could be attributed to the regulatory effect of gingerol on various enzymes directly or indirectly related to GSH synthesis or regeneration.

5.4.27.b) Effect on vitamins

Vitamin E is a lipophilic antioxidant which inhibits the propagation of peroxy and alkoxy radical intermediates of lipid peroxides by donating its labile hydrogen atom from phenolic hydroxyl groups and terminate the chain reaction (Walwadkar, 2006). Protective role of vitamin E against oxidative stress related diseases, is well documented (Bieri *et al.*, 1983). Apart from antioxidant property vitamin E, being a lipophilic molecule, can enter into the lipid bilayer and completely alter the membrane lipid biochemical property and preserve the structural integrity of cellular lipid by trapping reactive oxy-radicals (Sies, 1991). Among the water-soluble antioxidant vitamins, vitamin C plays a central role in the defense of free radical-induced cellular damage (Chan, 1993). Vitamin C can protect cell membranes and lipoprotein particles from oxidative damage by regenerating the antioxidant form of vitamin E (Beyer, 1994). In the present study a significantly lower level of vitamin C ($p<0.05$) and vitamin E ($p<0.05$) was found in group II rats in comparison to group I rats (Fig 5.27) and this can be explained by the excessive utilization of these antioxidants for quenching elevated levels of free radicals produced by CFA and lack of efficient antioxidant system in the inflamed tissue to restore these vitamins back into normal state. The present finding is in agreement with an earlier reported study by Vijayalakshmi *et al.*, (1997) who reported lower level of vitamin E and C in hepatic tissue of experimental rats induced with arthritis. An inverse relationship between intracellular accumulation of vitamin E and inflammation has been demonstrated by

Cinar *et al.*, (1998). The authors suggested that vitamin E could reduce the progression of inflammation by its regulatory effects on protein kinase C activity. Ascorbyl radical produced due to oxidation of vitamin C was reported to be directly proportional to the magnitude of inflammation (Simões, 2003). During the process of neutralisation of free radicals to non toxic form, both vitamin E and vitamin C are oxidized to their respective tocopheroxyl and ascorbyl radicals forms. Reduction of these radicals to their normal state depends on the status of antioxidant system inherently present in cell.

Dietary supplementation of CS oil and gingerol concentrate significantly increased the level of vitamin C ($p<0.05$) and vitamin E ($p<0.05$) in group III and IV rats compared to group II rats (Fig. 5.27). Fish oil rich in n-3 fatty acids demonstrated significantly higher activities of antioxidant enzymes like catalase, superoxide desmutase, glutathione peroxidase in liver of experimental animals (Crystal *et al.*, 1985). Polyunsaturated fatty acids were reported to elevate the activities of glutathione reductase enzyme responsible for maintenance of GSH pool in cell (Hsu *et al.*, 2001). Erdogan *et al.* (2004) showed that fish oils rich in n3 PUFA at hypotriglyceridemic dose enhanced the antioxidant defense in liver of experimental rats. In the present study higher levels of vitamins E and C observed in n-3 PUFA treated animals could be explained by the ability of PUFA to elevate the activities of antioxidant enzymes which in turn restore the GSH level and reduce the ascorbyl and tocopheroxyl radicals, produced during scavenging action on lipid peroxides, to their normal state in hepatic tissue of these animals. The free radical scavenging property of gingerol would have reduced the inflammatory disorder in the group IV animals, as observed in the study.

5.4.28 Effect of CFA-induced arthritis on enzymatic antioxidants

5.4.28.a) Effect on activity of glutathione related enzymes

Reactive oxygen species like hydrogen peroxide, lipid hydroperoxides, superoxides etc are implicated in several disorders. Intracellular defense system which detoxify these reactive free radicals and other xenobiotics include antioxidant enzymes such as glutathione peroxidase, glutathione reductase, , glutathione S transferase, superoxide dismutase, and catalase (Sindhu *et al.*, 2009; Mythilypriya *et al.*, 2009).

Glutathione and glutathione dependent enzymes such as glutathione-S-transferase (GST), glutathione peroxidase (GPX), glutathione reductase (GR) are of central importance in the detoxification of peroxide radicals and xenobiotics drugs (Ratheesh *et al.*, 2009). Glutathione peroxidase, catalase and super oxide desmutase are considered as primary antioxidant enzymes, since they are directly involved in scavenging of free radicals. Glutathione-S-transferase and glutathione reductase act as secondary antioxidant enzymes as they can detoxify the reactive oxygen species by decreasing the peroxide level and by maintaining the cellular pool of GSH respectively.

The significant decrease in the levels of glutathione S transferase ($p<0.001$), glutathione peroxidase ($p<0.001$) and glutathione reductase ($p<0.001$) observed in group II rats compared to group I rats may be due to the high demand for the antioxidants to detoxify the electrophiles produced by CFA (Fig. 5.28) This is in agreement with the earlier reported study by Vijayalakshmi *et al.*, (1997) who noticed an increased activity of GST and GPX (and hence a corresponding decline in their levels) in hepatic tissue of stress-induced rats as compared to control rats. Low levels of GST, GPX and GR in CFA-induced animals indicated the activation of antioxidant system to protect the tissue from electrophiles (Maiorino *et al.*, 1990) Activity of glutathione reductase (GR), the enzyme responsible for maintenance of GSH pool in cell, was elevated in arthritic tissues in comparison to normal human lung tissues (Vázquez *et al.* 1997) which is in line with the present finding of higher activity of GR and hence a corresponding decline in GR levels in hepatic tissues of group-II animals than that of group-I animals. The increase in the glutathione dependent antioxidant enzyme levels in treated animals (groups III and IV) shows the presence of functional defense mechanism against the oxidative stress in hepatic tissue of group II rats compared to normal controls. In the present study increased oxidative stress (Fig. 5.29); reduced level of glutathione essential for activities of antioxidant enzymes (Fig. 5.28) and histological studies (Plate. V.2) showing the damages of liver tissue caused by free radicals produced by CFA, indicate that the body's self-defense system is not sufficient to protect the hepatic tissues from the inflammatory effect of CFA in group II rats.

Supplementation of CS oil and gingerol significantly increased the level of GST ($p<0.05$), GPX ($p<0.05$) and GR ($p<0.05$) enzymes in groups III and IV rats compared to group II rats (Fig 5.28). Being highly unstable in nature n-3 PUFA in fish oils gets easily oxidized (Meydani, 1991) by the free radicals produced by CFA. However it is interesting to note that when free radicals produced by CFA causes inflammation (Ratheesh *et al.*, 2009), the antioxidants in fish oil and gingerol inhibits inflammation (Afshari *et al.*, 2007; Kota *et al.*, 2008). It can be suggested that it is the extent of oxidative stress which results from the disturbance in the equilibrium of lipid peroxides and antioxidants that decides the beneficial or harmful effect of lipid peroxides. Fish oil rich in n-3 fatty acids was reported to modulate the metabolic activation of adjuvants by altering the concentration of cytochrome P- 450 enzyme (Ammouche et al., 1993) and at the same time it increased the activity of NADPH-cytochrome C reductase, aniline hydroxylase, aminopyrine N- demethylase, glutathione reductase, glutathione S transferase, catalase, epoxide hydrolase and UDP-glucuronosyltransferase activities in rats (Ammouche *et al.*, 1993; Yang *et al.*, 1993; Kaur *et al.*, 1998). All the above enzymes are antioxidant or phase 2 enzymes, having potential to prevent inflammation induced by free radicals produced by CFA. Docosahexaenoic acids can upregulate the activities of gamma glutamyl cysteinyl ligase and glutathione reductase and enhance the antioxidant response in human fibroblast (Arab *et al.*, 2006). Polyunsaturated fatty acids may increase the antioxidant level in tissues by modulating eicosanoids pathway. Increased prostaglandin PGE_2 production is associated with higher oxidative stress which in turn can decrease the activity of glutathione peroxidase (Drew et al., 2005).

CS oil rich in n-3 PUFA and gingerol would have decreased the production of PGE_2 (Vázquez *et al.*,2004; Kota 2008) and this might have increased the activity of glutathione peroxidase and thereby reduced oxidative stress in arthritis. From the above studies it can be assumed that dietary fish oil and gingerol concentrate might enhance the levels of antioxidant enzymes in group III and IV rats by inhibiting the formation of PGE_2 eicosanoid or by up regulating the activities of other enzymes directly or indirectly associated with the antioxidant system.

5.4.28.b) Effect on activity of superoxide dismutase (SOD) and catalase

Superoxide dismutase (SOD) is the first line of defence antioxidant enzyme that protects cells from the oxidative damage mediated by superoxide radicals. SOD catalyses the dismutation of the superoxide radicals to hydrogen peroxide and oxygen (Mccord and Fridovich, 1969). Catalase is most abundant in liver and is responsible for the catalytic decomposition of H_2O_2 to oxygen and water. Metabolic activation of adjuvants by cytochrome P-450 dependent monoxidase augments oxidative stress by formation of H_2O_2 and superoxide anions (Farber and Gerson, 1984) and that could result in elevated activities of SOD and catalase. The levels of these enzymes in group II rats which were subjected to CFA-induced inflammations was significantly lower compared to control groups I and V. (Fig. 5.29). The present finding is in agreement with a previously reported study (Beno *et al.*, 1995), showing lower levels of catalase, SOD, and GPX in osteoclastic arthritis. Lower levels of SOD and catalase (Vijayalakshmi *et al.*, 1997) have been reported in arthritic tissues. Sometimes it is hypothesized that lower activities of SOD and catalase can expose the tissue to an extreme oxidative stress that helps in progression of an inflammatory disorder whereas higher activity of both the enzymes is thought to be responsiveness of body's defense mechanism to deleterious radicals produced excessively upon exposure to allergens.

CS oil and gingerol concentrate, in the present study, significantly ($p<0.05$) increased the level of SOD ($p<0.05$) and catalase ($p<0.05$) enzyme in group III and IV rats compared to group II rats (Fig. 5.29). This finding is in line with an earlier reported study by Crystal *et al.* (1992), who reported enhanced levels of superoxide desmutase and catalase activity in experimental mice fed with eicosapentaenoic acid at hypotriglyceridemic dose. Elevated levels of superoxide desmutase and catalase activity indicate the capability of CS oil and gingerol to protect the hepatic tissues from the harmful effect of free radicals produced by CFA.

5.4.29 Effect of CFA-induced arthritis on lysosomal enzymes

Lysosomes are a group of cytoplasmic organelles present in numerous animal tissues, characterized by their content of acid hydrolases (Essner and Novikoff, 1961). Higher activities of lysosomal enzymes in inflammatory exudates serves as a good marker to assess the intensity of inflammation and severity of necrotic damage in condition like CFA-induced arthritis. These hydrolytic enzymes are oozed out by

the rupture of the lysosomal membrane, initiating the synthesis of inflammatory mediators such as thromboxanes, prostaglandins and leukotrienes (Agha and Gad, 1995). Lysosomal membranes are reported to contain large amounts of glycoproteins, which play an important role in maintaining lysosomal structure and functions (Chen *et al.,* 1985). Oxygen free radicals generated during inflammation in addition to the direct damaging effect of CFA may also be responsible for the liver damage through the release of lysosomal enzymes (Kalra and Prasad, 1994).

In the present study, the levels of lysosomal hydrolases (β-glucuronidase, β-galactosidase, β-glucosidase, Cathepsin D, acid and alkaline phosphatases) were significantly ($p<05$) higher in liver tissues of Group II CFA-induced and untreated rats as compared to that of Group I and V control rats, indicating the severity of CFA-induced necrotic damage to the liver (Fig 5.30, Fig. 5.31, Fig. 5.32, Fig. 5.33). A parallel decline in the activities of these hydrolytic enzymes in lysosomal fraction of the liver tissue was also observed in the CFA-induced and treated groups III and IV. (Fig 5.30, Fig. 5.31, Fig. 5.32, Fig. 5.33). The disintegration of hepatic membrane noticed in the present study may be due to the increased leakage of the lysosomal hydrolases from the enclosed sacs into the cytosol. The present observation concurs with an earlier report (Ebenezar *et al.,* 2003a), that the cytosolic acid hydrolases liberated from lysosomes and from the sarcoplasmic recticulum induce the dysfunction and destruction of mitochondria, sarcolemma and other organelles. Measurement of these hydrolytic enzymes in systemic circulations is frequently used as an index of lysosomal membrane integrity (Ravichandran *et al.,* 1990). The higher levels of these enzymes in the hepatic tissues, as observed in the present study, may be either due to the infiltration of inflammatory cells or due to adjuvant-mediated lysosomal fragility by Ca^{2+} overload.

In the present study, supplementation of CS oil and gingerol extracts resulted in significant ($p<0.05$) reduction in the CFA-induced release of these hydrolytic enzymes from the lysosomes. This favorable protective effect of the fish oil components (squalene, ether lipids, vitamin E, n3 fatty acids – either individually or in combination) and of gingerol, may be related to its ability to exert stabilization on lysosomal membranes. Since the release of lysosomal enzymes is crucial in the pathogenesis of CFA-induced arthritis and related inflammation process, it is likely that the reduction of such enzyme release would prove beneficial, indirectly

confirming the salubrious effect of CS oil and gingerol. Earlier reports by Upasani and Balaraman (2001) indicated that lipophilic antioxidant molecules are capable of stabilizing the fragile lysosomal membranes by reducing inflammation process and inhibiting lipid peroxidation.

Squalene, in deep sea liver oils of sharks, has been reported to exert membrane stailization through forcing structural interactions with lipid membranes. The isoprenyl group of squalene plays a major role in this interaction process (Kohno *et al.*, 1995). Previous investigations have shown that squalene is capable of inhibiting oxidative deterioration of phospholipids associated with cellular membranes and lipoproteins particles (Dessi *et al.*, 2002). It is also possible to hypothesis that squalene is capable of stabilizing the layers of cellular and subcellular membranes like that of the action exerted by vitamin E through the formation of complexes with the fatty acids in the phospholipid bilayer membranes. Reports by Haines (2001) indicated that squalene plays a major role in the maintenance of the balance between the hydrophilic and hydrophobic clusters present inside the cell membrane. It is also capable of suppressing the effect of hydrolyzed products, which affect the membrane stability.

5.5 SUMMARY AND CONCLUSION

Rheumatoid arthritis is a systemic autoimmune disease that causes chronic inflammation of the joints and affects other organs of the body as well. Even though non-steroidal anti-inflammatory drugs (NSAIDS) and disease modifying anti-rheumatic drugs (DMARDS) are available today for treatment of inflammatory disorders they are associated with negative side effects. Natural remedies for arthritis are safer and better tolerated by the body than conventional drugs. These medications go beyond pain relief and look at the whole body with the aim of healing and reversing disease, rather than simply masking the symptoms. It has been reported that over 75% of patients suffering from rheumatoid arthritis believe that food plays an important role in their symptom severity and 20 to 50% try dietary manipulation as an attempt to relieve suffering. Clinical trials on those patients who relied on fish oil as a supplement of their diet have shown decreased joint tenderness, joint pain, swelling and morning stiffness.

Studies on the pharmacological properties of liver oils from sharks, inhabiting the waters beyond 600 m. depth of Indian EEZ are scanty. Shark liver oils such as those from *Centrophorus scalpratus* contain high fractions of health boosting unsaponifiable matter and unsaturated fatty acids that could render beneficial effects in combating inflammatory disorders. The exact mechanism underlying the anti-inflammatory effects of CS oil remain unclear. CS oil contains several bioactives like anti-inflammatory n3 pufa, vitamin E, the antioxidative and hypolipidemic hydrocarbon squalene, the anti neoplastic alkoxy glycerols and the ability of the oil to reduce CFA-induced inflammations in living systems may be due to the synergistic effect of all these components.

Gingerol has been used as a traditional anti-inflammatory agent since time immemorial but studies pertaining to its effect during CFA-induced arthritis, followed by its impact on metabolism of carbohydrates, lipids and proteins in the body, are scarce. In the present study gingerol was used in place of a standard reference drug owing to the natural anti-inflammatory potency of the substance and the absence of unwanted side-effects when taken at suitable dosages. Gingerol concentrate at 0.5g/kg bodyweight of the animal proved to possess maximum anti-inflammatory effect, confirmed by determining the acute toxicity of the substance with the help of

Karber's arithmetic method for evaluation. Consumption of ginger or its extracts would render beneficial effects within the body by boosting the immune status.

Though studies, pertaining to the action of CS oil and gingerol during induced inflammations within the body, are scarce, we propose the reduction in the release of inflammatory mediators to be the probable reason for the observed anti-inflammatory effects. Other probable mechanisms are focused around alteration in biosynthesis of eicosanoid metabolism, cell signaling process, gene transcription etc. Since scientific evidence of the antiarthritic effects of liver oils of *C.scalpratus* and gingerol is nil, the present study was aimed in determining the efficiency of the fish oil against Complete Freund's Adjuvant (CFA)-induced arthritis in male Albino rats and to compare the anti-inflammatory activity of the oil with a traditionally used anti-inflammatory substance gingerol. The salient findings of the study were as follows.

CFA induced inflammations raised the level of inflammatory mediators (cyclooxygenases, myeloperoxidases, nitrate and nitrites) and lymphocytes at the site of inflammation as indicated by the rise in paw sizes and higher edema on the 14^{th} day of the experiment, in the untreated arthritic animals. Both CS oil and gingerol effectively brought down the level of inflammatory mediators in the treated groups of arthritic animals as evident from their reduced paw edemas.

Loss of body weight and reduced rate of food intake were observed in all the groups administered with the adjuvant. Supplementation of CS oil and gingerol however, improved the appetite and body weights of the respective groups of animals. Higher level of RBC and WBC observed in arthritic rats indicated the greater oxygen demand and severity of inflammation in CFA-induced arthritis. A significant reduction in the level of WBC in experimental rats induced with inflammation and fed the fish oil and gingerol concentrate showed their anti-inflammatory effects.

Elevated levels of blood creatinine and urea in the diseased state and their reversion to normal condition upon treatment with CS oil and gingerol demonstrated the cyto-protective role of these substances towards CFA-induced inflammation. Significant increase in the levels of diagnostic marker enzymes like AST, ALT, LDH, in plasma and liver tissues revealed considerable damage of cellular system by CFA and leakage of these enzymes to circulatory system.

Histopathological and radiological studies confirmed greater fragmentation of joint tissues in CFA-induced and untreated animals. Supplementation of CS oil and gingerol concentrate reduced tissue damage in arthritic rats. This denotes the anti-inflammatory effect of CS oil and gingerol against the damage caused by arthritis.

Raised levels of protein observed in plasma and liver tissue of arthritic rats indicated enhanced synthesis of proteins during inflammation. Albumin, a major component of plasma protein, plays important role in transportation of biomolecules in body and significant reduction in the level of plasma albumin was observed in CFA-induced rats. Progression of inflammation is directly related to the production of immunoglobulins, an important member of family of globular proteins and this could be the reason for elevated level of globulins observed in the CFA-induced and untreated animals. Supplementation of CS oil and gingerol concentrate brought back the level of protein to near normal condition. This indicated that both the fish oil and gingerol may inhibit the synthesis of various growth factors, hormones, peptides and expression of various genes and their products responsible for progression of inflammation.

Elevated levels of glycoprotein components like hexose, hexosamine and sialic acid in plasma and liver tissues of CFA-induced animals showed greater synthesis of glycoproteins in their liver. High levels of proteins synthesized in liver can in part contribute to such elevation in glycoprotein level in plasma and liver of these animals. Any change in the levels of glycoprotein and its carbohydrate components indicates an alteration in cell membrane property and transport of metabolites across the membrane. Treatment with CS oil and gingerol concentrate resulted in a significant reduction in the level of glycoproteins in the arthritic rats.

Free amino acids play multiple roles in cellular metabolisms. The levels of gluconeogenic amino acids were higher in arthritic cases. Reduced activities of gluconeogenic enzymes like glucose-6-phosphatase and fructose-1,6-diphosphatase indicated a slow down in the gluconeogenesis process for synthesis of glucose from non carbohydrate sources. Animals treated with CS oil and gingerol showed lower level of gluconeogenic amino acids, ornithine and arginine. This demonstrated the inhibitory effect of the fish oil and ginger extract on protein

degradation induced by the adjuvant and utilisation of amino acids as source of energy.

Blood glucose and tissue glycogen levels were significantly reduced in CFA induced rats. Reduced rate of gluconeogenesis and faster utilisation of glucose for energy might be the reasons for such drop in the level of glucose in blood and depletion of glycogen in liver. Increased rate of gluconeogenesis and reduced rate of glycolysis observed in treated animals might maintain the homeostasis of glucose in blood and prevent glycogen degradation in rats during inflammation period. In the present study higher levels of hexokinase and aldolase enzymes indicated enhanced rate of glycolysis in rats during arthritic progression. The lower activities of glucose -6-phosphatase and fructose-1,6-diphosphatase showed a retardation of gluconeogenic pathway for synthesis of glucose from amino acids. Lower levels of hepatic ATP in CFA-induced and untreated animals showed the demand for high energy needed for the protein synthesis during repair of damaged tissues in inflammation. Supplementation of CS oil and gingerol however restored the activities of gluconeogenic enzymes to normalcy.

There were significant elevations in the cholesterol, triglyceride, free fatty acids and lipid peroxide levels in the CFA-induced and untreated group of rats. CS oil and gingerol feeding brought down their levels in the CFA-induced animals, displaying their hypocholesterolemic and immune boosting behaviour during periods of acute inflammations. The lipid lowering effect of the fish oil and gingerol might be explained due to higher rate of transfer of cholesterol to liver for its reduction to bile salts

Greater oxidative stress in CFA-induced rats indicated that the rise in activities of antioxidant enzymes within the system was not sufficient enough to protect the liver from the damages caused by the adjuvant. Supplementation of CS oil and gingerol showed significantly lower levels of GST, GPX, GR, SOD and catalase in CFA induced rats compared to control rats. Drop in levels of GSH, vitamin C and vitamin E manifested active participation of these antioxidants in neutralization of lipid peroxides in CFA-induced animals. Experimental groups with arthritis and treated with the fish oil and gingerol had higher levels of antioxidants like GSH, vitamin C and vitamin E, showing the immune boosting property of the substances.

Activities of membrane bound ATPases (Na^+ K^+ ATPase, Mg^{2+} ATPase, Ca^{2+} ATPase) were significantly reduced in hepatic tissue of arthritic rats. This indicated alterations in the integrity of cell membrane and its functioning by CFA. Feeding CS oil and gingerol restored the activities of ATPase significantly. Decreased level of sodium and potassium, increased levels of calcium observed in CFA-induced animals might be the effect of altered ATPase activity during inflammation. Treatment with n-3 PUFA resulted in preventing these adverse effects to a considerable extent.

The adjuvant induced inflammation in rats resulted in decreased stability of the cell membranes, which was reflected by the lowered activity of the hepatic lysosomal hydrolases with a concomitant increase in activity of these hydrolases in the respective tissues indicating the severity of inflammation. Supplementation of CS oil and gingerol significantly inhibited the release of these hydrolytic enzymes from the lysosomes. This might be due to the stabilizing effect of the fish oil components and of gingerol on the lysosomal membranes.

In conclusion, the findings of the present study indicated the anti-inflammatory effect of *Centrophorus scalpratus* liver oil and gingerol, supplemented at 3 and 1% levels respectively, in diet, against CFA-induced arthritis in swiss albino mice. CS oil rich in n-3 PUFA, squalene, monoalkyl glycerol ethers, vitamin E etc. and gingerol would have decreased the production of inflammatory series of prostaglandins and cytokines (Appel and Woutersen, 1995) and this would have boosted the antioxidant defense *in vivo* and thereby reduced oxidative stress in arthritis. From the above studies it can be assumed that dietary fish oil and gingerol concentrate might enhance the levels of antioxidant enzymes by inhibiting the formation of the pro-inflammatory mediators or by up regulating the activities of other enzymes directly or indirectly associated with the antioxidant system.

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1. **Mathen, M.**, Suseela, M., Asok Kumar, K. and Anandan, R. 2008 Analgesic and anti-inflammatory activities of liver oils of four shark species from the Indian EEZ *Journal of Food Lipids* (15), p 470-487.

2. **Mathen, M.**, Suseela, M., Asok Kumar, K. and Anandan, R. 2008 Lipid profile of liver oil of the sickle-finned chimaera (*Neoharriotta pinnata*) of the Arabian Sea. *Food* p 176-178.

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1. Chintu M.R., **Mathen, M.**, Suseela, M., Asok Kumar, K., Nair, P.G.V. Comparative evaluation of non-protein nitrogenous compounds in fishes of fresh and brackish water environments *Fishery Technology* - (MS 1786) to be published in 2010 ed. 47(2)

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1. **Mathen, M.**, Martin, X., Suseela, M., Asok, K., Asha, K.K., Anandan, R. Effect of mangrove root extracts on the wound healing and yeast induced hyper-pyrexia in experimental rats *8th Asian Fisheries Forum* – International Book of Abstacts (2007) p 350.

2. **Mathen, M.**, and Suseela, M., Coconut oil, fish oil and polyunsaturated fatty acid enriched diets on the health statuts of Albino rats – a comprehensive stydy. National Seminar (Conserve Fish 2009) *Book of Abstracts* p112.

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ANALGESIC AND ANTI-INFLAMMATORY ACTIVITIES OF LIVER OILS OF FOUR SHARK SPECIES FROM INDIAN EEZ

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ABSTRACT

The analgesic and anti-inflammatory properties of liver oils of four different sharks, namely Neohariotta raleighana, Centrosymnus crepidater, Apristurus indicus *and* Centrophorus scalpratus, *captured from the Arabian Sea and the Indian Ocean were evaluated. While the analgesic property was determined using the acetic acid-induced mouse writhings and hot-plate reaction time, the anti-inflammatory activity was evaluated using the formalin-induced rat-paw edema. The oils examined were found to possess significant ($P < 0.05$) analgesic activity against acetic acid-induced writhings and hot-plate reaction in mice. In the formalin-induced edema, a significant ($P < 0.05$) inhibition of inflammation was observed between the 2nd and 4th hour showing 58–65% inhibition. These results suggest that liver oils of sharks from Indian waters are effective as analgesic and anti-inflammatory agents. The role of lipid components (squalene, alkylglycerols and polyunsaturated fatty acids) on anti-inflammatory and antinociceptive properties is highlighted. Inhibition of the synthesis of prostaglandins and other inflammatory mediators which probably account for the properties is discussed.*

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PRACTICAL APPLICATIONS

Studies on the pharmacological properties of liver oils from sharks, inhabiting the waters beyond 600 m depth of the Indian Exclusive Economic Zone are scanty. Shark liver oils contain high fractions of health-boosting unsaponifiable matter and unsaturated fatty acids that could render beneficial effects. Results suggest that these oils possess excellent anti-inflammatory and peripheral antinociceptive effects that may contribute to its use in the treatment of arthritis and other inflammatory disorders.

INTRODUCTION

Oceans are unique resources that provide a diverse array of natural products (Marris 2006), primarily from invertebrates such as sponges, tunicates, bryozoans and mollusks, as well as from marine bacteria and cyanobacteria. While bioactive compounds of varied origin have been explored from deep sea resources worldwide, the discovery of natural drugs from the fishery resources of the Indian Exclusive Economic Zone (EEZ) is still in its infancy. Over the past few years, the perception of marine nutraceuticals (Shahidi 2007) to the health care professional and consumer has been popularized to fish oils from the greatest predators of the sea – the sharks.

Sharks are an important resource among the marine fish species caught in India. While the marine waters up to 50 m depth have been studied extensively, the waters beyond this depth remain unexplored. Statistics (Fowler *et al.* 2005) have shown that over 30,000 tons of pelagic shark and certain species like squalene sharks (inhabiting waters beyond 600 m) available in India's EEZ have hardly been exploited. While shark fishing gained momentum over the years, much of their commercial value has been limited to the sale and supply of shark fins. The nutraceutical values associated with the liver oils of sharks from the Indian EEZ remain unexplored.

Therapeutic use of shark liver oil is evident from its use for centuries as a remedy to heal wounds and fight flu (Neil *et al.* 2006). Japanese seamen called it *samedawa*, or "cure all." Shark liver oil is being promoted worldwide as a dietary supplement to boost the immune system, fight infections, to treat cancer and to lessen the side effects of conventional cancer treatment. These days, more emphasis is laid on the nutritive benefits of shark liver oils, especially on the omega 3 polyunsaturated fatty acids (PUFAs) (Anandan *et al.* 2007) and alkylglycerols (AKGs) (Pugliese *et al.* 1998) contained in them because of the high rise of inflammatory disorders such as arthritis, asthma and neurodegenerative diseases like Alzheimer's, Parkinson's and schizophrenia. Even though reports have confirmed the role of shark liver oils in lowering the

incidence of these diseases, the exact mechanisms involved in these disorders are yet to be explored.

Higher concentrations of AKGs in shark liver oils are now considered to be responsible for their high immune-boosting ability (Pugliese *et al.* 1998). These AKGs are essentially a class of lipids with an ether linkage and a glycerol backbone. In addition, shark liver oils also contain antioxidant vitamins and squalamine (Brunel *et al.* 2005), a substance which has shown a promising behavior toward fighting cancers of the breast, lung, brain and skin (melanoma specifically) by choking off the tumor's blood supply. The pharmaceutical values associated with shark liver oils are abundant; they form the active ingredients of many different formulations ranging from vitamin supplements to skin-based ointments and creams (Neil *et al.* 2006).

The aim of the present study was to evaluate the analgesic and anti-inflammatory effects of four different liver oils of sharks belonging to the Indian EEZ and to identify the components of the oil that is responsible for these activities. The analgesic and anti-inflammatory activities of liver oils from *Neoharriotta raleighana* (NR), *Centrosymnus crepidater* (CC), *Apristurus indicus* (AI) and *Centrophorus sculpratus* (CS) sharks caught from the Arabian Sea and the Indian Ocean were compared. As the information available with regard to these properties is relatively scanty, an attempt has been made to explore their ability as therapeutic agents.

MATERIALS AND METHODS

Materials

The shark species were caught beyond 600 m depth during Cruises 250 and 252 on the Fishery and Oceanographic Research Vessel Sagar Sampada from the southwest and eastern coasts of India. Expo model trawl nets were used to catch these deep sea fish species and they were immediately frozen at −20C onboard the vessel, and brought to the laboratory for further analyses. The catch details of the four different shark species, whose liver oils were analyzed for their analgesic and anti-inflammatory potentials, are shown in Table 1. All chemicals used were obtained from Merck (Darmstadt, Germany). Paracetamol and ibuprofen, the standard reference drugs used for the animal experiments, were purchased from Sigma-Aldrich Chemical Inc. (St. Louis, MO).

Methods

Oil Extraction. The livers of each of these sharks were excised and weighed separately. Lipid extraction was achieved by following the method of

TABLE 1.
DETAILS OF SHARK SPECIES COLLECTED SHOWING THEIR COMMON NAMES, LOCATION (LATITUDES AND LONGITUDES) AND DEPTH

S.No.	Scientific name (abbrev.)	Common name	Region	Lat (°N)	Long (°E)	Depth (m)
1	*Apristurus indicus* (AI)	Small-belly catfish	Azhikkal	12°04′	74°16′	735
2	*Centrophorus sculpratus* (CS)	Endeavour dogfish	Diglipur	13°21′	93°07′	695
3	*Centrosymnus crepidater* (CC)	Deep sea dogfish	Kasargode	12°25′	74°07′	740
4	*Neoharriotta raleighana* (NR)	Long-nosed ratfish	Alleppey	09°17′	75°38′	624

Folch *et al.* (1957). Briefly, minced liver was homogenized in a 2:1 (v/v) mixture of chloroform-methanol and filtered. Then, 20% water was added to this mixture and the layers were allowed to separate. The aqueous layer was discarded and the solvent was completely evaporated to obtain the oil. The oils were stored in amber-colored bottles under nitrogen at –60C. A portion of the oil was saponified (Hallgren and Larsson 1962) in a mixture of 150% potassium hydroxide (w/v) and absolute ethanol for 2 h in a water bath at 75C under nitrogen. The resulting mixture was extracted with ether, water-washed, dried over anhydrous sodium sulfate and finally condensed to a known volume. A small portion of the ether layer was air-dried to estimate the fraction of the unsaponifiable matter (USM) present in the oils.

Lipid Components. Aliquots of the ether extract or the diluted crude liver oil were analyzed using an Iatroscan MK-6s (M/s. Mitsubishi Kagaku Iatron Inc., Tokyo, Japan) to determine the abundances of individual lipid classes (HCs, alkoxyglycerols, triacylglycerols, fatty acids [FAs]; Bakes and Nichols 1995). Samples were applied in triplicate to silica gel SIII chromarods (5 μm particle size) using 1-μL disposable micropipettes. Chromarods were developed in a glass tank lined with pre-extracted filter paper. A nonpolar solvent system of hexane-diethyl ether (60:15, v/v) was used to resolve the lipid components. After development, the chromarods were oven-dried and analyzed immediately to minimize adsorption of atmospheric contaminants. The flame ionization detector (FID) was calibrated for each class of compounds (squalene, monopalmitoyl-rac-glycerol, oleic acid, tripalmitin). The peaks obtained via Chromatocorder were quantified and tabulated.

FA Methyl Esters. Aliquots of the ether extract were methylated using BF_3-methanol and the resulting FA methyl esters (Bakes and Nichols 1995) were subjected to gas chromatography (M/s. Thermo Electron Corporation, Milan, Italy) equipped with Perkin Elmer Elite 225 (Perkin Elmer Life and Analytical Services, Watham, MA) 50% cyanopropyl phenyl – 50% methyl

capillary column (30 m × 0.25 mm inner diameter) FID and a split/splitless injector. Nitrogen was the carrier gas. Samples were injected in splitless mode at an oven temperature of 110C. After 4 min, the oven temperature was raised to 240C at 2.7C/min. Peaks were quantified with Chromcard software by comparing retention time data with those obtained for authentic standards.

Experimental Animals. Wistar strain male albino rats (120–200 g) and mice (35–40 g) were used in the experiments. They were housed individually in polypropylene cages under hygienic conditions and were provided food and water *ad libitum*. The animals were maintained on a 12:12-h light : dark photoperiod under standard conditions of temperature and ventilation. The experiments were performed as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India, and with the approval of the Institutional Animal Ethics Committee.

Experimental Protocol. The analgesic and anti-inflammatory activities of the shark liver oils were assessed after checking their lethal dosages in rats and mice as animal models. Analgesic activity was determined using the acetic acid-induced writhing and hot-plate tests, while the anti-inflammatory activity was determined using the formalin-induced rat-paw edema test.

Toxicity Studies. Acute toxicity of the four shark liver oils was carried out on male albino rats and mice using Karber's arithmetical method for the determination of LD_{50} (lethal dose that causes mortality by 50%) (Turner 1965a). In this assay, increasing doses of the test substance, oil along with the vehicle dimethylsulfoxide (DMSO) (oil : DMSO, 4:1), were administered orally to groups of four animals at different doses (1.0–7.0 g/kg). The animals were observed for 1 week, the number of survivors was counted and the optimum average dosage was determined.

Acetic Acid-Induced Writhing Assay. In vivo determination of antinociceptive activity was carried out using the abdominal constriction test (Koster *et al.* 1959). The mice were divided into six groups of five animals each after initial screening. All shark liver oils were administered orally (50 mg/0.2 mL/animal) as a suspension in DMSO (oil : DMSO, 4:1) 1 h prior to the intraperitoneal administration of acetic acid (0.6% v/v). Ten minutes after the administration, the number of constrictions per animal was recorded at 20 min. Control animals received an equal volume of vehicle. The standard reference drug paracetamol was administered at 100 mg/kg body weight. Antinociceptive activity was reported as percent inhibition of constrictions compared with the placebo control group.

Hot-Plate Activity. The analgesic activity was investigated in male albino mice using the hot-plate test (Turner 1965b). The mice were divided into six groups of five animals each after initial screening. Each of the shark liver oils was administered orally at a dose of 50 mg/0.2 mL/animal as a suspension in DMSO (oil : DMSO, 4:1). Control animals received an equal volume of the vehicle. One group received the reference drug paracetamol at 100 mg/kg body weight. The animals were dropped gently on the hot-plate maintained at 53 ± 1C; this was done 5 min prior to the administration of the vehicle, oils and paracetamol, and at 30, 60, 90, 120, 150 min following administration. The time between placement and the first sign of paw licking or jumping was recorded as latency. The basal latencies were 6–10 s. A cut-off time of 30 min was established to prevent injury to the paws. The mean values were recorded.

Anti-Inflammatory Activity. The anti-inflammatory activity was determined, by the method of Hunskaar and Hole (1987), using the formalin-induced rat-paw edema test. Male albino rats weighing between 160–180 g were divided into five groups of six animals each. The oil along with the vehicle DMSO (oil : DMSO, 4:1) was administered orally at a dose of 1.5 g/kg body weight prior to the induction of inflammation by the subcutaneous injection of 0.1 mL sterile saline solution of 3.5% formalin in the right hind paw. The control group received sterile saline solution (1 mL 0.9% NaCl solution), while the reference drug ibuprofen at a dose of 100 mg/kg body weight was administered intraperitoneally to the standard group at least 30 min before the induction of edema. Paw sizes were measured with a calibrated screw gauge before the administration of formalin, then thereafter at 1, 2, 3 and 4 h after the injection of the inflammatory agent. The average size of the paw measured in millimeters was calculated from three measurements which did not differ by more than 1%. These individual measurements allowed us to determine the average paw size for each group (s_m) and then the percentage of edema by comparison with the average size obtained for each group before any treatment (s_o).

Percentages of inflammation-inhibition were obtained for each group using the following calculation:

$$\frac{[(s_m - s_o)\text{control} - (s_m - s_o)\text{treated}]}{(s_m - s_o)\text{control}} \times 100 \qquad (1)$$

where s_m is the mean paw size for each group after formalin treatment and s_o is the mean paw size obtained for each group before the treatment (Owolabi and Omogbai 2007).

Statistical Analyses. All data were expressed as mean ± SD and analyzed statistically by one-way analysis of variance using Duncan's test with a level of significance set at $P < 0.05$. The statistical software, SPSS for Windows version 16 (SPSS Inc., Chicago, IL), was employed for the analyses. Pearson's correlation test was performed between the various lipid components analyzed and the inflammation-inhibitory values to determine the factor(s) responsible for the observed pharmaceutical effects. A positive correlation was established when the level of significance was set at $P < 0.05$ or $P < 0.01$ as the case may be.

RESULTS

Acute Toxicity of the Oils

In the acute toxicity trial, no mortality was observed for doses (oral administrations) of up to 3.8 g/kg body weight for either mice or rats. No significant changes in the body weight were observed at this dose. However, 50% mortality was observed for both rats and mice at a dose of 6.2 g/kg body weight after 1 week. Based on these observations, the oils were administered at an optimum average dose of 1.5 g/kg body weight.

Lipid Composition of Oils

The lipid composition of oils extracted from the liver of four species of deep sea sharks found in southern Indian waters was determined. The oils of NR, CS and CC recorded high USM content of 78, 73 and 60%, respectively (Table 2). However, AI oil had the lowest content of USM (25%) among

TABLE 2.
LIPID COMPOSITION OF SHARK LIVER OILS (VALUES ARE MEAN ± SD)

Species (abbrev.)	Lipid (% tissue)	NSM (% lipid)	HC (% lipid)	AKG (% lipid)	FA (% lipid)
Apristurus indicus (AI)	70.58 ± 1.87	25.29 ± 5.71	20.11 ± 2.31	12.20 ± 5.51	68.83 ± 7.22
Centrophorus sculpratus (CS)	79.46 ± 2.27	73.62 ± 9.52	67.42 ± 5.84	18.52 ± 7.31	24.20 ± 6.67
Centrosymnus crepidater (CC)	77.43 ± 2.85	60.71 ± 8.69	52.76 ± 7.34	20.45 ± 6.77	28.21 ± 5.24
Neoharriotta raleighana (NR)	69.28 ± 1.33	78.01 ± 5.43	62.43 ± 5.29	21.13 ± 5.24	22.01 ± 4.85

NSM, nonsaponifiable matter; HC, hydrocarbons; AKG, alkylglycerols; FA, fatty acid. SD, standard deviation.

the four oils examined. AKGs and hydrocarbons (HCs), predominantly the isoprenoid squalene, were the major components of the USM. HC content varied significantly among the species analyzed. Oils of AI species recorded the lowest amount of squalene at 20.1%, while that of CS was 67.4%. Oils of CC and NR species contained 52 and 62% squalene, respectively. AKGs comprising both mono- and di-alkoxyglycerols were present in all shark species at levels between 12.2 and 21.1%. Polar lipids were either present in low abundance (<2%) or were not detected in the extracted oils.

The FA and total FA content of the four species of shark are given in Table 3. The total FAs ranged from 22 to 28% of the total lipid in NR, CS and CC species, whereas it was as high as 68% in the liver oils of AI species. The predominant FAs in all species were the monounsaturates (MUFA) ranging from 55 to 67% with 18:1(n-9), 20:1 and 22:1 being the major FAs. PUFA levels varied from 10 to 22% of the total FA content in oils.

Analgesic Activity of Oils

All the four shark liver oils (AI, CC, CS and NR) exhibited a highly significant ($P < 0.05$) analgesic activity when compared with the control group. Table 4 illustrates the antinociceptive activity of the four shark liver oils, in the acetic acid-induced constrictions in mice, when administered at 1.5 g/kg body weight. The analgesic behavior of AI, CC oils and that of the standard drug paracetamol were significantly different ($P < 0.05$) from each other and from CS and NR oils. Even though there was no significant difference in activity between CS and NR oils, they differed significantly ($P < 0.05$) from AI, CC oils and paracetamol. While mice treated with AI oil showed only a 29.5% inhibition, those treated with CC, CS and NR oils showed 52.7, 59.0 and 57.8% inhibitions, respectively ($P < 0.05$). Paracetamol at 100 mg/kg body weight showed 47.2% inhibition.

The analgesic activity for all the four oils in the hot-plate test, i.e., in the latency versus time test, was similar. However, only NR and CS oils exhibited an analgesic behavior (Fig. 1) similar to that of the standard drug paracetamol (latency time of 8.3 ± 0.5 s). NR, CS and CC oils showed a significant amount of ($P < 0.05$) analgesic activity from the 60th to 90th minute. While mice treated with AI oil showed significant ($P < 0.05$) analgesic behavior up to 90 min of its administration, its effect started to decline afterwards. The latency time for control mice was 2.6 ± 0.5 s, while NR, CS, CC and AI rats showed 9.3 ± 0.0, 8.6 ± 0.5, 8.0 ± 0.0 and 6.3 ± 0.5 s, respectively, from the 60th to 90th minute of the treatment. Of the four oils analyzed, only the animals treated with AI oil showed the least antinociceptive activity.

TABLE 3.
TOTAL FATTY ACID COMPOSITION OF LIVER OILS FROM DEEP-SEA SHARKS COLLECTED FROM INDIAN WATERS*

Fatty acid	Percentage composition (as % total fatty acid)			
	Apristurus indicus	*Centrosymnus crepidater*	*Centrophorus sculpratus*	*Neohariotta raleighana*
14:0	0.65	1.17	0.21	1.72
14:1	0.82	0.16	1.30	0.30
15:0	1.00	1.00	0.06	1.98
15:1	0.70	0.11	0.54	0.10
16:0	14.15	12.43	10.82	12.36
16:1	2.89	4.00	3.34	4.71
17:0	0.15	0.35	0.16	0.30
17:1	2.93	4.98	3.82	3.10
18:0	9.79	5.65	4.17	3.60
18:1(n-9)	16.34	24.11	33.43	27.68
18:2(n-6)	1.39	ND	1.68	2.46
20:0	0.21	0.47	0.31	0.40
20:1	23.85	15.99	15.87	11.35
20:3(n-3)	7.15	5.82	8.75	5.20
20:5(n-3)	0.62	0.88	1.87	4.53
22:0	0.32	0.37	0.28	0.67
22:1	10.17	14.01	7.46	6.22
22:6(n-3)	3.88	4.08	3.10	10.03
24:1	1.50	2.42	1.83	2.40
Others	1.50	1.99	1.01	0.88
Total	100.00	100.00	100.00	100.00
Total saturates	26.26	21.43	18.01	21.03
Total monounsaturates	59.20	65.79	68.59	49.96
Total polyunsaturates	13.04	10.79	15.40	22.22
Total fatty acid (mg/g)	687.82	282.71	242.34	220.10

* All values are expressed as percentage of the total fatty acids unless otherwise stated. gas chromatography results are subject to an error of ±1%.
ND, not detected.

Anti-Inflammatory Activity of Oils

The percentage inhibition in the formalin-induced rat-paw edema is shown in Table 5. In the formalin-induced inflammation, the oils showed a peak inhibition of inflammation at the third hour. Significant ($P < 0.05$) inhibition of inflammation was shown by the standard drug ibuprofen and the four shark liver oils within 1 hour from the onset of inflammation. Ibuprofen showed a maximum inhibitory activity at the second hour (64.5%). CS and NR oils showed 65.0% reduction in edema at the third hour while AI oil was able to reduce it by only 54.2%. At the fourth hour, while all the oils as well as the

TABLE 4.
THE EFFECT OF SHARK LIVER OILS AND PARACETAMOL (STANDARD REFERENCE DRUG) ON ACETIC ACID-INDUCED WRITHING TEST IN MICE

Treatment	Number of writhes	Inhibition (%)
Control	84.6 ± 1.52^{a}	–
Paracetamol	44.6 ± 3.05^{b}	47.27
AI	59.6 ± 2.08^{c}	29.51
CC	40.0 ± 2.00^{d}	52.75
CS	34.6 ± 2.08^{e}	59.05
NR	35.6 ± 3.05^{e}	57.83

Inhibition is reported as percent with respect to control. Oils of *Apristurus indicus* (AI), *Centrosymnus crepidater* (CC), *Centrophorus sculpratus* (CS) and *Neohariotta raleighana* (NR) were administered orally at 1.5 g/kg and paracetamol at 100 mg/kg animal weight. Values are mean number of writhes ± standard deviation ($n = 5$ per group).

^{a,b,c,d} Number of writhes with different superscripts are significantly different ($P < 0.05$).

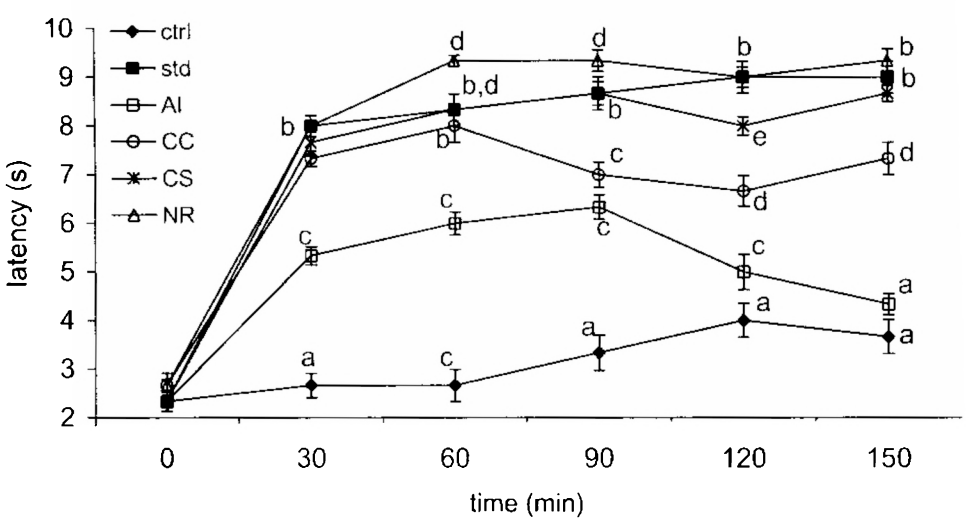

FIG. 1. EFFECT OF SHARK LIVER OILS ON THE HOT-PLATE REACTION TIME VERSUS THE BASAL LATENCY(S). OILS OF *APRISTURUS INDICUS* (AI), *CENTROSYMNUS CREPIDATER* (CC), *CENTROPHORUS SCALPRATUS* (CS) AND *NEOHARIOTTA RALEIGHANA* (NR) WERE ADMINISTERED ORALLY AT 1.5 g/kg ANIMAL; PARACETAMOL AT 100 mg/kg ANIMAL. DATA ARE EXPRESSED AS MEAN LATENCY ± SD VALUES

Latency values with different superscripts are significantly different ($P < 0.05$). Ctrl, control; std, standard; SD, standard deviation.

TABLE 5.
INHIBITORY EFFECTS OF SHARK LIVER OILS ON THE FORMALIN-INDUCED RAT-PAW EDEMA

Percentage inhibition				
Treatment	1 h	2 h	3 h	4 h
Ibuprofen	62.94 ± 0.75^{a}	64.58 ± 1.09^{a}	51.29 ± 3.18^{a}	55.63 ± 1.86^{a}
AI	20.97 ± 2.11^{b}	27.95 ± 0.65^{b}	54.23 ± 3.55^{a}	39.42 ± 0.94^{b}
CC	50.20 ± 1.49^{c}	58.30 ± 1.15^{c}	63.18 ± 1.40^{b}	61.17 ± 0.75^{c}
CS	56.18 ± 1.62^{d}	60.31 ± 0.60^{d}	65.72 ± 0.56^{b}	69.51 ± 0.78^{d}
NR	46.12 ± 0.49^{e}	58.11 ± 0.43^{c}	65.02 ± 5.81^{b}	48.68 ± 3.89^{e}

Values are mean percentage inhibition ± standard deviation ($n = 6$ per group). Oils of *Apristurus indicus* (AI), *Centrosymnus crepidater* (CC), *Centrophorus sculpratus* (CS) and *Neohariotta raleighana* (NR) were administered orally at 1.5 g/kg and ibuprofen at 100 mg/kg animal weight.

[a,b,c,d,e] For each hour, percentage inhibition values with different superscripts are significantly different ($P < 0.05$).

standard paracetamol started to show a decline in the inhibitory activity, only CS oil showed a peak inhibitory activity of 69.5% ($P < 0.05$).

DISCUSSION

In the preliminary toxicity studies, 50% mortality was observed at a dosage of 6.2 g/kg body weight. The oils were administered at a safer level of 1.5 g/kg body weight per animal. These oils did not cause any unwanted side effects in the experimental animals, indicating that they were safe for consumption.

Inhibition of acetic acid-induced writhing in mice suggested that the analgesic effect of the oils may be mediated via inhibition of the synthesis and release of prostaglandins (Koster *et al.* 1959). Writhes can be described as a wave of constriction and elongation passing caudally along the abdominal wall with twisting of the trunk and extension of the hind limb in mice (Surender and Mafumdar 1995). The results obtained here showed that the oils at 1.5 g/kg body weight had a higher rate of inhibition than the standard drug paracetamol. Of the four oils analyzed, those belonging to CS, NR and CC species had a better analgesic effect (59, 57 and 52%, respectively) than the standard drug paracetamol (47%) within 90 min from the onset of pain. The hot-plate test also confirmed our findings that the antinociceptive ability of the oils of NR, CS and CC were better off than AI oil.

In the anti-inflammatory study of shark liver oils, a significant inhibition of inflammation began from the second hour, peaked at the third hour and started declining from the forth hour onwards. This is in accordance with a

previously reported study that induction of inflammation involves three distinct phases of release of inflammatory mediators (Surender and Mafumdar 1995). The first phase being the release of histamine and serotonin lasting from the first to the second hour; the second phase being the release of kinins lasting from the second to the third hour while the third phase being the release of prostaglandins and lasting from the third to the fifth hour (Surender and Mafumdar 1995). Thus, it can be inferred that the mechanism through which the oils of CS, NR and CC elicited its effects might be through the inhibition of the synthesis of kinins and prostaglandins, as the oils had been effective at these phases of mediator release. AI oil showed the least inflammation inhibitory effect.

Inflammation is a normal part of the body's immune response to infection or injury. Activated white blood cells secrete a variety of inflammation-promoting compounds or rather inflammatory mediators including cytokines like interleukin-6 and C-reactive proteins (CRP), free radicals and eicosanoids like prostaglandins and leucotrienes to fight germs and to dispose off damaged cells (Ridker *et al.* 2000). It can be assumed that the mechanism of action of liver oils in the present study is via the inhibition of the synthesis of kinins and prostaglandins and this might be through the action on cyclooxygenase (COX) enzyme. COX-2 is responsible for the biosynthesis of prostaglandins under acute inflammatory conditions (Nantel *et al.* 1999). This inducible COX is believed to be the target enzyme for the anti-inflammatory activity of nonsteroidal anti-inflammatory drugs (NSAIDs; Lau *et al.* 1993) which reduce the level of inflammatory mediators and alleviate the pain in the body.

The behavior of shark liver oils from CS, NR, CC and AI species in the descending order of anti-inflammatory activity was 69, 65, 63 and 54%, respectively, as was similar to the standard drug ibuprofen (64%) in the formalin-induced rat-paw edema test, thus confirming their antinociceptive profile and that their action might be similar to that of NSAIDs. Earlier reports have shown that NSAIDs inhibit the activity of COXs (Lau *et al.* 1993) and lower the levels of myeloperoxidase (Faurschou and Borregaard 2003) in the tissues and that they attenuate the pain response in the second phase but not in the first phase of the formalin test in rats.

Antinociceptive and anti-inflammatory components present in the shark liver oils are yet to be explored. Shark liver oils comprise mainly 1-O-alkylglycerols, which constitute about 10–30% of the unsaponifiable matter of the oils (Hallgren and Larsson 1962). These AKGs are indeed responsible for reducing pain or inflammation in the body (Pedrono *et al.* 2004). The exact mechanism by which they function has not been fully understood but it has been proposed that they work by either inhibiting the synthesis, release or action of inflammatory mediators, namely histamine, serotonin and prosta-

glandins that might be involved in inflammation. It has been reported that naturally occurring AKGs have potent biological activities on various cells or systems (Devaraj and Jialal 2000). Shark liver oils also contain high proportions of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (James *et al.* 2003). It has been shown that these long-chain n-3 PUFAs (James *et al.* 2003) lower the incidence of inflammatory diseases such as asthma and arthritis (Shahidi and Senanayake 2006). These dietary FAs are known to reduce the levels of arachidonic acid metabolites and lower the formation of pro-inflammatory compounds, like prostaglandins and leukotrienes, by blocking their activity (Olivera *et al.* 2004). Early studies reviewed by Stamp *et al.* (2005) and Calder (2006) attributed the anti-inflammatory effects of fish oils to competition with arachidonic acid for production of inflammatory eicosanoids. Anti-inflammatory effects of EPA and DHA have been studied by several workers (Arita *et al.* 2005; Lukiw *et al.* 2005; Hudert *et al.* 2006). EPA and DHA contained in fish oils provide nutrients needed to build anti-inflammatory prostaglandin series 1 and 3 (Simopoulos 1991). In addition, shark liver oils are rich in antioxidants like vitamin E (Devaraj and Jialal 2000), which reduce inflammation by decreasing CRP levels and by blocking the activity of tumor necrosis factor-alpha series 2-prostaglandins and COXs (James *et al.* 2003). Antioxidants are well-known to alleviate the inflammatory processes mediated by allergic substances. They also curb inflammation by quenching hazardous molecules called free radicals, which stimulate inflammation (Vittala and Newhouse 2004). Sharks inhabiting waters beyond 600 m depth are believed to possess reasonably high content of the HC squalene (Ko *et al.* 2002), yet another antioxidant with potent pharmaceutical values. Its role as an antilipidemic agent (Qureshi *et al.* 1996) and membrane stabilizer has been reported (Sabeena *et al.* 2004).

Pearson's correlation test was used to determine the influence of the HC squalene and AKGs upon anti-inflammation (Table 6). Significant correlations were observed between nonsaponifiable matter (NSM) and anti-inflammation for all the four oils used in the study ($P < 0.01$ for CS, CC and AI, $P < 0.05$ for NR). HC and AKG contributed significantly toward anti-inflammation in CS, NR and AI oils, whereas it was the AKGs which were responsible for the observed anti-inflammatory activity in CC oils ($P < 0.01$). Significant correlations were also observed between HC squalene and antinociception (Table 6): $P < 0.01$ for oils of CC, NR and AI species, $P < 0.05$ for CS species. AKG component of NSM significantly influenced analgesic responses in oils of CC, NR and CS species ($P < 0.05$). Pearson's correlation test proved a significant correlation on the influence of FAs upon anti-inflammation ($P < 0.05$). Saturated FAs and MUFAs showed a negative correlation coefficient with anti-inflammation/antinociception which meant that as their levels increased the anti-inflammatory responses decreased. Positive correlations

TABLE 6.
PEARSON'S CORRELATION COEFFICIENT TEST TO DETERMINE THE INFLUENCE OF VARIOUS LIPID COMPONENTS UPON ANTI-INFLAMMATION AND ANTINOCICEPTION

Oils		Lipid components						
		NSM	HC	AKG	FA	SatFA	MUFA	PUFA
AI	P. corr[a]	1.000†	1.000†	1.000†	1.000*	−0.777	−0.756	0.982
	P. corr[b]	1.000†	0.499	1.000†	1.000*	−0.997	−0.372	0.541
CC	P. corr[a]	1.000†	0.492	1.000†	1.000*	−0.996	−0.365	0.548
	P. corr[b]	1.000*	1.000†	1.000†	1.000†	−0.783	0.849	0.408
CS	P. corr[a]	1.000†	1.000†	1.000†	1.000*	−0.777	−0.756	0.982
	P. corr[b]	0.999*	1.000*	0.497	1.000†	0.406	−0.282	0.596
NR	P. corr[a]	1.000*	1.000†	1.000†	1.000*	−0.780	0.851	0.404
	P. corr[b]	1.000†	0.499	1.000†	1.000*	−0.997	−0.372	0.541

* Correlation is significant at the 0.05 level (2-tailed).
† Correlation is significant at the 0.01 level (2-tailed).
P.corr[a], Pearson's correlation coefficient with respect to anti-inflammation.
P.corr[b], Pearson's correlation coefficient with respect to antinociception.
NSM, nonsaponifiable matter; HC, hydrocarbons; AKG, alkylglycerols; FA, fatty acid; SatFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

were observed between levels of PUFA and anti-inflammation, thus confirming their roles in lowering inflammation (Table 6). The cumulative effects of the various lipid components (squalene, AKGs and PUFAs) studied are indeed responsible for the observed anti-inflammatory and antinociceptive effects of the extracted shark liver oils (Ko *et al.* 2002; Pedrono *et al.* 2004; Arita *et al.* 2005).

The results of the present investigation indicated that shark liver oils belonging to CS, NR and CC species showed a better analgesic and anti-inflammatory profile than that of AI oil. We propose that the high NSM content to be responsible for the observed findings. The liver oils of NR, CS and CC contained a high fraction of NSM (average 70%); the AKGs, HCs and vitamin E could play a major role in lowering the incidence of inflammatory diseases by blocking the activity of prostaglandins and leukotrienes. AI oil recorded the lowest fraction of NSM (25%) and hence exhibited lower effects.

In conclusion, liver oils extracted from the sharks, namely CS, NR, CC and AI, possessed excellent anti-inflammatory and peripheral antinociceptive effects, contributing to its use in the treatment of arthritis and other inflammatory disorders. AKGs, long-chain PUFAs, vitamin E and squalene present in shark liver oils play a major role in reducing the level of inflammatory mediators during an inflammation. The bioactive potentials of marine lipids from creatures inhabiting the Indian EEZ and the ability of these oils to interfere with the inflammatory mediators deserve further investigation.