Bioactivity Profile of Polyunsaturated Fattyacid extracts from *Sardinella longiceps and Sardinella fimbriata* - A Comparative Study

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By

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November – 2010

**********Dedicated to

My beloved son Tejas Praveen

****

Declaration

I hereby do declare that the thesis entitled " Bioactivity Profile of Polyunsaturated Fattyacid extracts from *Sardinella longiceps* and *Sardinella fimbriata* – A comparative study" is a genuine record of research work done by me under the guidance of Dr. C.K. Radhakrishnan, Professor Emeritus, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology and that no part thereof has been presented for the award of any degree, diploma, associateship, or other similar title of any University or Institution.

Kochi – 16 **CHITRA SOM. R.S**
November 2010

Certificate

I hereby declare that the thesis entitled ***"Bioactivity Profile of Polyunsaturated Fattyacid extracts from Sardinella longiceps and Sardinella fimbriata – A comparative study"*** *is an authentic record of research work carried out by Ms. Chitra Som. R.S under my supervision in the School of Marine Sciences, Cochin University of Science and Technology in partial fulfilment of the requirements for the degree of Doctor of Philosophy and that no part thereof has been presented for the award of any degree, diploma or associateship in any University.*

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Abstract

The oceans have proved to be an interminable source of new and effective drugs. Innumerable studies have proved that specific compounds isolated from marine organisms have great nutritional and pharmaceutical value. Polyunsaturated fattyacids (PUFA) in general are known for their dietary benefits in preventing and curing several critical ailments including Coronary heart disease (CHD) and cancers of various kinds. Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) are two PUFA which are entirely marine in origin – and small Clupeoid fishes like sardines are known to be excellent sources of these two compounds. In this study, we selected two widely available Sardine species in the west coast, Sardinella longiceps and Sardinella fimbriata, for a comparative analysis of their bioactive properties. Both these sardines are known to be rich in EPA and DHA, however considerable seasonal variation in its PUFA content was expected and these variations studied. An extraction procedure to isolate PUFA at high purity levels was identified and the extracts obtained thus were studied for anti-bacterial, anti-diabetic and anti-cancerous properties.

Samples of both the sardines were collected from landing centre, measured and their gut content analysed in four different months of the year – viz. June, September, December and March. The fish samples were analyzed for fattyacid using FAME method using gas chromatography to identify the full range of fattyacids and their respective concentration in each of the samples. The fattyacids were expressed in mg/g meat and later converted to percentage values against total fatty acids and total PUFA content. Fattyacids during winter season (Dec-Mar) were found to be generally higher than spawning season (June-Sept). PUFA dominated the

profiles of both species and average PUFA content was also higher during winter. However, it was found that S. longiceps had proportionately higher EPA as compared to S. fimbriata which was DHA rich. Percentage of EPA and DHA also varied across months for both species – the spawning season seemed to show higher EPA content in S. longiceps and higher DHA content in S. fimbriata. Gut content analysis indicate that adult S. fimbriata is partial to zooplanktons which are DHA rich while adult S. longiceps feed mainly on EPA rich phytoplankton. Juveniles of both species, found mainly in winter, had a gut content showing more mixed diet. This difference in the feeding pattern reflect clearly in their PUFA profile – adult S. longiceps, which dominate the catch during the spawn season, feeding mostly on phytoplankton is concentrated with EPA while the juveniles which are found mostly in the winter season has slightly less EPA proportion as compared to adults. The same is true for S. fimbriata adults that are caught mostly in the spawning season; being rich in DHA as they feed mainly on zooplankton while the juveniles caught during winter season has a relatively lower concentration of DHA in their total PUFA.

Various extraction procedures are known to obtain PUFA from fish oil. However, most of them do not give high purity and do not use materials indicated as safe. PUFA extracts have to be edible and should not have harmful substances for applying on mice and human subjects. Some PUFA extraction procedures, though pure and non-toxic, might induce cis-trans conversions during the extraction process. This conversion destroys the benefits of PUFA and at times is harmful to human body. A method free from these limitations has been standardized for this study. Gas Chromatography was performed on the extracts thus made to ensure that it is substantially

pure. EPA: DHA ratios for both samples were derived - for S. longiceps this ratio was 3:2, while it was 3:8 for S. fimbriata.

Eight common strains of gram positive and gram negative bacterial strains were subjected to the PUFA extracts from both species dissolved in acetone solution using Agar Well Diffusion method. The activity was studied against an acetone control. At the end of incubation period, zones of inhibition were measured to estimate the activity. Minimum inhibitory concentration for each of the active combinations was calculated by keeping $p < 0.01$ as significant. Four of the bacteria including multi-resistant Staphylococcus aureus were shown to be inhibited by the fish extracts. It was also found that the extracts from S. fimbriata were better than the one from S. longiceps in annihilating harmful bacteria.

Four groups of mice subjects were studied to evaluate the anti-diabetic properties of the PUFA extracts. Three groups were induced diabetes by administration of alloxan tetra hydrate. One group without diabetes was kept as control and another with diabetes was kept as diabetic control. For two diabetic groups, a prescribed amount of fish extracts were fed from each of the extracts. The biochemical parameters like serum glucose, total cholesterol, LDL & HDL cholesterol, triglycerides, urea and creatinine were sampled from all four groups at regular intervals of 7 days for a period of 28 days. It was found that groups fed with fish extracts had marked improvement in the levels of total LDL & HDL cholesterol, triglycerides and creatinine. Groups fed with extracts from S. fimbriata seem to have fared better as compared to S. longiceps. However, both groups did not show any marked improvement in blood glucose levels or levels of urea.

Cell lines of MCF-7 (Breast Cancer) and DU-145 (Prostate Cancer) were used to analyse the cytotoxicity of the PUFA extracts. Both cell lines were subjected to MTT Assay and later the plates were read using an ELISA reader at a wavelength of 570nm. It was found that both extracts had significant cytotoxic effects against both cell lines and a peak cytotoxicity of 85-90% was apparent. IC_{50} values were calculated from the graphs and it was found that S. longiceps extracts had a slightly lower IC_{50} value indicating that it is toxic even at a lower concentration as compared to extracts from S. fimbriata.

This study summarizes the bioactivity profile of PUFA extracts and provides recommendation for dietary intake; fish based nutritional industry and indigenous pharmaceutical industry. Possible future directions of this study are also elaborated.

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ABBREVIATIONS

Acronym	Expansion
AA	Arachidonic Acid
ALA	Alpha-Linolenic Acid
AOAC	Association of Analytical Communities - known as AOAC International now
AOE	Anti-Oxidant Enzyme
CHD	Coronary Heart Disease
COX	Cyclooxygenase. An enzyme in human body
CPT-11	Irinotecan. A cytotoxic quinoline alkaloid used in treatment of cancer
CVD	Cardio-Vascular Disease
DHA	DocosaHexaenoic Acid
DISC	Death-Inducing Signalling Complex
DMEM	Dulbecco's Modified Eagle Medium
DU-145	A human prostate cancer cell line
EFA	Essential Fatty Acid
EPA	EicosaPentaeonic Acid
FA	FattyAcid
FAME	Fatty Acid Methyl Ether - known as FAME Method
GC	Gas Chromatography
HDL	High-density Lipoprotein - known as HDL Cholesterol
hK2	Hexokinase 2. An enzyme in human body
IC50	Half maximal inhibitory concentration
LDL	Low-density Lipoprotein - known as LDL Cholesterol
LOX	Lipooxygenase. An enzyme in human body
MCF-7	A human breast cancer cell line
MDA-MB	A set of human breast cancer cell lines
MHA	Mueller Hinton Agar – known as MHA medium
MIC	Minimum Inhibitory Concentration
MRA	Magnetic Resonance Angiogram

MRSA	Methicillin-Resistant Staphylococcus aureus
MTT	3- (4, 5- dimethylthiazol-2yl)-2, 5- diphenyltetrazolium bromide)
MUFA	Mono-Unsaturated FattyAcid
MX-1	A human breast cancer cell line
NSAID	Non-Steroidal Anti-Inflammatory Drugs
PSA	Prostate Specific Antigen
PUFA	Poly-Unsaturated FattyAcid
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
SFA	Saturated FattyAcid

Chapter 1
Introduction

Contents

The issues that plague world health have swerved with the times. New pharmaceuticals drugs, research into the lifecycle of pathogens, disease prevention and vaccination have helped eradicate major health risks posed by infections, and many diseases are believed to be eradicated. The report of World Heath Organisation (WHO) on global patterns of health risks renders plenty of evidence that the major health risks we face today do not lie with any particular infection but are the consequences of an unhealthy lifestyle and perverted eating habits.

As stated in the report of WHO, the top five contributors to world's health problems are high blood pressure, tobacco use, high blood glucose, physical inactivity, overweight and obesity. Next up on the list is high cholesterol. These factors are responsible for raising the risk of chronic diseases, such as heart disease and cancers. They affect countries across all income groups: high, middle and low. Internationally, health experts are now asserting the importance of a healthy lifestyle that involves a conscientious diet and meticulous exercise regimen. 'Prevention is better than cure' as the saying goes.

The food that traditionally forms the staple of the local diet goes far in ensuring the health of a population - preventing the onslaught of the said maladies, and exerting a positive influence where they have made their ominous presence already felt. It is no secret that the longevity and wellness enjoyed by the Japanese, particularly Okinawans, are a result of their centuries-old dietary habits which include plenty of seaweed and fish. On an average, a Japan national consumes half a pound of fish each day. Japanese women also command the lowest rate of obesity in modern cultures.

Another popular model that illustrates the extent to which diet influences the health of a popular is the diet of Alaskan Eskimos. These communities that survive in some of the most strenuous climates on the planet and eat a diet that is nearly 70% fats still manage to astonish their western meat-eating counterparts with an extremely low incidence of cardiac illnesses and also joint and skin diseases. Research reveals that their diet includes lot of fish, rich in the two major ω-3 Fatty Acids - Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA).

The role of these essential fatty acids in promoting cardiac health stems from the fact that they can improve the performance of heart and bring down cholesterol levels and blood pressure. And in so doing they protect us against Cardiovascular Diseases (CVD). One cannot overemphasize the import of this in healthcare: according to WHO Fact Sheets CVDs is the number one killer in the world accounting for 29% (17.1 million) deaths globally in 2004. By lowering blood pressure in hypertensive individuals PUFAs can axe almost half of all strokes and ischemic heart diseases. The physical inactivity brought about by our lifestyle and nature of employment is the fourth most threatening health risk as it causes around 21–25% of breast and colon cancer. It is a well known fact that PUFAs can lower the risk of cancer and assist in its treatment. So their presence in our diet can go a long way in eliminating the risk of cancer, and treatments can be augmented by an inclusion of PUFA supplements as well.

PUFAs are also essential for the proper development of brain. A markedly positive influence of these bio-molecules has been noted on neurological disorders like Attention Deficit Hyperactive Disorders in Children, brain disorders like Alzheimer's and mental illnesses like depression and bipolar disorder. Researchers are also optimistic about the assistance PUFAs can provide in the treatment of diseases like lupus and psoriasis.

PUFAs are absolutely essential to the body in performing its cellular functions effortlessly. Cell to cell signalling and biochemical functions at molecular levels are mediated and perfected by the presence of these fatty acids in our body. They also form precursors of substances with hormonal regulator properties. Bolstering our immune system, both EPA and DHA

help to check abnormal immune responses in asthma, rheumatoid arthritis, kidney inflammation and eczema. Both gestating and lactating mothers and their babies can benefit from an inclusion of these fatty acids in the diet, which helps proper brain development and good vision.

Fish, being high in PUFA content and low in harmful cholesterol, is an ideal source of EPA and DHA for regular consumption. Fishes vary in the abundance of these molecules. If one is hoping to include more omega 3 fatty acids in the diet the best choices are sardines, mackerel, salmon, herring and menhaden. Sardines, are one of the most abundant and cheaply available fishes across the Indian coast line, has long been an integral part of the diet in Kerala, Coastal Karnataka and Tamil Nadu.

With many countries of the Indian Ocean belt – almost 500 million people - face a serious deficiency of proteins. Sardines can be utilized as an affordable source of excellent proteins. Sardine fishery, if put to proper use, and underpinned by spreading awareness, can bring down underweight in children, which is the major global contributor to increasing Disability-Adjusted Life Years (DALY) - the number of years lost due to ill-health, disability or early death. Sardines have now achieved added significance because of the discovery that it is a fish exceptionally rich in EPA and DHA. A regular inclusion of the fish in the diet can contribute a great deal to healthcare and help us grapple more effectively with cardiac illnesses, diabetes and cancer that affect a large segment of our population.

A steady supply of fresh sardines is possible only across our coastal belt. For the benefit of inland populations an effective processing method can be developed which will ensure that they get the valuable components in the

fish intact. This will also guarantee a longer shelf life and perennial availability.

In keeping with the trend of the day, brands of everyday food such as fruit juices, breads, spreads, margarines, snack bars, yogurt, fish products, eggs and children's beverages enriched with DHA and EPA have invaded market shelves. It is a quick and effective method to deliver the necessary fatty acids to the body, even for strict vegetarians. Trials to produce PUFA-enriched eggs by feeding marine PUFA to egg laying hens have yielded encouraging results. Even fast food like hot dogs and frankfurters can be absolved from the infamy of 'greasy foods' by enriching them with PUFA. It helps people to eat healthy even when they are on the move.

Review of literature

1.1 Clupeoids and Sardines

Sardines are a group of small fishes classified under three genus; *Sardina*, *Sardinops* and *Sardinella*; together consisting of 23 species (Table 1) under the widely categorized group of fishes known as Clupeoids. Clupeoid fishes are small teleosts which are relatively 'unevolved' and typically are less than 20 or 30 cm in length; they have no barbules and are frequently laterally compressed, with a series of hard scutes along the ventral surface of the body. Most are soft-bodied, difficult to handle, and covered with rather deciduous scales. Their flesh is often very oily, and the oil content normally varies seasonally, even in low latitude species (Longhurst 1971). These fishes are commercially extremely important – in fact clupeoid fishes like Herrings, Pilchards and Sardines form the main stay of economy of the European maritime nations – as aptly coined by the

famous French expression '*la crise sardinere*' refering to the disastrous effect of the failed sardine fishery and its impact on the nation. While only a single genus (Clupea) of clupeoids is important in high latitudes and the cold temperate regions, in the warm temperate mid-latitudes there are approximately 10 important genera, dominated by *Sardina, Sardinops, Engraulis, Brevoortia, Etlzmidium and Opisthonema*. In low latitudes more than 25 genera occur, many of which are important of which *Sardinella* being the prime (Longhurst 1971).

One of the most significant characteristics of clupeoids from a behavioural and fisheries viewpoint is their occurrence in dense and often very large schools containing many hundreds of thousands of fish which may weigh up to more than 100 tons (Longhurst 1971). Such large schools generally occur in the open ocean, particularly for pelagic sardines and frequently have a diagnostic shape and shoaling behaviour which enabled experienced fishermen to identify them. Clupeiformes also congregate in smaller, less-organized shoals, particularly during spawning seasons. In addition to schooling, some clupeoid fishes may migrate inshore or across latitudes on a seasonal basis (Longhurst 1971).

Most Clupeiformes filter feed by straining water through their long and numerous gill rakers. They consume plankton, particularly small crustaceans and the larval stages of larger crustaceans and fishes. The species which have a diet in which phytoplankton appears to be the preferred component, feed by filtering large diatoms and other phytoplanktons from the water by means of elongated gill rakers which form a filtration sieve. Though few in number, from a fisheries point of

view these species are some of the most interesting, both because of their occurrence in great quantity and also because they represent the most efficient possible utilization of the results of primary production. Indian Oil-sardine *Sardinella longiceps* (Peterson 1956; Nair 1960) fall into this category.

The Indo-Pacific region also contains the greatest number and diversity of species of clupeoids in the tropical oceans; along the mainland of Asia from China to the Red Sea, down the coast of eastern Africa, and through the Indonesian, the Philippine, and the Australian archipelagos. In Indian waters, the clupeoids are chiefly represented by Sardines, Anchovies, White-taits among which Indian Oil Sadrine (*Sadinella longiceps*) ranks as the most valuable and forms the backbone of the fishery of the west coast of India. At least three other species of Sardines can also be found in the south-west coast of India where this study was conducted viz. *Sardinella fimbriata, Sardinella gibbosa, Sardinella jussieu*; listed in decreasing order of abundance. The present study concentrates on two widely available Sardines in this area – *S. longiceps* and *S. fimbriata* and attempts to compare their fatty acid profile with their corresponding variations in bioactivity.

Sardines of the World

Table 1: Sardines of the World

Common Name	Scientific Name	Distribution
European Pilchard	*Sardina pilchardus*	NE Atlantic: Iceland (rare) and N. Sea, southward to Bay de Gorée, Senegal. Mediterranean (common in the W. part and in Adriatic Sea, rare in the E. part), Sea of Marmara and Black Sea
South American Pilchard	*Sardinops sagax*	Indo-Pacific: S. Africa to the E. Pacific. Three lineages: S. Africa (*ocellatus*) and Australia (*neopilchardus*), Chile (*sagax*) and California (*caeruleus*) and Japan (*melanostictus*)
White Sardinella	*Sardinella albella*	Indo-West Pacific: Red Sea, Persian Gulf, E. African coasts, Madagascar E. to Indonesia and the Arafura Sea, N. to Taiwan and S. to Papua New Guinea.
Bleeker's blacktip Sardinella	*Sardinella atricauda*	W. Pacific: Indonesia
Round Sardinella	*Sardinella aurita*	E. Atlantic: Gibraltar to Saldanha Bay, S. Africa. Also known from the Mediterranean and Black Sea. W. Atlantic: Cape Cod, USA to Argentina. Bahamas, Antilles, Gulf of Mexico and Caribbean coast
Deepbody Sardinella	*Sardinella brachysoma*	Indo-West Pacific: Madagascar (but apparently not elsewhere in the W. Indian Ocean), Madras, Indonesia, N. Australia
Fiji Sardinella	*Sardinella fijiense*	W. Pacific: Papua New Guinea and Fiji. Reported from New Caledonia
Fringescale Sardinella	*Sardinella fimbriata*	Indo-West Pacific: S. India and Bay of Bengal to the Philippines, also E. tip of Papua New Guinea
Goldstripe Sardinella	*Sardinella gibbosa*	Indo-West Pacific: Persian Gulf, East Africa and Madagascar to Indonesia, north to Taiwan and Korea south to the Arafura Sea and northern Australia.
Taiwan Sardinella	*Sardinella hualiensis*	NW Pacific: Taiwan, possibly S. to Hong Kong
Brazilian Sardinella	*Sardinella janeiro*	W. Atlantic: Gulf of Mexico, Caribbean, W. Indies S. to Brazil and N. Uruguay
Mauritian Sardinella	*Sardinella jussieu*	W. Indian Ocean: W. coasts of S. India, from Bombay S. to Sri Lanka; also to Madagascar and Mauritius. NW Pacific: Taiwan, Hong Kong and Viet Nam

Bali Sardinella	*Sardinella lemuru*	E. Indian Ocean: Phuket, Thailand; southern coasts of E. Java and Bali; and W. Australia. W. Pacific: Java Sea, Philippines, Hong Kong, Taiwan Island, S. Japan
Indian Oil Sardine	*Sardinella longiceps*	Indian Ocean: N. and W. parts only, Gulf of Aden, Gulf of Oman, but apparently not Red Sea or the Persian Gulf, E. to S. part of India, on E. coast to Andhra; possibly to the Andaman Islands.
Madeiran Sardinella	*Sardinella maderensis*	E. Atlantic: Gibraltar to Angola; single specimen recorded from Walvis Bay, Namibia. Also known from the Mediterranean (S. and E. parts, also penetrating Suez Canal).
Marquesan Sardinella	*Sardinella marquesensis*	E. Pacific: endemic to the Marquesan Islands. Introduced into Hawaiian waters.
Blacktip Sardinella	*Sardinella melanura*	Indo-West Pacific: Gulf of Aden S. to Madagascar and Mauritius and E. to the Arabian Sea and NW India (apparently not found S. of Bombay nor in N. Bay of Bengal); then from Indonesia (but not in S. China Sea) to Samoa. Reported from the Penghu Islands
East African Sardinella	*Sardinella neglecta*	W. Indian Ocean: Somalia, Kenya, and Tanzania
Richardson's Sardinella	*Sardinella richardsoni*	NW Pacific: Hainan Island, Hong Kong, China.
Yellowtail Sardinella	*Sardinella rouxi*	E. Atlantic: Senegal to Congo and perhaps S. of Angola.
Sind Sardinella	*Sardinella sindensis*	W. Indian Ocean: Arabian Sea, from Gulf of Aden to the Persian Gulf and Bombay
Freshwater Sardinella	*Sardinella tawilis*	Endemic to Lake Taal (= Lake Bombon), Luzon, Philippines
Japanese Sardinella	*Sardinella zunasi*	W. Pacific: S. coasts of Japan S. to about Taiwan

Source: ***www.fishbase.org***

1.1.1 Taxonomy

Taxonomic positions of the two test species, *Sardinella longiceps* and *Sardinella fimbriata* are given below

Kingdom Animalia

- **Phylum Chordata**
- **Class Actinopterygii**
- **Order Clupeiformes**
- **Family Clupeidae**
- **Genus Sardinella**
- ***Sardinella longiceps* (Valenciennes, 1847)**
- ***Sardinella fimbriata* (Valenciennes, 1847)**

Indian Oil-Sardine - Sardinella longiceps

Fringescale Sardine - Sardinella fimbriata

Sardinella longiceps is identified by its sub-cylindrical elongated body with its ventral profile evenly convex. It can be conclusively separated from all other clupeids in the northern Indian Ocean by its longer head and lower gill rakers. Caudal fin is well forked, lobes pointed; two large alar scales can be seen at the base, colour bluish green back with golden reflections, abdomen silvery with pinkish tinge and a faint golden spot behind gill opening are other in-hand diagnosis (Whitehead 1985).

Sardinella fimbriata can be identified by its compressed, flattened body and conclusively by its total number of scutes which varies consistently from 29 to 33. Vertical striae on scales do not meet at center, hind part of scales have a few perforations and somewhat produced posteriorly. A dark spot at dorsal fin origin also can be seen (Whitehead 1985).

Sardinella longiceps is an extremely valuable commercial fish and is also the most important clupeoid fishery of the whole of western Indo-Pacific. Stocks of this species extend around the whole perimeter of the northern part of the Indian Ocean from the Gulf of Aden to the Bay of Bengal and also occur in the Indonesian archipelago and the Philippines. Malabar region is considered as the zone of maximum abundance. The landings of this important species in India have reached as much as 200 thousand tons per annum in some years (Mohanty *et. al*.). During the Second World War, people of Kerala purely sustained themselves on the traditional dish of sardine and tapioca to save them from the bitter famine. However, the fishery is susceptible to irregular and large-scale fluctuations in resource availability and hence it has been studied intensively by fishery

biologists in India, beginning in the early 1920s with the work of Hornell and his associates. When Central Marine Fisheries Research Institute (CMFRI) was established in 1947, its top priority area was to study the Indian Oil Sardine. Since then, investigations on systematics, fishery, food and feeding, growth, distribution, reproduction, nutritional value and processing were extensively carried out which enriched our knowledge on this species. The shoals of Indian Oil Sardines become available to the fishery towards the end of June, when populations of adults with mature gonads appear near the coast and progressively move northwards as the season advances; these fish have mature gonads and spawn during their first few months in the coastal region. As the season advances, a second wave of shoals arrives in the coastal region and becomes available to the fishery; these are younger, immature fish and their availability reaches a peak during the months of October to December. Approximately the same cycle of events is repeated annually on the east coast of India. The arrival of the first wave of adult sardines at the coast generally coincides with the onset of the southwest monsoon; at this time there is a very strong seasonal bloom of phytoplankton, principally of the diatom *Fragillaria sp.* and it is supposed that the spawning migration is timed to coincide with this; the arrival of the second wave of such shoals, at a peak in October to December coincides with a second phytoplankton bloom. Nair (1960) has shown that the stomach contents of this species are dominated by phytoplankton, largely a single species of the diatom *(Fragillaria oceanica)* and dinoflagellates. He has suggested that the large and important fluctuations in the availability of the species from year to year may be dependent upon the nature and timing of the annual bloom of this diatom.

S. fimbriata is comparatively a less important lesser Sardine in terms of the trade and the species has an average annual landing of around 50000 tonnes in India (Mohanty *et al*.). This species is found in the local fisheries of Philippines, in the Visayan Sea in Indonesia, along the south-east coast of Bay of Bengal and southern coast of the Arabian Sea, and along the north coast of the Australian continent. This species occurs in commercially significant quantities in the southern part of Indian coast. These are mostly zooplankton-feeders and where their diet has been investigated (Ronquillo 1960) it is evident that they subsist upon a mixed diet, dominated by crustacea of various sorts, according to the relative availability from place to place and season to season. Spawning season extends from August to February (Bennet 1965) with juveniles appearing in the catch almost at the same time as *S. longiceps*.

1.2 Marine Lipids and Polyunsaturated Fattyacids

Marine lipids from sardines come under two categories of fattyacids; Saturated Fattyacids (SFA) and Unsaturated Fattyacids (USFA). Unsaturated Fattyacids are characterized by the presence of at least one double bond between the carbons. Unsaturated fattyacids consist of monounsaturated fattyacids (MUFA) and polyunsaturated fattyacids (PUFA). There are two classes of PUFAs, ω3 and ω6. The distinction between ω3 and ω6 fattyacids is based on the location of the first double bond, counting from the methyl end of the fattyacid molecule. ω3 and ω6 fattyacids are also known as essential fattyacids (EFAs) because humans, like all mammals, cannot make them and must obtain them in their diet (Bendich and Deckelbaum 2005).

The base origin of most marine lipids are from phytoplankton which are rich in PUFA(Klein Breteler *et al.* 1999). However, zooplanktons are also known to assimilate PUFA with higher levels of unsaturation. This extra level of unsaturation is partly achieved in zooplankton from their microplankton based diet. Microplanktons are known to preferentially increase the level of unsaturation in the food chain by converting the PUFA obtained from phytoplankton to higher degrees of unsaturation (Kleppel *et al*. 1998, Klein Breteler *et al.* 1999). Two important naturally occurring ω3 fatty acids that are entirely marine based, are Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) (Klein Breteler *et al.* 1999). EPA and DHA are essential as structural components of all the cell walls. They are necessary for proper brain and eye development, and are required for the proper functioning of the immune, reproductive, respiratory and circulatory systems (Simopoulos 1991).

1.2.1 Eicosapentaenoic Acid (EPA)

EPA, systematically called all-cis-5, 8, 11, 14, 17-icosapentaenoic acid (Figure 1), is a carboxylic acid with a 20-carbon chain with five *cis*-double bonds (sometimes denoted as C20:5(n-3)). This FA is involved in the production of eicosanoids, which are hormone-like substances which act as vasodilators and anti-platelet aggregators. EPA is a precursor to the eicosanoids known as series 3 prostaglandins and thromboxanes and series 5 leukotrienes (Arthur 1999).

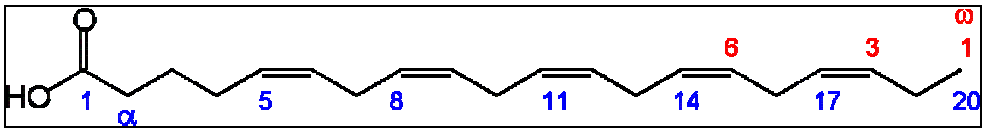

Figure 1: EPA Structure

1.2.2 Docosahexaenoic acid (DHA)

DHA, systematically called as *all-cis*-docosa-4,7,10,13,16,19-hexa-enoic acid (Figure 2), is a carboxylic acid with a 22-carbon chain and six cis double bonds (denoted sometimes as C22:6(n-3)). DHA is an important component of our brain and eyes. It is fundamentally important in the neurological growth and development of children, and for their eyesight (Arthur 1999).

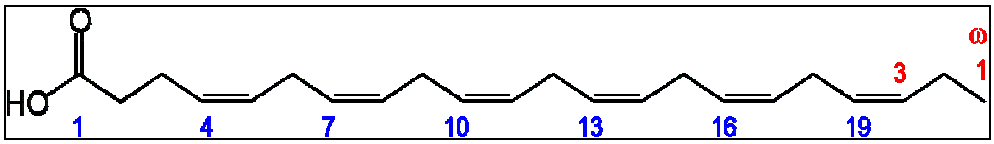

Figure 2: DHA Structure

1.2.3 Metabolism

Omega 6 fatty acids are represented by linoleic acid (LA) and the corresponding ω3 fatty acids by ά-linolenic acid (ALA). LA is plentiful in nature and is found in the seeds of most plants except for coconut, cocoa, and palm. ALA on the other hand is found in the chloroplast of green leafy vegetables. Both EFAs can be metabolized to longer-chain fatty acids of 20 and 22 carbon atoms. LA is metabolized to arachidonic acid (AA) and ALA, to EPA and DHA, increasing the chain length and degree of unsaturation by adding extra double bonds to the carboxyl group. Humans and animals except carnivores such as lions and cats can convert LA to AA and ALA to EPA and DHA (de Gomez & Brenner 1975). There is competition between ω3 and ω6 fattyacids for the desaturation enzymes. However, both 1-4 and 1-6 desaturase prefer ω3 to ω6 fattyacids. There is some evidence that 1-6 desaturase decrease with age. Premature infants, hypertensive individuals,

and some diabetics are limited in their ability to make EPA and DHA from ALA. These findings are important and the role of fish oils which are natural sources of EPA and DHA is quite significant in these cases (Simopoulos 1991). AA is found predominantly in the phospholipids of grain-fed animals. LA, ALA, and their long-chain derivatives are important components of animal and plant cell membranes. In mammals and birds the ω3 fattyacids are distributed selectively among lipid classes. ALA is found in triglycerides, in cholesteryl esters, and in very small amounts in phospholipids. EPA is found in cholesteryl esters, triglycerides, and phospholipids. DHA is found mostly in phospholipids. In mammals, including humans, the cerebral cortex, retina, and testis and sperm are particularly rich in DHA. DHA is one of the most abundant components of the brain's structural lipids. DHA, like EPA, can be derived only from direct ingestion or by synthesis from dietary EPA or ALA (Simopoulos 1991).

It has been reported that conversion of ALA to EPA and further to DHA in humans is limited, but varies with individuals. Women have higher ALA conversion efficiency than men, probably due to the lower rate of utilization of dietary ALA for beta-oxidation. This suggests that biological engineering of ALA conversion efficiency is possible (Hussein *et al.* 2005). Goyens *et al.* (2006) suggest that it is the absolute amount of ALA, rather than the ratio of $n-3$ and $n-6$ fattyacids, which affects the conversion. However, ALA-feeding studies and stable-isotope studies using ALA, which have addressed the question of bioconversion of ALA to EPA and DHA, have concluded that in adult men conversion to EPA is limited (approximately 8%) and conversion to DHA is extremely low (<0.1%). In women fractional conversion to DHA appears to be greater (9%), which

may partly be a result of a lower rate of utilisation of ALA for beta-oxidation in women. In this context, direct intake of sufficient quantities of these ω3 FA is essential for the stable metabolism of the body (Simopoulos 1991).

1.3 Sardines and PUFA

Clupeid fishes are known to be seasonal feeders. They store great reserves of energy for maintenance during the times when food is scarce. Fishes store their energy as lipids and these compounds are burnt when energy has to be expended. They are also useful in maintaining stability, permeability and fluidity of the cell membranes. Fat reserves and fattyacid composition of the fishes can vary with age, sex and season. This has been proved in several species of clupeids including *S. longiceps* (Gopakumar 1965) apart from other *Sardinops* (Gamez-Mezza *et al.* 1999, Shirai *et al.* 2002) and *Sardina* (Bandarra *et al.* 1997).

Easy availability of these species in the western coast in great quantities roughly all through out the year means a ready availability of enormous amounts of these essential FAs for human consumption. This brings in a huge commercial implication for the fishery industry as this can potentially supplement a viable nutritional and pharmaceutical industry solely based on Marine PUFA.

1.4 PUFA and Nutrition

Scientists were first alerted to the many benefits of fish oils in the early 1970s when Danish physicians observed that Greenland Eskimos had an exceptionally low incidence of heart disease and arthritis despite the fact

that they consumed a high-fat diet. Intensive research soon discovered the secret that two of the fats (oils) they consumed in large quantities, EPA and DHA, were actually highly beneficial. More recent research has established that fish oils (EPA and DHA) play a crucial role in the prevention of atherosclerosis, heart attack, depression, and cancer.

Seemingly minor differences in their molecular structure make the two EFA families act very differently in the body. While the metabolic products of ω6 FA promote inflammation, blood clotting, and tumor growth, the ω3 FA act entirely opposite (Caygill *et al.* 1996). Although both ω3s and ω6s are needed, it is becoming increasingly clear that an excess of ω6 FA can have dire consequences. Many scientists believe that a major reason for the high incidence of heart disease, hypertension, diabetes, obesity, premature aging, and some forms of cancer is the profound imbalance between our intake of ω6 and ω3 FAs. Our ancestors evolved on a diet with a ratio of ω6 to ω3 of about 1:1. A massive change in dietary habits over the last few centuries has changed this ratio to something closer to 20:1 and this spells trouble (Simopoulos 1991).

Recognizing the unique benefits of EPA and DHA and the serious consequences of a deficiency the US National Institutes of Health recently published Recommended Daily Intakes of fattyacids. It recommends a total daily intake of 650 mg of EPA and DHA, 2.22 g/day of ALA and 4.44 g/day of LA. Saturated fat intake should not exceed 8% of total calorie intake or about 18 g/day.

1.4.1 Pregnancy & Child Birth

An adequate intake of DHA and EPA is particularly important during pregnancy and lactation. During this time the mother must supply all the baby's needs because it is unable to synthesize these essential fattyacids itself. DHA makes up 15 to 20% of the cerebral cortex and 30 to 60% of the retina (Gal *et al.* 2005). There is some evidence that an insufficient intake of ω3 fattyacids may increase the risk of premature birth and an abnormally low birth weight (Carlson 1999, Cunnane *et al.* 2000, Makrides *et al.* 2000). There is also emerging evidence that low levels of omega-3 acids are associated with hyperactivity in children (Mitchel *et al.* 1987). The constant drain on a mother's DHA reserves can easily lead to a deficiency and it is believed that pre-eclampsia and postpartum depression could be linked to a DHA deficiency. Experts recommend that women get at least 500-600 mg of DHA every day during pregnancy and lactation (Carlson 1999).

1.4.2 Depression

The human brain is one of the largest "consumers" of DHA. A normal adult human brain contains more than 20 grams of DHA. Low DHA levels have been linked to low brain serotonin levels which again are connected to an increased tendency to depression, suicide, and violence (Edwards *et al.* 1998). Studies have shown that countries with a high level of fish consumption have fewer cases of depression (Hibbeln 1998). Researchers at Harvard Medical School have successfully used fish oil supplementation to treat bipolar disorder (Stoll *et al.* 1999) and British researchers report encouraging results in the treatment of schizophrenia (Laugharne *et al.* 1996).

1.4.3 Cardiac Disorders

Danish researchers have concluded that fish oil supplementation may help prevent arrhythmias and sudden cardiac death in healthy men (Christensen *et al.* 1999). An Italian study of 11,000 heart attack survivors found that patients supplementing with fish oils markedly reduced their risk of another heart attack, a stroke or death. A group of German researchers found that fish oil supplementation for two years caused regression of atherosclerotic deposits (von Schacky *et al.* 1999) and American medical researchers report that men who consume fish once or more every week have a 50% lower risk of dying from a sudden cardiac event than do men who eat fish less than once a month (Siscovick *et al.* 1995). Fish oil supplementation (10 grams/day) reduces the number of attacks by 41% in men suffering from angina (Salachas *et al.* 1994). It is found that fish oil supplementation reduces the severity of a heart attack and supplementation started immediately after a heart attack reduces future complications (Eritsland *et al.* 1994). Bypass surgery and angioplasty patients reportedly also benefit from fish oils and clinical trials have shown that fish oils are beneficial for heart disease patients (Singh *et al.* 1997). Fish oils are especially important for diabetics who have an increased risk of heart disease. It is found that supplementing with as little as 2 grams/day of fish oil (410 mg of EPA plus 285 mg of DHA) can lower diastolic pressure by 4.4 mm Hg and systolic pressure by 6.5 mm Hg in people with elevated blood pressure (Appel *et al.* 1993).

1.4.4 Rheumatic Disorders

Fish oils are particularly effective in reducing inflammation and can be of great benefit to people suffering from rheumatoid arthritis or ulcerative colitis. Daily supplementation with as little as 2.7 grams of EPA and 1.8 grams of DHA can markedly reduce the number of tender joints and increase the time before fatigue sets in (Kremer 2000). Some studies have also noted a decrease in morning stiffness (Fortin *et al.* 1995) and clinical trials concluded that arthritis patients who took fish oils could eliminate or sharply reduce their use of NSAIDs and other arthritis drugs (Kremer *et al.* 1995).

1.4.5 Cancer

There is also considerable evidence that fish oil consumption can reduce the risk of breast and prostate cancer (Chavarro *et al.* 2008) and help slow their progression (Caygill *et al.* 1996). Daily supplementation with fish oils has been found effective in preventing the development of colon cancer (Mehta *et al.* 2008). There is now also considerable evidence that fish oil consumption can delay or reduce tumor development in breast cancer. Studies have also shown that a high blood level of omega-3 fattyacids combined with a low level of ω6 acids reduces the risk of developing breast cancer (Soto-Guzman *et al.* 2010). Daily supplementation with as little as 2.5 grams of fish oils has been found effective in preventing the progression from benign polyps to colon cancer (Fernandez-Banares *et al.* 1996) and Korean researchers reported that prostate cancer patients have low blood levels of omega-3 fattyacids (Yang *et al.* 1999). Greek researchers report

that fish oil supplementation improves survival and quality of life in terminally ill cancer patients (Gogos 1998).

1.4.6 Omega 3 Enriched Products

Omega 3 fattyacids are being increasingly promoted as important dietary components for health and disease prevention. These fattyacids are naturally enriched in fatty fish like salmon and tuna and in fish-oil supplements. An increasing number of foods that are not traditional sources of ω3 fattyacids, such as dairy and bakery products, are now being fortified with small amounts of these fattyacids (Surette 2008).

In conclusion, the direct and indirect nutritional advantages of ω3 fattyacids have been recognised by the medical community and increasing presence of ω3 enriched food products is a testimony to this fact.

1.5 PUFA and Pharmaceuticals

Omega 3 oils, though called 'miracle food' of the 21st century, are not a 'miracle drug' in itself. Its use in pharmaceutical industry is always in combination with a more direct drug and the presence of these FA induce a favourable condition in the patient's body for the real drug to be effective.

FA in combination with drugs for the treatment of diseases is an area of immense interest because it opens a new field in pharmaceutical research - ω3 fattyacids in the control of metabolic and autoimmune disorders, that includes CVD, arthritis, nephrites, psoriasis, ulcerative colitis and cancer (Simopoulos *et al.* 1991). Preliminary data from animal and human studies suggest that the concurrent ingestion or administration of ω3 fattyacids with

drugs leads to potentiation of drug effects, as with propranolol, which may lead to a decrease both in the dose of ω3 fattyacids and in the drug dose or, as with cyclosporine, to a decrease in toxicity of the drug. By partially replacing the fattyacids of phospholipids in the cell membranes, ω3 fattyacids modify enzymes, receptors, and other proteins (Simopoulos *et al.* 1991). Additional studies suggest that the incorporation of ω3 fattyacids by cell membranes is enhanced in the presence of olive oil and linseed oil, emphasizing once again the importance of nutrient interactions (Cleland *et al.* 1991). Cyclosporin is used widely in organ transplantation and in many individuals its use leads to impairments in renal function and increased thromboxane formation. It was noted that the use of fish oil instead of olive oil as the vehicle for its administration in rats led to attenuation of the cyclosporine nephrotoxicity (Elzinga *et al.* 1987) without affecting thromboxane synthesis (Walker *et al.* 1989).

There is much scope for research in finding out new combinations of drugs with ω3 FA and delving deep into the causes for the interactions that happen in the body. Research in these aspects is still in budding stage. Fishery biology, fishing techniques, taxonomy, size distribution, nutrient value and processing of Sardines have been extensively worked out in research institutions like Centre Marine Fisheries Research Institute and Central Institute of Fisheries Technology. However, there have been hardly any investigations relating to the industrial applications of this fish commodity despite being cheap and available round the year. Hence, the present work attempts to fill this gap.

1.6 Research Objectives

The present study revolves primarily around 3 specific intentions.

a) Explicate the importance of Sardines found in the west coast of India.
b) Examine the preferential bioactivity of EPA and DHA.
c) Evaluate the pharmaceutical applications of marine PUFA from widely available sources.

As the fattyacid profile of sardines is expected to change across seasons, the first target was to analyze the seasonal change in all kinds of fatty acids for the two study species. As per those results, a good extraction technique was to be standardized to obtain a substantially pure polyunsaturated fattyacid extract which can be suggested for use in clinical research. Based on these objectives, the study is crystallized into following chapters.

Chapter 2 deals with the seasonal variation of fattyacids in *S longiceps* and *S fimbriata*, with emphasis on how EPA and DHA varies across seasons in the two species of sardines. An explanation is attempted to elucidate why the quantity of these fatty acids vary across seasons in relation to their feeding habits.

Chapter 3 elaborates the extraction procedure of polyunsaturated fattyacids from *S longiceps* and *S fimbriata* and compares on how this particular procedure fares with other known procedures for extracting polyunsaturated fatty acids

Chapter 4 deals with the activity of the polyunsaturated fattyacid extracts against a selected set of gram positive and gram negative bacteria. Results obtained are analysed in the light of contemporary research and compared to evaluate the validity of the findings.

Chapter 5 elaborates an invivo study on how this polyunsaturated fattyacid extracts influence the levels of various biochemical parameters in diabetes induced mice tested against controls. Recovery profiles for both species for different biochemical parameters are compared and results explained relating them with contemporary research findings.

Chapter 6 details the cytotoxic activity of the polyunsaturated fattyacid extracts on two cancer cell lines, MCF-7 and DU-145. Differences in cytotoxic activity between the species is explained based on their PUFA profiles.

Chapter 7 summarizes the present study with recommendations for a sardine based pharmaceutical/ nutritional venture.

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Chapter 2

Seasonal analysis of fattyacids in *Sardinella longiceps* and *Sardinella fimbriata*

Contents

2.1 Introduction

Fattyacids in sardines are known to show seasonal fluctuations in their composition and yield. Earlier studies in temperate regions on species like *Sardinops sagax* (Gamez-Mesa *et al.* 1999), *Sardina pilchardus* (Bandarra *et al.* 1997) and *Sardinops melanostictus* (Shirai *et al.* 2002) have clearly demonstrated a seasonal fluctuation influenced by temperature of sea water, food availability and sexual state of the animal. There has been a seasonal study in the Indian seas for *S. longiceps* (Gopakumar 1965), however this study did not delve at the granularity of individual fattyacids but considered PUFA as one component. There has been no seasonal fattyacid profiling on *S. fimbriata*, except the information available for a

single sampling period on the species (Reena *et al.* 1997). They catalogued the fatty acid composition of 31 marine fish species including these two species.

In the present investigation, fattyacid composition of the two species of sardines is compared four seasons. Though phylogenetically close, the feeding pattern of these two sardines are known to be slightly different; *S. longiceps* favouring phytoplankton diet (Nair 1953) while *S. fimbriata* being more partial to zooplanktons (Chacko 1956). Hence, the fattyacid profiles of these two fishes are expected to show deviations. Specific focus is on the variation of EPA and DHA composition of these fish oils as this is of high relevance in the subsequent bioactivity studies. Seasonal variations of these two important PUFA were analysed keeping in mind the commercial implication of such a variation for pharmaceutical and nutritional industry. Potential reasons for such a variation across seasons and across species in relation to the chief factors influencing the fattyacid composition is also elaborated.

2.2 Materials and Methods

2.2.1 Fish samples

Freshly caught samples of *S longiceps* and *S .fimbriata*, were collected from the Kaalamukku landing centre (9°58'55''N, 76°14'33''E) at Kochi. Samples were washed in sterile water and brought to the laboratory in an ice box. Fishes were identified for their maturity stages (Antony Raja 1971) and their lengths measured and their stomach content analyzed (Wallace 1981). Sampling was done on the 15th day of four months

representative of four seasons in the Indian tropics (McKnight and Hess 2000) – September (post-monsoon), December (winter), March (summer) and June (monsoon).

2.2.2 Analysis of fattyacids:

Fattyacids were analyzed according to the method of AOAC (1975). In this method, fatty acids were made volatile by converting them into methyl esters. The esters were identified and quantified by GC by comparing with a set of standard esters. Lipid content of the tissues was estimated by the method of Folch *et al.* (1957). Methyl esters of fattyacids (FAME) from animal and vegetable origin having 8-24 atoms are separated and detected by gas chromatography. Methyl esters of the fattyacid thus obtained were separated by gas liquid chromatography equipped with a capillary column and a flame ionization detector. Fattyacids separated were identified by the comparison of retention times with those obtained by the separation of a mixture of standard fattyacids. Measurement of peak areas and data processing were carried out by Thremo Chrom card software. Individual fattyacids were expressed as mg/g and then converted to percentage of total fattyacids.

2.2.3 Extraction of Total Lipids (Folch *et al.* 1957)

One g of tissue was subjected to lipid extraction using chloroform-methanol mixture (2:1).The lipid extracts were transferred to a separating funnel and added with, 20% of water and left overnight. It was drained through filter paper containing anhydrous sodium sulphate and was collected in round bottom flask and evaporated to dryness in an evaporator. The lipid in the round bottom flask was made up to 10ml with

chloroform,taken into a pre-weighed vial and allowed to dry in warm temperature to constant weight. Total lipid content was calculated from the difference in weight and the result expressed as mg/g of total lipid of fresh tissue.

Satellite Imagery of the collection site (Courtesy: Google maps)

2.2.4 Fattyacid Methyl Ester Method (AOAC 1975)

Lipid weighing about 300- 500mg was taken in a round bottom flask,and was added with 6ml of methanolic NaOH and boiling chip.. The condenser was attached and refluxed under nitrogen (10min) until fat globules disappeared. To this, 6 ml of Borone trifluoride (BF_3) solution was added through condenser and continued boiling for 2minutes. Heat was removed and 15ml saturated NaCl solution is added. Stoppered flask was shaken vigorously for 15 sec while solution was still tepid. Aqueous phase was transferred to 250ml separating funnel and extracted with two 30ml portions of petroleum ether. The combined extracts were washed with 20ml portion of water, dried over anhydrous sodium sulphate, filtered and solvent evaporated. The content was made up to 1ml with PE and separated by gas liquid chromatography.

Table 2: Gas Liquid Chromatography Characteristics

Type and Dimensions of Coloumn	**10% OV275 on ChromosorbHP (30m long and 0.54mm diameter)**
Detector	Flame Ionization Detector
Injector temperature	260°C
Detector temperature:	275°C
Column temperature	250°C
Carrier gas	Nitrogen
Flow rate of Carrier Gas	0.8ml/min

2.4.5 Presentation of Measures

Individual fattyacids were expressed in mg/g tissue. This is a measure of absolute yield and is relevant for the viability of a nutritional industry. Additionally, the same values were expressed in percentage of total FA/PUFA and compared. This is a relative yield and is an indication of the quality of the product. It is emphasized that both measures are complimentary to each other and serve different purposes. Specific focus has been given to important fattyacids in fish oil and aggregates like Saturated Fattyacids (SFA), Mono-unsaturated Fattyacids (MUFA) and Poly-unsaturated Fattyacids (PUFA).

2.3 Results

Table 3: Sampling Details of *S. longiceps and S. fimbriata*

Season	Notes on Catches and Sampling	*S. longiceps (length in mm)*	*S. fimbriata (length in mm)*
September	Mostly Running (6) or Partially spent ones (7a) in the catch. Rarely Immatures (1). Selected samples were in stage 6.	178.8±5.11	142.6±3.91
December	Mostly immatures (1) and rarely Spent (7b) or Spent Resting (2b). Selected samples were in stage 1.	131.4±3.13	105.2±5.71
March	Mostly immatures (1) and developing virgin (2a) stages. Selected samples had both 1 and 2a stages	138.6±5.85	111.2±10.63
June	Mostly maturing (4) and sometimes mature (5). Selected samples were in stage 4	165.6±7.3	129.2±5.54

Table 3 provides all details on the sampling of both species, the maturity period of the fishes used for experiments and their lengths expressed as a mean and SD.

Total FA content in mg/g meat for both species across seasons is illustrated in Figure 3. *S. longiceps* has a higher concentration of FA across all four seasons. The statement is also valid for all the three variants of FA. FA concentration is highest during December (165.71 mg/g and 90.38 mg/g) and lowest during June and September for both species.

In both species, PUFA dominate the profile followed by SFA and MUFA (Figure 4 & 5). It is also clear that the concentration of PUFA peaks during the winter (December-March) and falls during the spawning (June-September). However, MUFA has a complementary increase during this period.

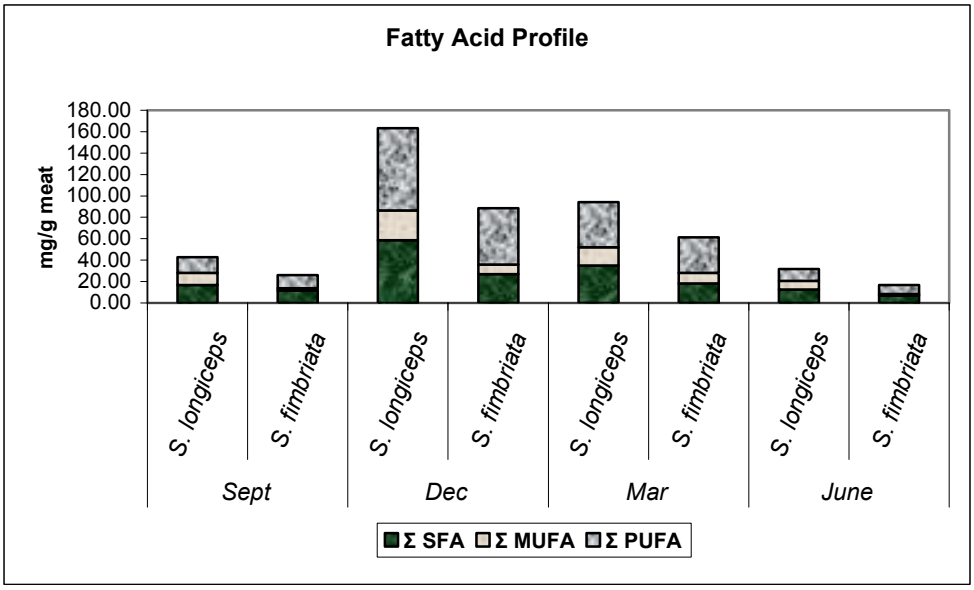

Fig. 3: Seasonal variation in fattyacid profile of *S. longiceps and S. fimbriata*

The seasonal trend of seven dominant Fattyacids in these sardines are shown (Table 4).

Table 4 : Variation of Important Fattyacids in *S. longiceps and S. fimbriata* across seasons

		In percentage of total fatty acids									
		Sardinella longiceps					*Sardinella fimbriata*				
FA	**FA Name**	**Sept**	**Dec**	**Mar**	**June**	**Avg**	**Sept**	**Dec**	**Mar**	**June**	**Avg**
C14	Tetradecanoic Acid	10.93	12.33	7.68	13.18	11.03	4.62	7.77	3.94	6.43	5.69
C16	Palmitic Acid	17.14	15.63	21.87	16.68	17.83	22.81	14.58	15.43	19.46	18.07
C16:1	Palmitoleic Acid	7.40	2.41	8.30	5.53	5.91	3.14	3.93	7.58	2.41	4.27
C18	Stearic Acid	7.85	5.31	6.19	5.97	6.33	11.90	4.76	7.25	9.66	8.39
C18:1n-9	Oleic Acid	18.30	14.18	8.99	19.20	15.17	4.15	5.32	7.74	7.02	6.06
C20:5n-3	Eicosapentaenoic Acid	22.39	22.98	19.96	21.72	21.77	8.26	14.43	18.23	6.94	11.96
C22:6n-3	Docosahexaenoic Acid	6.86	14.88	18.42	6.22	11.59	31.27	37.26	31.82	32.02	33.09
	Σ SFA	38.33	35.43	36.35	39.30	37.35	43.87	29.56	29.12	40.44	35.75
	Σ MUFA	25.91	16.75	17.79	25.15	21.40	7.47	9.97	15.55	9.56	10.64
	Σ PUFA	33.77	46.35	44.08	34.22	39.61	46.16	58.45	53.30	48.69	51.65
	% EPA in PUFA	66.31	49.59	45.29	63.47	54.95	17.89	24.69	34.19	14.25	23.16
	% DHA in PUFA	20.32	32.10	41.78	18.17	29.27	67.75	63.75	59.70	65.77	64.07

Palmitic Acid (C:16) is the dominant SFA in both species followed by Tetradecanoic Acid (C14) and Stearic Acid (C18) respectively. Among MUFA, Oleic Acid (C18:1n-9) seems to be the most dominant followed by Palmitoleic Acid (C16:1) while among PUFA, EPA was the dominant FA in *S. longiceps* while DHA showed dominance in the FA of *S. fimbriata* and there was a noticeable trend across seasons in both these PUFA.

Though December happens to be the month with maximum yield for both the PUFA in the Sardines, the relative concentrations of EPA and DHA in terms of PUFA seems to vary much across seasons (Fig 6 & 7). Months of June and September seems to have a higher concentration of EPA and

lower concentrations of DHA in *S. longiceps*. Similarly during the same months, the concentration of DHA is higher in *S. fimbriata*.

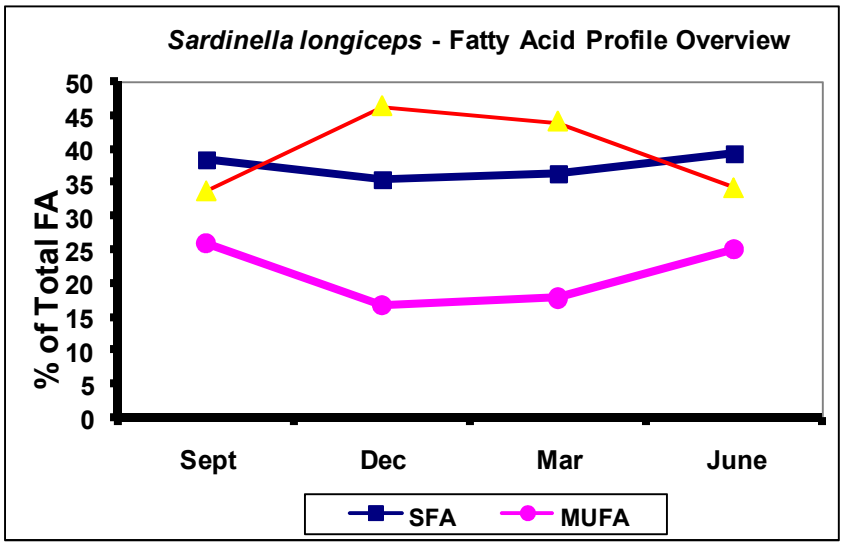

Fig. 4: Seasonal variation of different types of fattyacids present in *Sardinella longiceps*

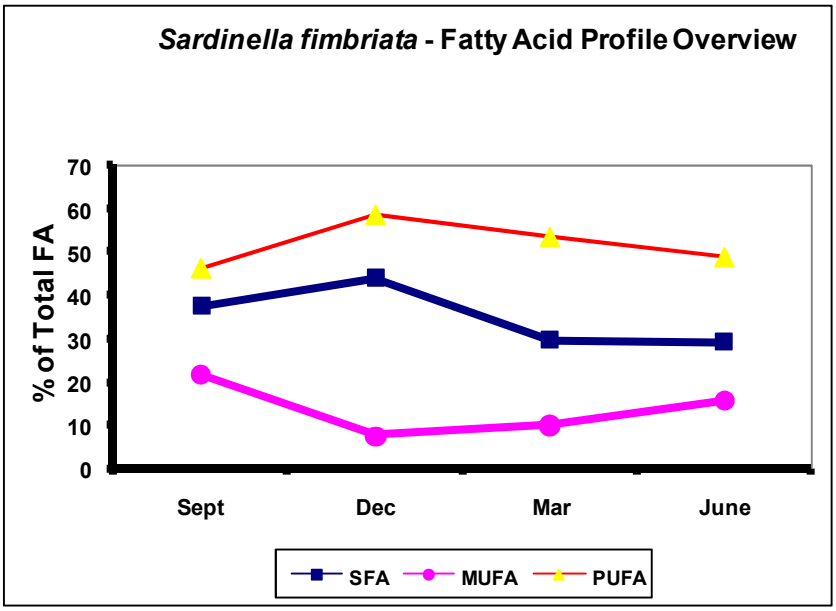

Fig. 5: Seasonal variation of different types of fattyacids present in *Sardinella fimbriata*

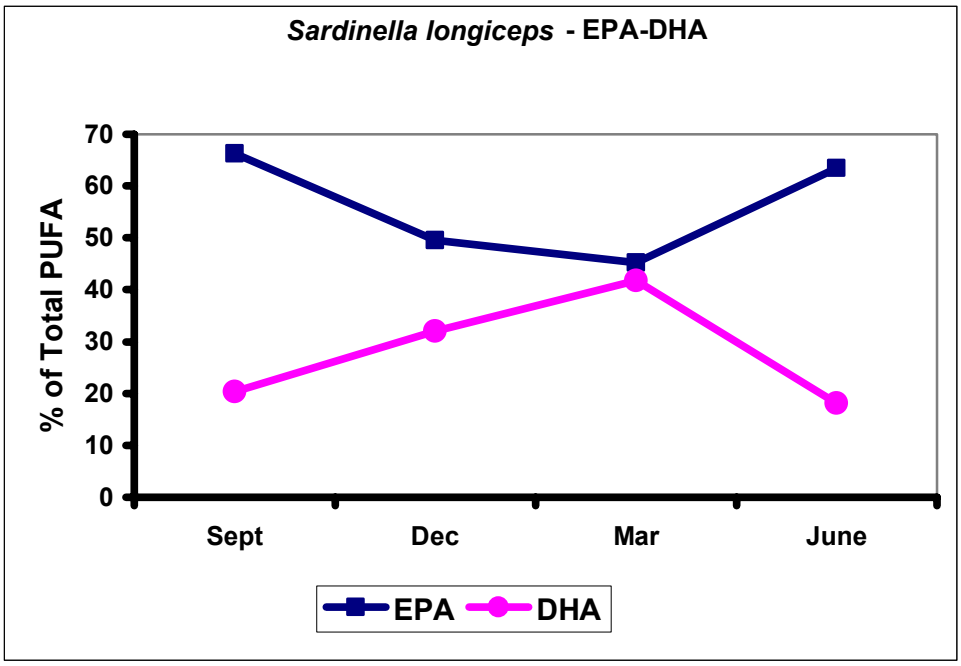

Figure 6: Variations in EPA and DHA in *Sardinella longiceps*

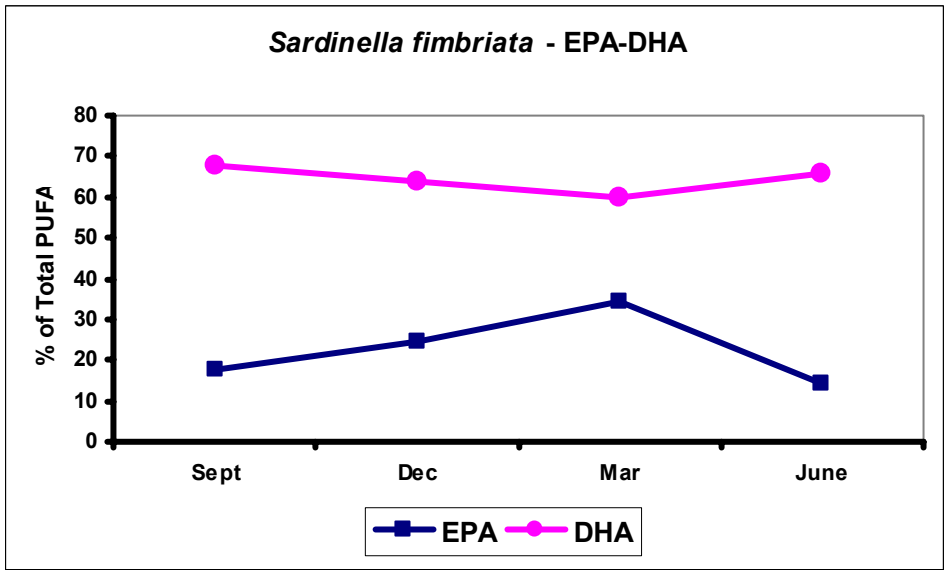

Fig. 7: Variations in EPA and DHA in *Sardinella fimbriata*

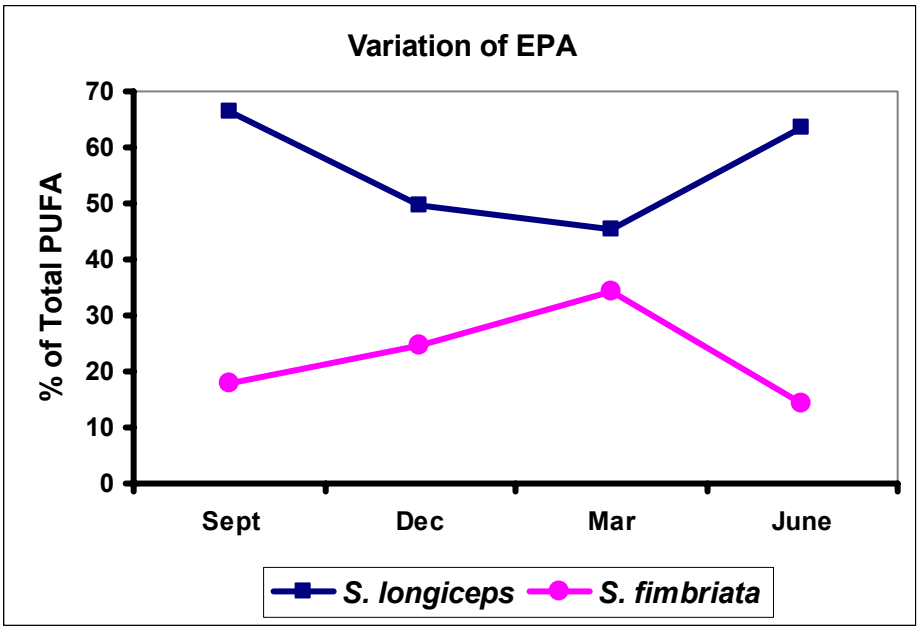

Fig. 8: Variation in EPA concentration in *S. longiceps and S. fimbriata*

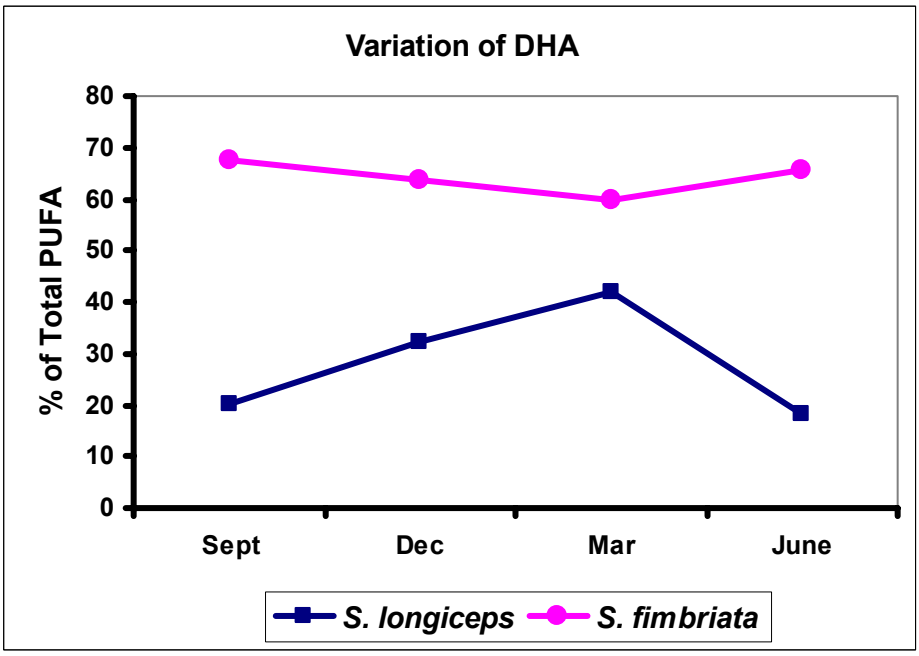

Fig. 9: Variation in DHA concentrations in *S. longiceps and S. fimbriata*

Hence, in both species, the DHA-EPA concentrations are complementary – when DHA values are less, EPA values are more and vice versa. Among the species, EPA values and DHA values are themselves complementary – when EPA values of *S. longiceps* are high, it is low for *S. fimbriata* and vice versa; the same holds good for DHA too (Figure 8 & 9).

Bray-Crutis analysis of similarity indices (Zar 1984) for both species clearly indicates two clusters in terms of fatty acid variations (Figure 10 & 11). Fatty acid composition of June and September months seems to be more similar and divergent from the months of March and December.

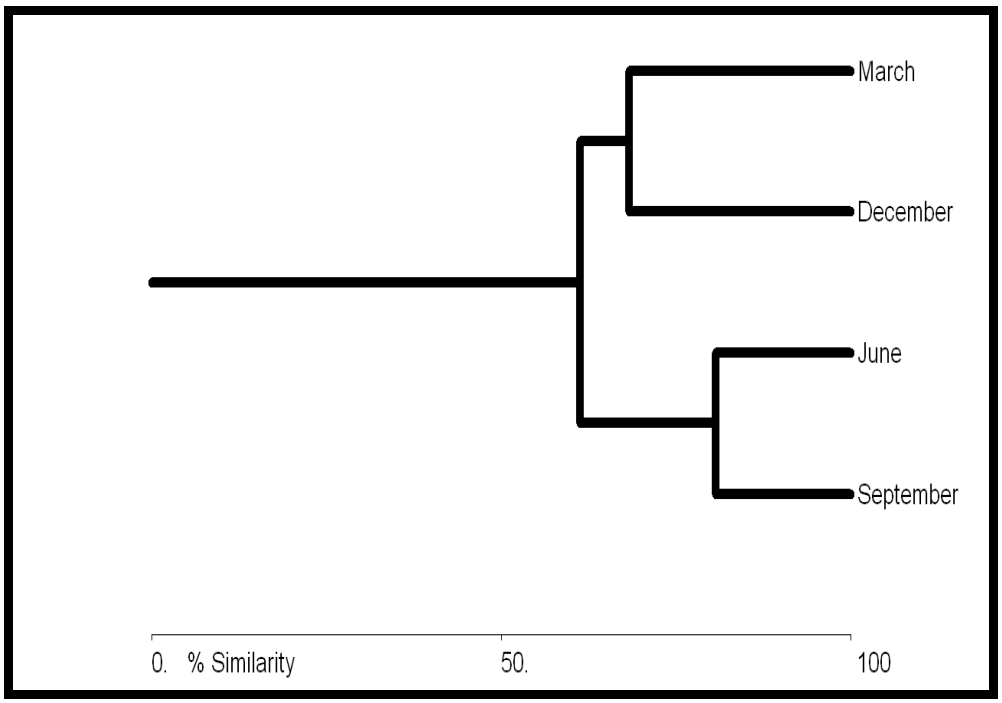

Fig. 10: Bray-Crutis similarity index for FA from *Sardinella longiceps*

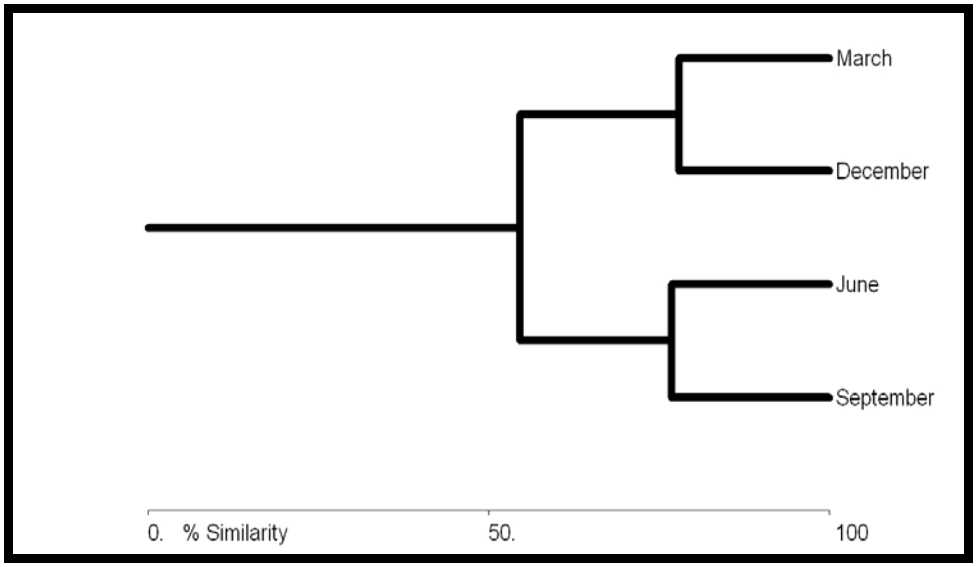

Fig. 11: Bray-Crutis similarity index for FA from *Sardinella fimbriata*

2.4 Discussion

Total FA content in mg/g meat for both species are highest during December-March and lowest in June-September. June to September happens to be the spawning period for both species (Hornell and Nayudu 1924, Chidambaram and Venkataraman,1946). This could be attributed to the result of fat mobilization associated with gametogenesis (Hady and Keay 1972). During winter, the sea water temperature falls to 25-26 °C as compared to 30-31°C and hence FA concentration in fishes like sardines increase to sustain the lowered temperature. This has also been observed in several prior studies on Sardines elsewhere (Gamez-Mesa *et al.* 1999, Bandarra *et al.* 1997, Shirai *et al.* 2001) including one on *S. longiceps* in the tropic coasts (Gopakumar 1965). A secondary reason could be the dominance of juveniles and immatures in the catch during winter – these individuals are heavy feeders and accumulate large quantities of fat during

winter. The high concentrations of FA and particularly PUFA in the winter are an interesting nutrition aspect as the PUFA intake per sardine goes up significantly. The same is also important from an industrial production perspective as their yield would be significantly better during winter.

In both species, PUFA dominate the profile and peaks during winter and falls during spawning while MUFA has a complementary increase during the spawning. This inverse relation has also been noted in prior studies in a variety of fishes in the west coast (Reena *et al.* 1997), however its biochemical significance is unknown.

Palmitic Acid (C:16), the most dominant SFA in both species, has no noticeable trend across seasons and this seems to be true for other species of sardines too (Bandarra *et al.* 1997). This compound is also hypothesized as not influenced by the diet (Ackman 1964, Ackman 1966). Among MUFA, Oleic Acid (C18:1n-9) seems to be the most dominant, and this is in accordance with findings by Ackman (1982) who pointed out that the main MUFA detected in marine lipids usually contained 18 carbon atoms. The difference in the EPA and DHA content between *S. longiceps* and *S. fimbriata* of these two species of sardines is also present in the values tabulated in prior studies (Reena *et.al.* 1997) though its relevance was not highlighted then.

The variation in the EPA and DHA amount in both species can be explained by the maturity stage prevalent in the catch during the season and its dietary patterns. The dietary patterns of the catches were confirmed in current study by analysis of gut contents of these fishes prior to the experiments. Months of June and September are characterized by the

presence of adults in the catch during the spawning season of *S. longiceps*. Adult *S. longiceps* are exclusive phytoplankton feeders (Nair 1953) and hence have an EPA-rich diet. However, immatures found abundantly during the winter are carnivorous with varying amounts of zooplankton entering into its diet. The gill-rakers of the immatures are either imperfectly developed or under developed and their carnivorous tendency is actually an indirect selection for the large sized items by their inefficient filtering mechanism while the predominantly phytoplanktonic diet of the adult is due to their efficient sieving of the minute organisms (Bensam 1964). Hence, it is the presence of zooplankton in the diet of immatures that increases the DHA content during winter. The situation is directly the opposite for *S. fimbriata*. Adults found during June-September are exclusive zooplankton feeders (Chacko 1956) and hence PUFA concentrations show high DHA content during this period. However, the immatures found during December-March intake varying amount of phytoplankton also (Basheeruddin and Nayar 1961) and hence EPA reaches higher levels for this species in winter. Hence, in both species, the DHA-EPA concentrations are complementary – when DHA values are less, EPA values are more and vice versa.

The variation in DHA-EPA composition in these two *Sardinella* species is a classical case of trophic upgradation in the seas. Interestingly, this has a large influence on the nutritional aspects of dietary fish intake in humans - food products based on *S. fimbriata* would enhance DHA intake in its consumers. Zooplanktons, the main food source of *S. fimbriata*, feed on microplanktons – and the microplanktons in turn feed on phytoplankton which also happens to be the chief food of *S. longiceps*. However, these

microplanktons, also known as heterotrophic protists, are known to trophically improve poor algal quality for subsequent use by higher trophic organisms (Klein Breteler *et al.* 1999). As an intermediate prey, they improve the quality and quantities of the types of fattyacids in the food web there by forming fattyacids like DHA which have higher levels of unsaturation (Kleppel *et al.* 1998; Klein Breteler *et al.* 1999). Thus, higher concentrations of DHA in zooplankton are a consequence of this preferential assimilation in planktonic food webs by the microplanktons. Hence, it has to be realized that the fisheries of the zooplankton feeding *S. fimbriata* are of great importance in food and nutrition.

The result that EPA and DHA values are complementary among the species is particularly interesting from the perspective of a pharmaceutical industry as the result has direct implication on the quality of the fattyacids extracts. For EPA rich drugs, the industry has to base its operations on *S. longiceps* while *S. fimbriata* can be a source for DHA rich drugs. Across seasons, the quality of the PUFA extract also varies and hence additional processes need to be put in place to maintain a steady concentration of EPA or DHA, as the case may be.

Bray-Crutis analysis clearly indicated two clusters in terms of fattyacid profile - one cluster during the spawning period (June-September) dominated by mature individuals and another during the winter period (December-March) dominated by immature individuals. Despite variations in the yield, the fact that the fattyacid profile across season shows a high degree of similarity (> 50%) is encouraging as it provides a minimum

assurance on the levels of various individual fattyacids present in the extract through out the year.

2.5 Conclusion

In summary, the two dominant sardines, *S. longiceps and S. fimbriata* in the Cochin coast represent an excellent source of essential fattyacids like EPA and DHA. Their PUFA profiles are complementary and suite the pharmaceutical and food processing industries to harness this resource and generate specialized drugs or PUFA-enriched food products. Though there is a strong seasonal variation in their FA profile, mainly between the spawning season and winter season, this knowledge about the variations in its key constituents across seasons will greatly help in the design of such an industry. Reasons for the seasonal fluctuations in FA profile are attributed to their respective food habits, lifecycle stages of these fishes and the temperature of the sea water.

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Chapter 3

Extraction of Polyunsaturated Fattyacids from *Sardinella longiceps* and *Sardinella fimbriata*

Contents

3.1 Introduction

Poly-unsaturated Fattyacids (PUFA) in general are known to be difficult to synthesize and can only be obtained by extraction from natural fats or fatty oils in which they naturally occur. Many of these unsaturated fatty acids are known to have therapeutic potential. Two particular polyunsaturated fattyacids which have been shown to have therapeutic efficacy, and which are difficult to obtain in pure form and high quantities, are (all-Z)5,8,11,14,17-eicosapentaenoic acid, hereinafter referred to as EPA, and (all-Z)-4,7,10,13,16,19-docosahexaenoic acid, hereinafter referred to as DHA. Hence, extraction of substantial pure PUFA from natural oils like fish oils and obtaining industrially viable amounts of EPA and DHA is a much-researched problem. Therapeutic importance of these extracts additionally mandates the purity of the entire process without any compromises on even traces of unwanted substances. Due to the commercial

implications of such a process, most of the techniques are patented. The method described in this chapter is based on US Patent 4792418 (Rubin and Rubin 1989) after normalizing to the current lab conditions.

One of the first methods for isolating PUFA (Abu-Nasr *et. al.* 1954) discloses isolation of methyl eicosapentaenoate and ethyl docosahexaenoate starting with cod liver oil acids, using preliminary concentration by precipitation of the pure complexes followed by chromatographic separations. However, this technique does not give high enough purity and chromatographic separations require undesirably high amounts of solvent. Later, another method was described for isolation of EPA and DHA from squid liver oil by saponifying with ethanolic potassium hydroxide, extracting the fatty acids with ether and methylating (Teshima *et. al.* 1978). The crude fatty acid methyl ester is purified by column chromatography on Silica Gel 60 and then the EPA is separated from the DHA by column chromatography on a mixture of silver nitrate and silica gel. The problem with this technique is that there are often traces of silver left in the final product which is extremely undesirable in a food or pharmaceutical for human consumption. Furthermore, very high amounts of solvent are necessary in order to conduct the column chromatography. Other disclosures of the use of column chromatography to separate and purify, to some extent, EPA are described in Japanese Kokai No. 56-115736 and Russian 973,128.

Another method of obtaining high purity EPA is disclosed in British patent publication No. 2,148,713. This publication describes a process in which the double bonds of the unsaturated fatty acids, in a mixture of fatty

acids, are iodinated, followed by saponification of the iodinated oil, extraction of the fattyacids from the saponification mixture, methylation of the iodinated fattyacids, separation of the fattyacids by column chromatography, and then deiodination of the desired fractions. This process permits excellent resolution of the fattyacids upon eventual column chromatography, and protects the fattyacids from oxidation during processing. When used to separate EPA from its natural source such as cod liver oil, a yield of over 90% and a purity of 96-100% may be obtained.

One of the major requirements of PUFA with therapeutic properties is that it must be in a particular cis-trans isomeric form as present in the natural compound. However, it has been found, that this method induces a substantial amount of cis-trans conversion, so that the product obtained of a purity of 96-100% is not pure all cis- EPA.

Hence, all these methods did not yield PUFA with sufficient purity and in many cases it also required extreme physical and chemical conditions as to cause some degree of degradation of the fattyacids, formation of peroxides, and/or conversion of at least some of the cis- bonds to the trans-form. Furthermore, some of them use materials which are not on the generally recognized as safe (GRAS) list of the U.S. Food and Drug Administration. In order for the final product to be used in foods and drugs it is important that there be no non-GRAS substances in the final product.

Various techniques of separation have been used with respect to fattyacids in general. Among these known techniques are separation by

means of urea complexes and separation by means of low temperature fractional crystallization (Markley 1964).

The separation technique utilizing urea involves the formation of crystalline inclusion compounds, also called adducts or complexes, between urea and various straight chain organic compounds. Inclusion compounds are combinations of two or more compounds, one of which is contained within the crystalline framework of the other. The components of an inclusion compound are each capable of separate existence, and they have no obvious way of uniting chemically. They are held together by secondary valence forces and by hydrogen bonds. Inclusion compounds differ from the conventional hydrogen-bonded systems, however, because the size and shape of the "host" and "guest" molecules are critically important in the former but may play little or no part in the latter. It is known that the more saturated a long chain fattyacid, the more readily is a urea complex formed. Thus, saturated and most mono-unsaturated compounds may be separated from polyunsaturated compounds by treatment with urea.

Low temperature fractional crystallization has been used for separation of fattyacids and monoesters, and also for the separation of glycerides of natural fats and other lipid substances. The technique involves dissolving the fattyacids in a solvent and then lowering the temperature in order to cause crystallization of the various fatty acids from the solvent. Often sub-zero (°C.) temperatures are used. This technique has many limitations, however. It is difficult to obtain great degrees of purity when separating mixtures of many fattyacids, and there are problems of mutual solubility of various acids.

Rubin and Rubin (1989) suggested a method for separating EPA and DHA from marine animal oil in substantially 100% purity, without cis-trans conversion, using only materials generally recognized as safe and capable of being used in an industrial process. Hence, this method for obtaining PUFA extracts is followed for further pharmaceutical experiments mentioned in the subsequent chapters.

3.2 Materials and Methods

This section details the complete process of extraction after the freshly sampled fish are brought to the lab.

3.2.1 Preparation of the Extract

The fishes that were brought to the laboratory in an icebox and it was washed with sterile water. The internal organs were removed and the meat cut into slices. A 1:1 (25mg) mixture of ascorbyl palmitate and gamma tocopherol was sprayed immediately all over the slices. Meat was mixed in a blender for 2 minutes. Blended meat was centrifuged at 10,000rpm for 15 minutes. Oil phase got separated and the same was mixed in a 1:1 ratio with a 1% aqueous solution of hydrochloric acid to remove all methylamines. After mixing well, the phases were allowed to separate and the oil phase removed by a separating funnel. Oil was washed with distilled water to remove any remaining acid.

3.2.2 Saponification

A known quantity of fish oil was mixed with 16ml of 95% ethanol and 4ml of water. To that, 4.6 gm of Potassium hydroxide was added.

Partial hydrolysis was performed by heating the mixture in a flask with continuous stirring for 15-20 minutes at 40°C. Mixture is cooled and poured into crushed ice and 40 ml of water added. The mixture was shaken with 4 ml ethyl ether to wash of the unsaponified materials such as cholesterol, vitamin, A, D and hydro carbons. The organic solvent, ethyl ether, was removed with a separatory funnel. Aqueous phase acidified with 28ml of 4 molar sulphuric acid. The free fattyacids were separated into a separate organic phase. The aqueous phase was discarded.

3.2.3 Urea Complexing

Free fattyacids were dissolved in 20ml of ethanol. A known amount of Urea was dissolved in 20ml ethanol with heating to obtain a urea solution. Fattyacids in ethanol was mixed with urea solution, the mixture was stirred together and cooled. Inclusion crystals of urea and saturated fattyacids appeared. These crystals were filtered out.

3.2.4 Low temperature Fractional Crystallisation

The supernatant was further cooled to -20°C overnight. At this temperature all the saturated FA and most of the monounsaturated FA formed crystals with urea. These crystals were also filtered out. The supernatant, which included mostly polyunsaturated fattyacids, was evaporated to remove the solvent. The remains from the supernatant were dissolved in 50ml ethyl ether to which was added 5% hydrochloric acid and separated the organic phase. Organic phase (the PUFA extract) was then washed with distilled water and separated.

As December happened to be the month of maximum yield of fattyacids, the extracts of that season were subjected to Gas Chromatography described in Chapter 2 and their PUFA profile estimated. This happens to be the same extract which shall be used for bioactivity studies as detailed in the subsequent chapters.

3.3 Results and Discussion

From analysis of gas chromatography, it can be found that the extraction has yielded substantially pure PUFA with a preponderance of DHA and EPA (Table 5) as against their smaller relative percentage in crude extracts. It is found that the percentage of EPA in *S. longiceps* is about 50% while that of DHA is 32%. This is in accordance with the result obtained in Chapter 1 that about 83% of total PUFA from a winter sample in *S. longiceps* consists of EPA and DHA alone. Similarly, only 25% of the total PUFA is EPA in *S. fimbriata* while DHA is substantially higher (64%). This result is also in accordance with the result from previous chapter that more than 88% of PUFA from a winter sample consists of EPA and DHA in this fish.

Table 5: EPA and DHA content of Extract and Crude Oil

	S. longiceps	***S. fimbriata***
Crude Extract (% EPA)	22.98	14.43
Crude Extract (% EPA in Total PUFA)	49.59	24.69
Purified Extract (% EPA)	50.10	25.32
Crude Extract (% DHA)	14.88	37.26
Crude Extract (% DHA in PUFA)	32.10	63.75
Purified Extract (% DHA)	31.89	64.54

3.4 Conclusion

It can be seen that the extracts prepared using this methodology have substantial amounts of EPA and DHA in them and hence can be considered as ideal samples for further comparative experiments like anti-microbial, anti-diabetic and anti-cancer studies in later chapters. Moreover, the method chosen (Rubin and Rubin 1989) further ensures that there are no cis-trans conversions or unwanted harmful impurities.

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Chapter 4

Anti-bacterial studies of PUFA extracts from *Sardinella longiceps* and *Sardinella fimbriata*

Contents

4.1 Introduction

Several bioassay reports have indicated the presence of antimicrobial compounds in marine flora and fauna (Ely *et al.* 2004, Mariana *et al.* 2000). The antibacterial activities of fattyacids in general have been documented in several pioneering studies (Kabara *et al.* 1972, Willet *et al.* 1966, Miller *et al.* 1977, Sun *et al.* 2003). Significant antibacterial properties have been observed in fattyacids from marine sources such as algae (Rosell *et al.* 1977, Kellam *et al.* 1988, Ikawa 2004, Reichelt and Borowitzka 1984) and diatoms (Findlay *et al.* 1984, Viso *et al.* 1987, Desbois *et al.* 2008). Moreover, there have been sporadic studies on the antibacterial activity of the products and by-products of marine fishes. Subsequently, antimicrobial characteristics have been reported in the epidermal mucus of fishes (Subramanian *et al.* 2008, Hellio 2002) and fish skin gelatins as well (Gó mez-Estaca *et al.* 2009).

It was Rybin *et al* (1999) who documented fish oil from a sardine species *Sardinops melanostica* inhibiting microbial growth. However, studies on antibacterial activity of PUFA extracts from fishes have been very few till date. EPA, one of the major constituent omega-3 fattyacids found in marine sources exhibits antibacterial activity against a wide range of bacteria (Desbois *et al.* 2008, Shin *et al.* 2006). Thompson *et al.* (1994) in their experiments on *Helicobacter pylori,* with an extensive range of fattyacids having different unsaturation levels, concluded that inhibitory effects increase with level of unsaturation; however they did not go beyond EPA in their unsaturation levels of fattyacids. Later, DHA having a higher unsaturation level than EPA, was demonstrated to have an inhibitory effect on Gram negative bacteria that surpasses that of EPA (Shin *et al.* 2007).

The objective of this study was to determine the antibacterial property of PUFA extracts from two different species of Sardines, viz. *S. fimbriata* and *S. longiceps*, found in the same area with in their ranges. Comparison of their respective activity profile is also attempted. Screening of such new fish species for antibacterial activity and searching for novel antibacterial drugs is important due to the constant generation of antibiotic-resistant strains of pathogenic bacteria.

4.2 Materials and Methods

4.2.1 Extract Preparation and Determination of PUFA Composition

Freshly caught samples of the fishes were subjected to the same procedure documented in Chapter 2 to obtain a mixture of substantially pure PUFA. The composition of PUFAs in the above mixture was directly analysed by Gas Chromatography (GC) adopting the fattyacid methyl ester

(FAME) method mentioned in Chapter 3 (3.3) and individual fattyacids were expressed as a percentage of total fatty acids.

4.2.2 Antibacterial Assay

The following bacteria were used for antibacterial study - *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Klebsiella pnuemoniae, Enterobacter aerogenes, Proteus vulgaris, Salmonella enterica* and *Pseudomonas aeruginosa*. These bacteria have been reported to cause infections and health hazards among human beings. Bacteria were cultured in nutrient agar at 37°C and maintained on nutrient agar slants. Each bacterial strain was then transferred into a separate test tube containing nutrient broth to reactivate them by culturing overnight at 37°C. Agar well diffusion method (Perez *et al.* 1990) was used to screen the antibacterial activity of fish extracts. Invitro antibacterial activity was screened using Mueller Hinton Agar (MHA) obtained from Himedia (Mumbai). The MHA plates were prepared by pouring 20ml of molten media into sterile petri plates. The plates were allowed to solidify for 5min and three wells of 10mm diameter were cut in all the plates. The bacterial strains were inoculated separately in peptone water. It was incubated at 37°C for 4hrs (till it became turbid) and then the inoculum suspension was swabbed uniformly on the medium. To these plates, 100µl of different dilutions of fatty acid extracts from the two fishes in acetone were introduced. For each bacterial strain, controls were maintained where acetone alone was used. The plates were incubated at 37°C for 24 h. Zones of inhibition were measured at the end of the incubation period. The experiment was repeated four times for each variant and the mean and SD calculated. Photographs were also taken showing the peak activity of fish extracts on bacterial cultures.

4.2.3 Statistical analysis

The results were analyzed using 1-way ANOVA (Zar *et al.* 1984) against the control followed by Fisher's LSD test and $p<0.01$ was considered as significant.

The Minimum Inhibitory Concentration (MIC), which is the lowest dilution of the test agent at which no visible growth occurred, was calculated for the inhibited bacteria keeping $p< 0.01$ as significant.

4.3 Results

Of the eight bacterial cultures, four of them showed a positive activity towards the fish-oil extracts in comparison with pure acetone control. Two of them showed no activity while two others showed a negative activity in comparison with the acetone extract. Results are summarized in Table 6. It may be noted that both gram-positive bacteria used in the study showed positive activity.

Table 6: Summary of Antibacterial activities of PUFA extracts from *S. longiceps* and *S. fimbriata*

Bacteria	Type of Bacteria	Type of Activity	
		S. longiceps	***S. fimbriata***
Staphylococcus aureus	Gram +ve	Postive	Postive
Enterococcus feacalis	Gram +ve	Positive	Positive
Escherichia coli	Gram –ve	Positive	Positive
Klebsiella pneumoniae	Gram –ve	Negative	Negative
Enterobacter aerogenes	Gram –ve	Negative	Negative
Pseudomonas aeruginosa	Gram –ve	Positive	Positive
Proteus vulgaris	Gram –ve	None	None
Salmonella enterica	Gram –ve	None	None

Both extracts showed maximum inhibitory effect on *P aeruginosa* while *S. fimbriata* extract also showed remarkable inhibition of *E. feacalis*. Both *S. aureus* and *E. coli* showed marginal response with both extracts though *S. fimbriata* extract showed a higher inhibitory effect between the two. *K. pneumoniae* and *E. aerogenes* showed an inhibitory response to pure acetone control solution but also demonstrated significant negative activity with increased concentrations of fish-oil extracts. *P. vulgaris* and *S.enterica* showed no response either to acetone or to various concentrations of fish-oil extracts. Details of the values for both species are shown in Table 7 and Table 8. Values of high significance ($p < 0.01$) are marked. For details, please see Appendix.

Table 7: Antibacterial activity profile of *Sardinella fimbriata*.

Bacteria	Inhibition Zone diameter in mm (mean + SD)						
	Concentration (% in Acetone solution)						
	0%	**5%**	**10%**	**20%**	**30%**	**50%**	**80%**
S. aureus	13.75 ± 0.50	13.75 ± 2.22	13.75 ± 1.71	14.25 ± 0.50	14.25 ± 2.22	18.00 ± 0.82*	21.75 ± 2.06*
E. feacalis	10.00 ± 1.15	10.25 ± 1.71	10.25 ± 0.96	13.25 ± 0.96*	15.00 ± 0.82*	21.50 ± 2.38*	24.00 ± 1.41*
E. coli	13.50 ± 2.08	13.50 ± 1.29	13.75 ± 1.71	13.75 ± 0.50	14.50 ± 0.58	16.25 ± 0.96*	18.00 ± 1.15*
K. pneumoniae	21.75 ± 1.50	21.75 ± 1.26	21.75 ± 1.89	15.75 ± 1.26	14.00 ± 3.37	10.25 ± 2.50	9.75 ± 1.71
E. aerogenes	15.25 ± 0.96	15.25 ± 0.50	13.75 ± 0.96	11.50 ± 1.00	10.75 ± 1.26	7.75 ± 1.26	6.75 ± 1.50
P. aeruginosa	12.50 ± 0.58	13.25 ± 1.50	23.00 ± 3.56*	24.00 ± 2.16*	24.75 ± 2.22*	26.25 ± 1.71*	29.00 ± 0.82*

* $p < 0.01$

Table 8: Antibacterial activity profile of *Sardinella longiceps*

Bacteria	Inhibition Zone diameter in mm (mean + SD)						
	Concentration (% in Acetone solution)						
	0%	5%	10%	20%	30%	50%	80%
S. aureus	13.75 ± 0.50	13.75 ± 1.26	14.00 ± 0.82	14.00 ± 0.82	14.25 ± 1.71	16.25 ± 0.96*	19.25 ± 0.96*
E. feacalis	10.25 ± 1.71	10.25 ± 0.50	10.25 ± 0.96	10.25 ± 0.96	10.50 ± 0.58	10.75 ± 1.26	11.00 ± 0.82
E. coli	13.75 ± 2.50	14.00 ± 1.63	14.00 ± 0.82	14.00 ± 0.82	14.00 ± 1.41	15.00 ± 1.41	15.00 ± 0.82
K. pneumoniae	22.25 ± 1.71	22.25 ± 1.26	22.00 ± 0.00	18.50 ± 1.29	17.50 ± 1.00	12.75 ± 1.89	9.75 ± 1.26
E. aerogenes	15.50 ± 0.58	15.25 ± 1.26	15.25 ± 0.50	12.50 ± 1.00	10.75 ± 1.26	10.00 ± 0.82	9.75 ± 0.50
P. aeruginosa	12.50 ± 1.29	13.25 ± 0.96	13.00 ± 0.82	15.00 ± 0.00*	18.00 ± 0.82*	20.00 ± 0.82*	21.25 ± 0.96*

* $p < 0.01$

A comparison of the activity profile at high (80%) and mid (50%) concentrations indicate that *S.fimbriata* extracts are significantly better than *S.longiceps* ones for *P. aeruginosa* and *E. feacalis*. At high concentrations, this is also true for *E.coli*. At mid-concentration *S.fimbriata* extracts demonstrated a higher activity of borderline significance ($p<0.05$) for *S. aureus*. The trend of activity for both species based on high and mid concentration along with their significance values are illustrated in Figure 12 and Figure 13. It is clear that *S. fimbriata* extracts had an overall higher inhibitory action on all the test species as compared to *S. longiceps*. Images 1-4 illustrate the peak activity detected in each of the four bacterial strains. MIC, calculated with a significance of $p<0.01$, is summarized in Table 9. *S. fimbriata* showed remarkable lower levels of MIC on inhibited strains. MIC was lowest for *P aeruginosa* among the four inhibited bacterial strains.

Table 9: Minimum Inhibitory Concentration of PUFA extracts for Antibacterial activity

Bacteria	Concentration (% in Acetone solution)	
	S.fimbriata	***S.longiceps***
S. aureus	50	50
E. feacalis	20	NA
E. coli	50	NA
P. aeruginosa	10	20

The PUFA extracts were analyzed by GC to identify the fatty acids present in the extract. The major compounds identified were unsaturated fatty acids ranging from C20 to C24 with a preponderance of C20:5 (EPA) and C22:6 (DHA) PUFA. GC analyses of the PUFA from the fish *S. longiceps* showed an EPA content of 55.54% and a DHA content of 32.52%. The GC analyses of the PUFA from the fish *S. fimbriata* gave a much lower EPA content of 24.02% but a correspondingly higher DHA value of 65.82%.

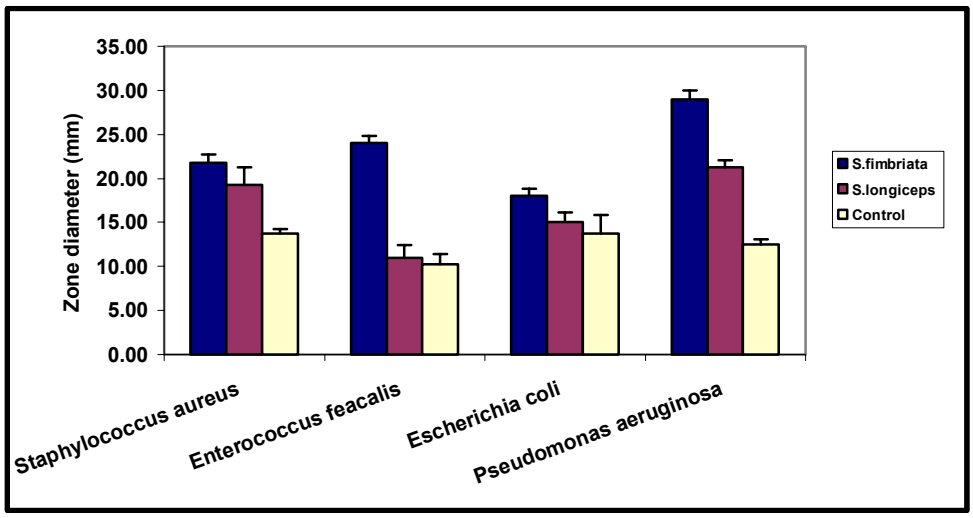

Figure 12: Comparison of antibacterial activity at highest concentration

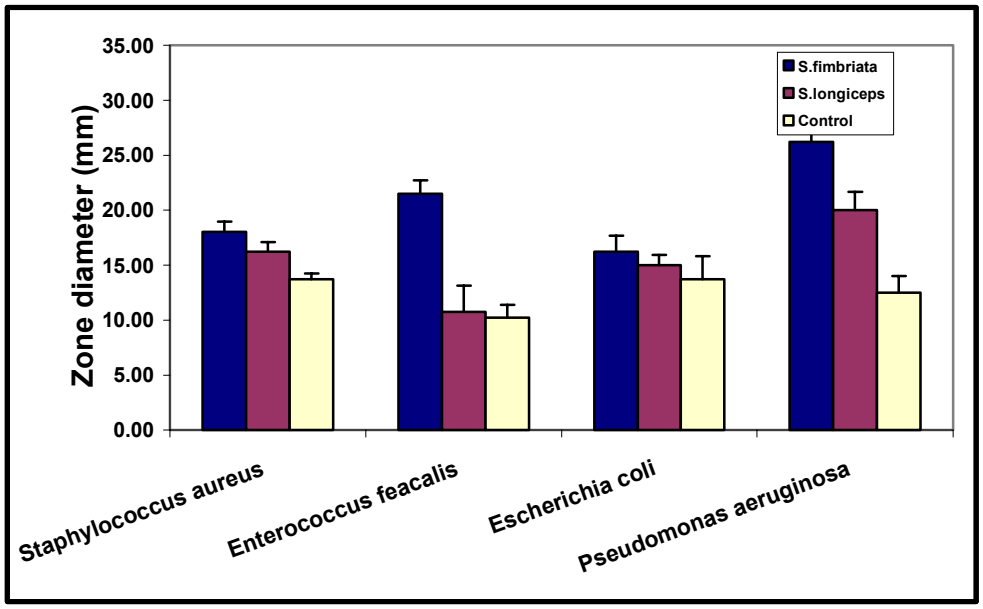

Figure 13: Comparison of antibacterial activity at mid-concentration

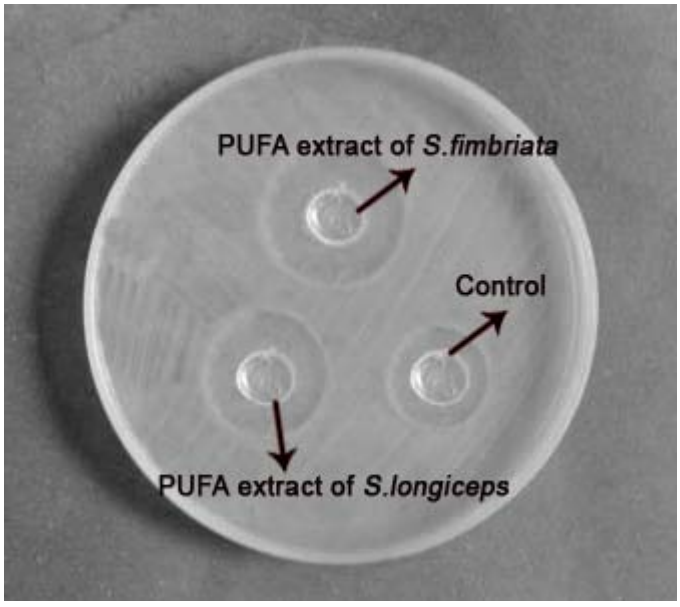

Image 1: *Staphylococcus aureus*. Acetone control showed an activity of 14mm, *S. fimbriata* extract at 80% concentration showed an activity of 22mm while *S. longiceps* extract at same concentration showed an activity of 19mm.

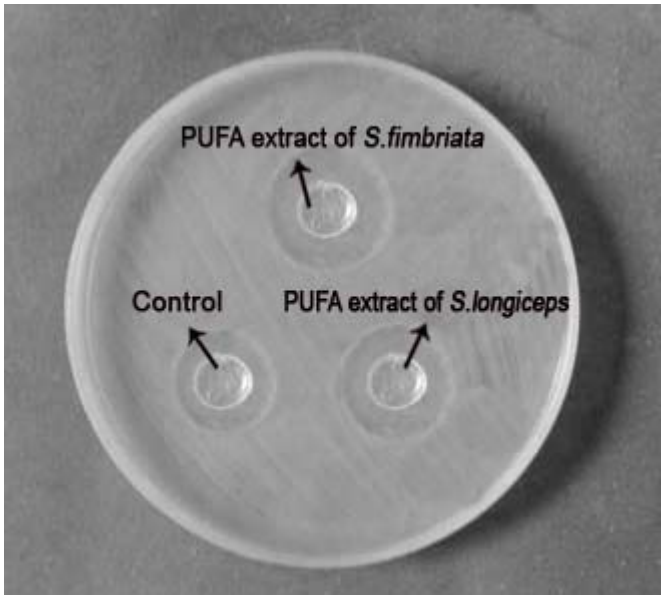

Image 2: *Escherichia coli*. Acetone control showed an activity of 14mm, *S. fimbriata* extract at 80% concentration showed an activity of 18mm while *S.longiceps* extract at same concentration showed an activity of 15mm.

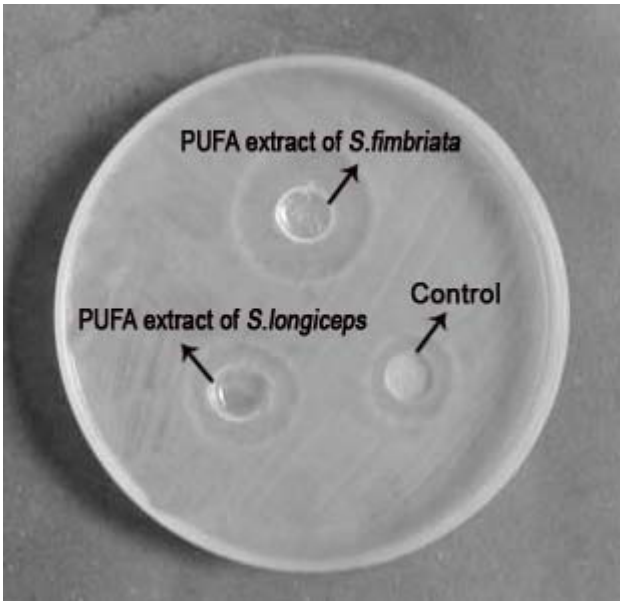

Image 3: *Enterococcus feacalis*. Acetone control showed no activity (10mm), *S. fimbriata* extract at 80% concentration showed an activity of 25mm while *S. longiceps* extract at same concentration showed an activity of 11mm.

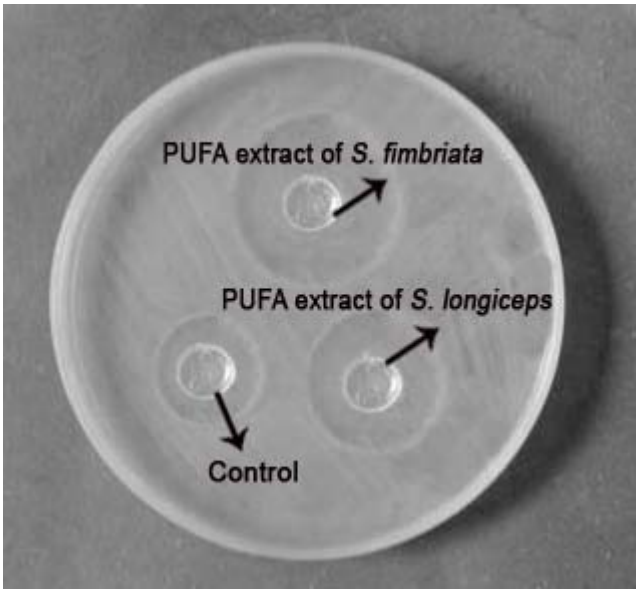

Image 4: *Pseudomonas aeruginosa*. Acetone control showed an activity of 13mm, *S. fimbriata* extract at 80% concentration showed an activity of 28mm while *S. longiceps* extract at same concentration showed an activity of 21mm.

4.4 Discussion

The results showed that the DHA-rich *S. fimbriata* extracts have an overall higher activity against all the four bacterial strains as compared to *S. longiceps*. It tallies with the generalization by Thompson *et al.* (1994) that inhibitory effects on certain bacterial strains increase with levels of unsaturation. Higher activity shown by DHA-rich extracts on Gram negative bacterial strains in this study also matches with contemporary studies on DHA (Shin *et al.* 2007). However, PUFA extracts from both species showed inhibitory activity against both gram-positive and gram-negative bacterial strains. This is congruent with previous results on EPA and DHA showing activity against a range of both gram-positive and gram-negative bacteria

(Desbois *et al.* 2008, Shin *et al.* 2006, Shin *et al.* 2007). However, it is noteworthy that bacterial strains that showed either negative or no activity were all gram-negative (Table 6).

Long chain fatty acids are well-known to be inhibitory on gram positive bacteria even at low concentrations (Kabara *et al.* 1977, Kodicek 1949). However, gram negative bacteria are known for their complex lipopolysaccharide layer as compared to the former. But, PUFA are known to have inhibitory effect on these strains as compared to saturated fattyacids as they are readily incorporated into the outer cell membranes of these organisms, where they significantly increase membrane fluidity. It is possible that by opening up permeability channels, the concentration gradients necessary between the organism and its environment may be dissipated resulting in fatality of the organism (Thompson *et al.* 1994).

According to Shin *et al.* (2006), EPA can reduce the viability of *P. aeruginosa*. In their experiment, scanning electron microscopy (SEM) study of bacterial cells clearly exhibited the antibacterial effect of EPA evidenced by the damages found in the outer membrane of the cells when treated with EPA. Shin *et al.* (2007) later opinioned that DHA is even more potent against this bacterium. High positive results in this study for inhibiting cultures of *P. aeruginosa* could also be due to high DHA and EPA concentrations in both the extracts. Higher DHA concentration of *S. fimbriata* correlates with greater inhibitory effect on this bacterium.

Algal extracts with high EPA concentrations are known to show high levels of inhibitory effects on *S. aureus* (Desbois *et al.* 2008). This is fairly

in line with the results of the current study where both extracts in mid-concentrations (50%) itself showed significant ($p<0.01$) activity. Studies by Shin. *et al.* (2006) also exhibited inhibitory actions of EPA on this bacterium. It was also found that EPA and DHA have similar antibacterial action on this bacteria (Shin *et al.* 2007).

Algal extracts (Tuney *et al.* 2006, Kandhasamy and Arunachalam 2008) from marine sources are known to show moderate inhibitory activity on *E. coli* as also apparent in this study. Only extracts from *S. fimbriata* showed significant activity on *E. coli* and the activity even at high concentration is not that prominent. DHA is known to have a better inhibitory effect as compared to EPA against this bacterium (Shin *et al.* 2007).

PUFA (Shin *et al.* 2007) and marine algal extracts (Desbois *et al.* 2008) are also known to show moderate activity on *E. feacalis*. No comparative studies have conducted specifically on this Gram positive bacterium and hence the response is not clear if DHA or EPA has a higher inhibitory effect. In the current study, DHA-rich *S. fimbriata* extract demonstrated a significantly greater action on *E. feacalis*.

It may be worth highlighting that the growth media also seem to play an important role in the determination of the antibacterial activity. Muller-Hinton agar appears to be the best medium to explicate the antibacterial activity (Lin *et al.* 1999) and the same was used in the present study.

4.5 Conclusion

In conclusion, widely available marine fishes like sardines serve as a rich source of DHA and EPA and have the potential to be an excellent source of pharmaceuticals that target microbes which mar human and animal life. Fish oil extracts from *Sardinella fimbriata* have higher concentrations of DHA than EPA and hence seem to have greater potential in inhibiting the growth of several strains of pathogenic bacteria including several gram negative strains and this result has been supported by earlier studies. The onus is now on the pharmaceutical industry to harness this cheap source to transform these raw materials by efficient industrial techniques to create potent drugs to be used as anti-microbial agents.

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Chapter 5

Anti-diabetic studies of PUFA extracts from *Sardinella longiceps* and *Sardinella fimbriata*

Contents

1.1 Introduction

Diabetes mellitus is a chronic metabolic disorder with a worldwide incidence of 5% in general, with a suffering population of over 246 million and its proliferance is increasing steadily with changing life styles (Anonymous 2006). It is generally associated with complications like hypercholesterolemia, hypertriglyceridemia, atherosclerosis, coronary heart disease, renal malfunctioning and hypertension (Simopoulos 1991).

Diabetes can arise from a failure of the pancreas to secrete insulin. Insulin makes possible adequate utilization of glucose by the tissues at normal blood sugar levels. When sufficient insulin is not secreted by the beta cells of the Islets of Langerhans in the pancreas, higher concentrations of blood sugar are produced and this partially restores a more normal rate of glucose utilization.

5.1.1 Lipids and Diabetes

Lipid is a name given to compounds that are insoluble in water, and this property means that a lipid has to be transported bound to other molecules, to enable it to be transported in an aqueous environment. There are four major groups of lipids in human body viz. Cholesterols, Triglycerides, Phospho-lipids and Fatty acids (Thomas 2000).

Cholesterol is present in the diet and is required by all cells. Cells can synthesize cholesterol, which is the precursor for the steroid hormones mostly made in the adrenal glands and the gonads. Triglyceride is the major lipid found in the diet and it is broken down to yield glycerol and fattyacids.

Lipid is transported in the blood in small particles called lipoproteins, synthesized in the liver and gut. These particles are a complex of triglyceride, cholesterol, phospholipids and proteins. There are five main types of lipoprotein, classified according to size and density. The five lipoproteins, in size order are Chylomicrons, Very low density lipoprotein (VLDL), Intermediate density lipoprotein (IDL), Low density lipo protein (LDL) and High density lipoprotein (HDL). Of which HDL, LDL and VLDL are of clinical significance

VLDL is a medium sized particle, containing mainly triglyceride, are synthesized in liver. The main function of VLDL is to transport lipids synthesized in the liver to parts of the body which require triglyceride as an energy source or for storage.

LDL is a small particle rich in cholesterol derived from the metabolism of VLDL. They contain an important apolipoprotein called apo B-100, which is responsible for recognizing an LDL receptor on the surface of cells.

HDL is the smallest of the lipoproteins but the densest, and contains the highest protein concentration. The role of HDL is to remove cholesterol from peripheral cells and plasma, transporting the cholesterol to the liver for reprocessing or excretion.

The relative levels of LDL and HDL have been shown to be important in assessing the risk of developing atherosclerosis. There is much research showing links between the development of heart disease and the presence of lipid in the blood. The major findings have shown that increased LDL correlates with an increased risk of heart disease while increased HDL correlates with a decreased risk of heart disease.

5.1.2 Kidney Disease and Diabetes

Diabetic condition in a long term can result in kidney disease (Simopoulos 1991). Disease of the kidney often manifests itself with symptoms relating to biochemical changes that can be detected in the blood. The two important measures of renal function are the urea and creatinine

concentrations (Thomas 2000). Urea is the end product of amino acid metabolism, formed from the break down of proteins. It is very water soluble and is freely filtered by the glomerulus and passes into the kidney tubules. Creatinine is derived from phosphocreatine found in muscle. Creatinine is filtered by the glomerulus and passes through the nephron without reabsorption taking place. An increase in the amount of urea and creatinine shows impaired kidney function.

The characteristic feature of diabetes mellitus is a diminished ability to utilize glucose at ordinary blood sugar levels. This produces a diminished glucose tolerance with a raised blood sugar, which is often well above the renal threshold for glucose. There is also increased mobilization of fat. More fat is used for supplying the energy requirements of the body. As a result, blood fat and cholesterol are also increased.

5.1.3 PUFA and Diabetes

Poly-unsaturated Fattyacids (PUFA), particularly eicosapentaenoic acid (EPA; 20:5n23) and docosahexaenoic acid (DHA; 22:6n23) present in marine sources, have been found to have healing effects against several of these complications (Simopoulos 1991). Fish oil or fish oil supplemented diets, PUFA extracts from fish oils or substantially pure EPA or/and DHA have all been used in several invivo experiments. Though the utility value of fish oils in the treatment of diabetes is univocal, there is considerable disparity between results of most of these studies. Disparity subsumes in several factors; response of fish oil or EPA/DHA on normal humans and humans suffering from ailments varies considerably. Responses of PUFA extracts on the lipid profile of mice are sometimes different from that of

humans (De Caterina *et al.* 2007). It is also established that EPA and DHA have divergent effects on total cholesterol and triglycerides with exact nature of their action still unknown (Hansena *et al.*1998). However, in all these studies, profiling of blood glucose, total, LDL and HDL cholesterol and triglycerides seems to be the most widely used strategy to prove beneficial or adverse effects.

Studies have also shown that Omega-3 fattyacids offer a direct or indirect reno-protective effect in diabetes patients (Holm *et al.* 2001, van der Heide *et al.*1993). Diet supplemented with Omega-3 fattyacids from plant sources is known to prevent diabetic renal injury and can even reverse kidney damage in mice subjects (Garman *et al.*2009). Hence, two additional parameters serum urea and serum creatinine were considered worth monitoring.

Hypocholesterolemic effect of fish oil from *S. longiceps* has also been reported (Sen *et al.*1977). However, there has not been any PUFA estimation done for the equally prolific *S. fimbriata*. The purpose of this study was to determine and compare the hypolipidimic and anti-diabetic properties of PUFA extracts from these two widely available sardines in Cochin coast obtained from the same area in its range. A comparison of their recovery profile is also attempted.

5.2 Materials and Methods

5.2.1 Extract Preparation and Determination of PUFA Composition: Freshly caught samples of the fishes were subjected to the procedure documented in Chapter 2 to obtain a mixture of substantially pure PUFA. The composition of PUFAs in the above mixture was directly analysed by Gas Chromatography (GC) adopting the fatty acid methyl ester (FAME) method mentioned in Chapter 3 (3.3) and individual fattyacids were expressed as a percentage of total fatty acids.

5.2.2 Experimental Animals

Adult male albino mice (230-260 g) were obtained from the animal house of College of Veterinary and Animal Sciences, Mannuthy and housed at 22±2 in an air-conditioned chamber. Animals were maintained throughout the study at 24-28 °C, were fed a standard laboratory rat diet and water *ad libitum* and maintained in specious polypropylene cages and well ventilated animal house with 12 hr dark and light cycle. The experimental protocol has been approved by the animal ethics committee.

5.1.3 Induction of experimental diabetes

Alloxan tetra hydrate (Sigma) was dissolved in sterile distilled water. Diabetes was induced in 18 mice by intra-peritoneal injection of 185 mg/kg (5%) of Alloxan tetra hydrate (Kavitha *et al.* 2007). The mice were fasted 12hrs before and after the alloxan injection. The mice with blood glucose above 250 mg/dl, which lasted for at least one week, were selected for the experiment.

5.1.4 Study design

The mice were randomly divided into four groups of eight numbers each and the groups were labeled I-IV as follows.

Group I : **Standard Control (C).** Normal mice with no extra diet components.

Group II : **Diabetic Control (DC).** Mice induced with diabetes with no extra diet components.

Group III : ***Sardinella longiceps* Group (SL)**. Diabetic mice orally administered with PUFA extract of *Sardinella longiceps* (1ml) daily using intra gastric tube for 28 days.

Group IV : ***Sardinella fimbriata* Group (SF)**. Diabetic mice orally administered with PUFA extract of *Sardinella fimbriata* (1ml) daily using intra gastric tube for 28 days.

5.1.5 Blood sampling

The blood was collected from orbital plexus and serum was separated by immediate centrifugation of blood samples using semi ultra cooling centrifuge at 3000 rpm for 5 minutes at room temperature. This was repeated on the 0^{th}, 7^{th}, 14^{th}, 21^{st} and 28^{th} day of the experiment from each individual mouse in the set. The following bio-chemical parameters, viz. glucose, total cholesterol, HDL cholesterol, LDL Cholesterol, triglycerides, urea, creatinine, were estimated for each of the samples.

5.1.6 Analysis of Biochemical Parameters

Fasting blood glucose was estimated by glucose oxidase-peroxidase method (Marks 1959). Serum was analysed and estimated for total cholesterol (Zak 1957), HDL (Burstein and Scholnick 1972) and LDL Cholesterol (Warnick *et al.* 1990) levels and triglycerides (Rice 1970). Urea and Creatinine levels were estimated using procedures of Chaney and Marbach (1983) and Jaffe Reaction (John and Keith 1994) respectively. For each day, all parameters were expressed as a Mean ± SD across 5 samples in each set.

Estimation of Blood Glucose (Marks 1959)

To 1.1ml of 0.9% NaCl, 0.4ml of 5% $ZnSO4,7H_2O$ solution and 0.4ml of 0.3N NaOH were added. To this 0.1ml of blood was added, mixed well, centrifuged and separated the supernatant. 1ml of the supernatant was transferred to a test tube. Into other two test tubes were added 1ml of water (blank) and 1ml of standard glucose solution. 3ml of glucose oxidase reagent was added to each at half minute intervals. This was mixed gently for not more than ten seconds and absorbance read in a Shimadzu-UV spectrophotometer-1601 at 625nm. The values were expressed as mg glucose/dl.

Estimation of Total Cholesterol (Zak 1957)

0.1ml serum was added to 10ml of the Ferric chloride- acetic acid reagent in a glass stoppered centrifuge tube, Mixed well and kept for fifteen minutes for the proteins to flocculate. This was centrifuged and 5ml of the clear supernatant was transferred to a glass stopperd centrifuge tube. To two other tubes, 5ml of cholesterol standard & 5ml of ferric chloride- acetic acid reagent were added separately. 3ml of sulphuric acid was added to all the three tubes, stoppered tightly and mixed by repeated inversion. The stopper was loosened carefully and kept for twenty minutes and then absorbance read at 560nm. The values were expressed as mg /dl.

Estimation of Total Triglycerides (Rice 1970)

0.1ml of serum, standard and distilled water (blank) are added in 3 stoppered centrifuge tubes. 3.9ml isopropanol was added to each test tube, mixed well and then 400mg activated alumina added. This was shaken in a vortex mixer for 15min and centrifuged at 4000rpm for 5min. 2ml of supernatants were transferred to another 3 test tubes. 0.6ml of saponification reagent (5.0g of potassium hydroxide dissolved in 60ml distilled water and 4.0ml isopropanol) was added, stoppered and incubated at 60ºC for 15 minutes and then cooled and 1ml metaperiodate solution added. This was mixed and 0.5ml acetyl acetone reagent added, incubated at 50ºC for 30minutes, cooled and absorbance read at 405nm. The value of triglyceride in plasma was expressed as mg per dl.

Estimation of high-density lipoprotein fraction (HDL) (Burstein and Scholnick 1972)

To 0.2ml of serum, 0.09 ml of heparin-manganous chloride reagent was added and mixed well. This was allowed to stand at RT for 10 minutes and then centrifuged at 4000 rpm for 10 minutes. The supernatant represented HDL fraction. 0.1ml of supernatant was taken for the estimation of cholesterol

Estimation of low-density lipoprotein (LDL) Friedewald equation (Warnick *et al* 1990)

- LDL = Total cholesterol - HDL - VLDL
- VLDL= Triglyceride/5

Estimation of Urea (Chaney and Marbach 1983)

To 0.02ml of serum, 0.2 ml of urease enzyme reagent was added. Urease reagent, 0.20 ml was added to two other test tubes, standard and blank and 0.02ml of urea solution was added to the standard. This was kept in a water bath at 37ºC for 15minutes. 5ml of the phenol-nitroprusside solution was added, mixed, and followed with 5ml of hypochlorite reagent in all the three test tubes. This was kept at 37 ºC for 15minutes and then absorbance read at 630nm. The values were expressed as mg per dl.

Estimation of Creatinine – Jaffe Reaction (John and Keith 1994)

To 4.5ml of tungstic acid reagent 0.5ml of serum was added. This was allowed to stand for 5mts and centrifuged at 3000rpm. To three test

tubes, 3ml of supernatant (protein free solution), 0.1ml creatinine standard and 3ml distilled water were added separately. One ml creatinine reagent and 0.5ml NaOH were added to all the 3 test tubes. This was allowed to stand for 5mts and absorbance read at 540nm. The values were expressed as mg/dl.

5.1.7 Statistical Procedure and Analysis

The results were analyzed using pair-wise 1-way ANOVA against diabetic control and $p<0.01$ was considered as significant (Zar 1984). Recovery percentage of biological parameters were calculated using the formula

Recovery % = (Diabetic Control – Recovered Value) ÷ (Diabetic Control – Standard Control) * 100

5.3 Results

Anti-diabetic effects of PUFA on various parameters of blood are summarized in Table 9; values obtained for each parameter in each set across 5 samples are expressed as Mean±SD. With the possible exception of the means of the response of serum glucose for both the fish extracts, all the other means were found to be different with significance of $p < 0.01$ (See Appendix)

Table 10: Bio-chemical parameters analysed for all the sets

Set	Measurement	Start(mg/dl)	End (mg/dl)
Control Set	**Glucose**	81.20±0.84	80.60±0.55
	Total Cholesterol	72.00±1.22	71.20±0.45
	Triglycerides	82.00±1.22	81.00±1.00
	Urea	39.20±0.84	39.20±0.45
	Creatinine	0.24±0.05	0.26±0.05
	HDL Cholesterol	39.60±0.55	39.60±0.55
	LDL Cholesterol	16.00±1.12	15.40±0.73
Diabetic Control Set	**Glucose**	322.60±2.51	320.00±1.00
	Total Cholesterol	181.20±0.84	180.80±0.84
	Triglycerides	251.60±1.34	250.80±0.84
	Urea	128.00±1.22	128.00±1.00
	Creatinine	3.02±0.08	2.96±0.05
	HDL Cholesterol	20.00±1.00	19.60±0.55
	LDL Cholesterol	110.88±1.39	111.04±0.95
Diabetic Set administered with PUFA extract from *Sardinella longiceps*	**Glucose**	321.40±2.07	311.00±1.00
	Total Cholesterol	182.00±1.22	129.00±1.87
	Triglycerides	251.60±1.52	183.60±0.89
	Urea	129.20±0.84	122.40±0.55
	Creatinine	2.96±0.05	2.44±0.05
	HDL Cholesterol	19.80±0.84	30.40±0.55
	LDL Cholesterol	111.88±1.96	61.88±1.23
Diabetic Set administered with PUFA extract from *Sardinella fimbriata*	**Glucose**	322.00±2.45	310.40±0.89
	Total Cholesterol	182.20±0.84	117.60±2.51
	Triglycerides	251.80±0.84	166.40±0.55
	Urea	128.60±0.55	120.40±0.55
	Creatinine	2.98±0.08	2.28±0.08
	HDL Cholesterol	20.00±1.00	34.00±1.00
	LDL Cholesterol	111.84±1.61	50.32±1.62

5.3.1 Effects on Serum Glucose

Serum Glucose levels quadrupled in alloxan-induced mice at the start of the experiment and remained so through out the experimental period. However, groups administered with both fish extracts showed a small decrease in levels of blood glucose from the 7th day sample itself (Figure. 14). Though the decrease was statistically significant ($P < 0.01$), the percentage decrease does not indicate a recovery worth further assessment. Hence, both fish extracts do not largely impact the serum glucose level in diabetes induced animals.

5.3.2 Effect on Cholesterol and Triglycerides

Total Cholesterol levels more than doubled and triglyceride levels tripled in alloxan-induced mice at the start of the experiment and remained so through out the experimental period. However, groups administered with both fish extracts showed a significant recovery in total cholesterol and triglycerides (Figure. 16,17). About 10-15% of improvement of total cholesterol and triglycerides was noted after every seven day interval when the sampling was done.

In terms of its components, LDL Cholesterol level (Figure. 19) also reduced significantly across the experiment for groups administered with extracts. Recovery values shot up by 15% after the 7th day of administration of the drug for both the species but after that recovery with S. longiceps extract was slower by about 5% as compared to that with S. fimbriata extract for every next 7th day of sampling. HDL Cholesterol (Figure. 18) which came down drastically in diabetic control improved significantly towards the end of the experiment. This also showed a remarkable improvement after the first 7 days itself with about 20% recovery for both

species. Thereafter, *S. fimbriata* extract showed a slightly better recovery than *S. longiceps* extract.

Recovery plots for all four showed that sets treated with extracts from *S. fimbriata* was recovering better than the ones treated with extracts from *S. longiceps* and this became more apparent towards the end of the experiment (Fig. 20,21,22,23).

LDL and HDL Cholesterol levels almost recovered 60% in one month after being treated with the extract of *S. fimbriata*. Triglycerides and total cholesterol levels recovered by 50% for this particular fish extract. Recovery was obvious in all these parameters from the first collection after drug administration (7^{th} day) itself. A minimum of 35-40% recovery in all these parameters were observed with both species of fishes after a month and recovery curves indicated that the sets were still improving with good chances of reaching total normalcy. In summary, it can be concluded that there is considerable positive impact on cholesterol and triglycerides of diabetic mice subjects due to the administration of the fish extracts.

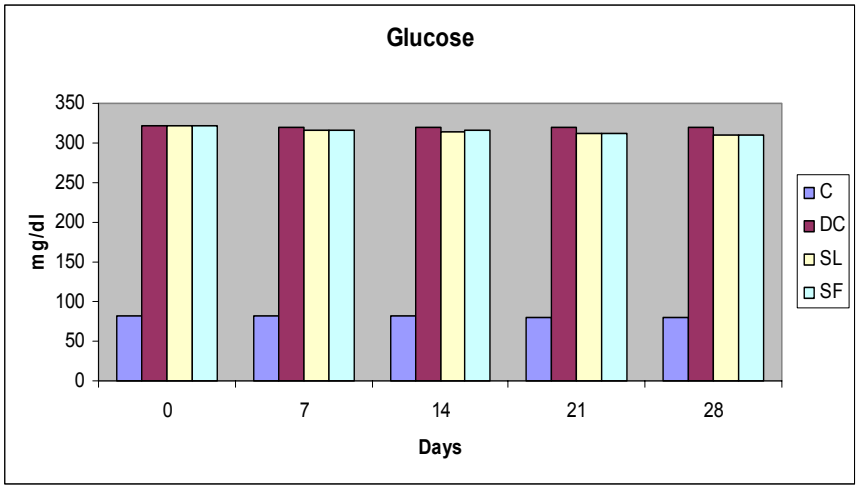

Fig. 14: Glucose Variation in the four experimental groups

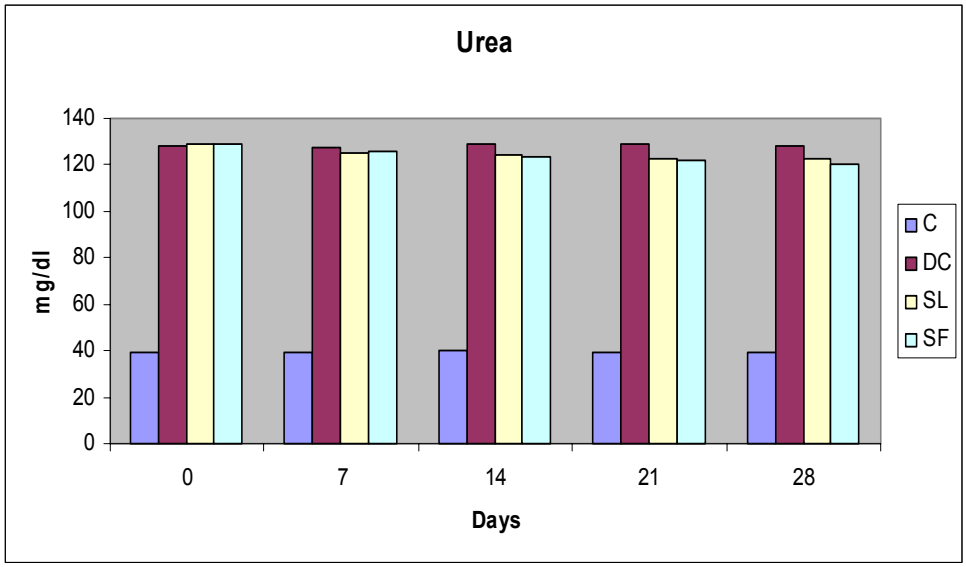

Fig. 15: Urea Variation in the four experimental groups

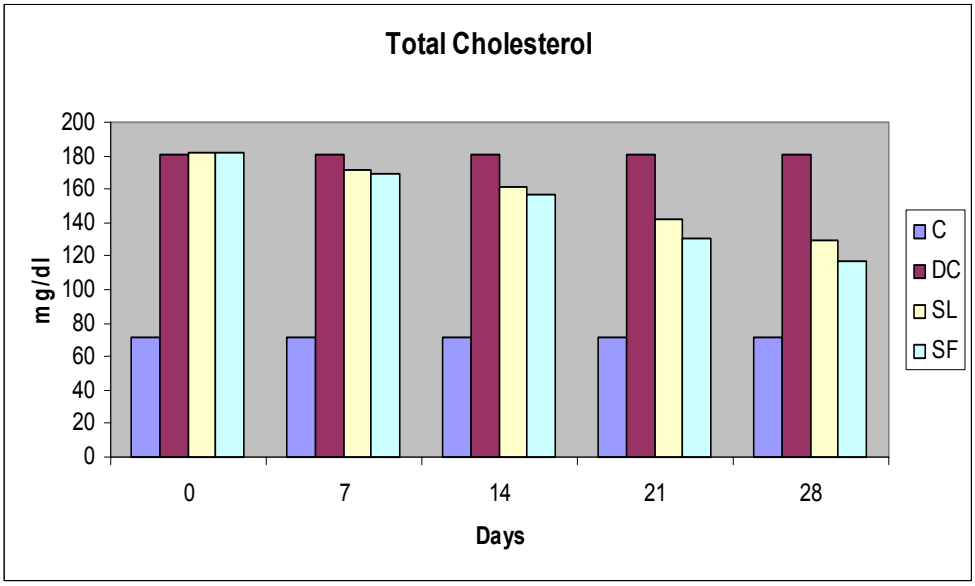

Fig. 16: Total Cholesterol Variation in the four experimental groups

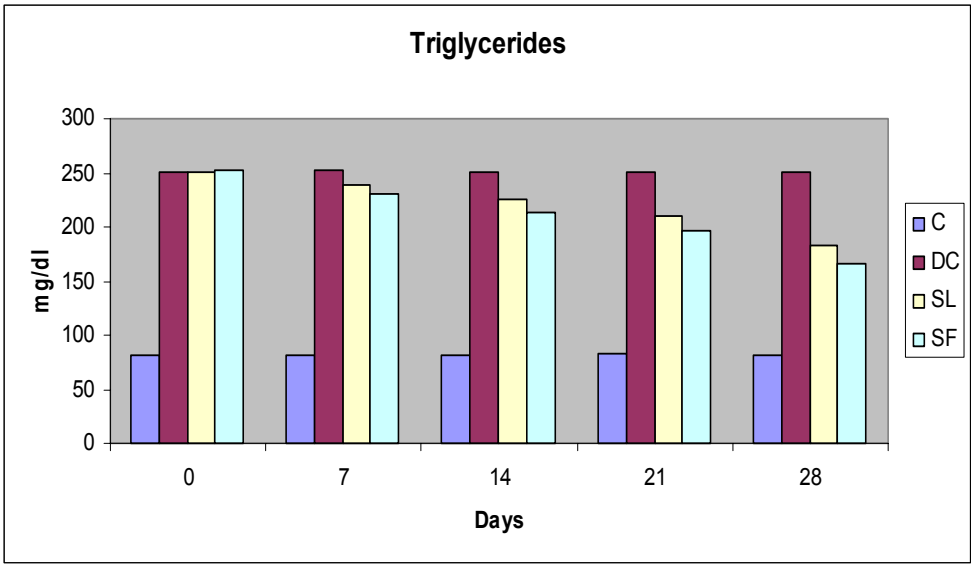

Fig. 17: Triglycerides Variation in the four experimental groups

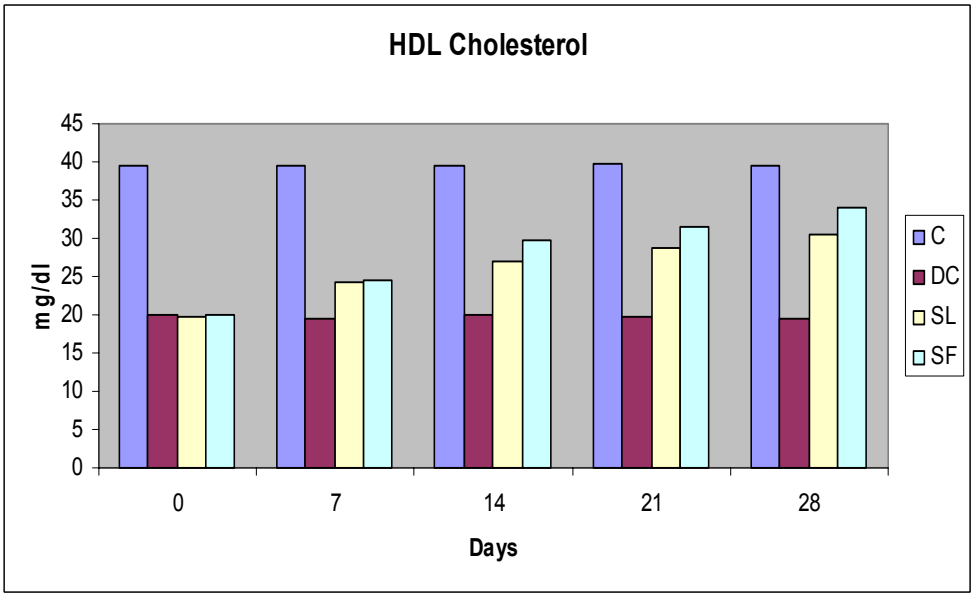

Fig. 18: HDL Cholesterol Variation in the four experimental groups

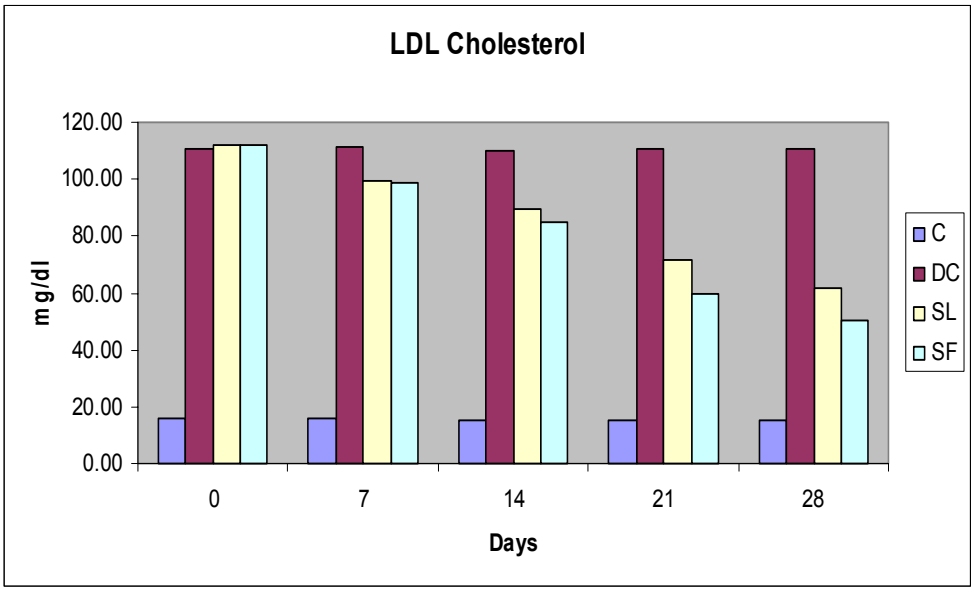

Fig. 19: LDL Cholesterol Variation in the four experimental groups

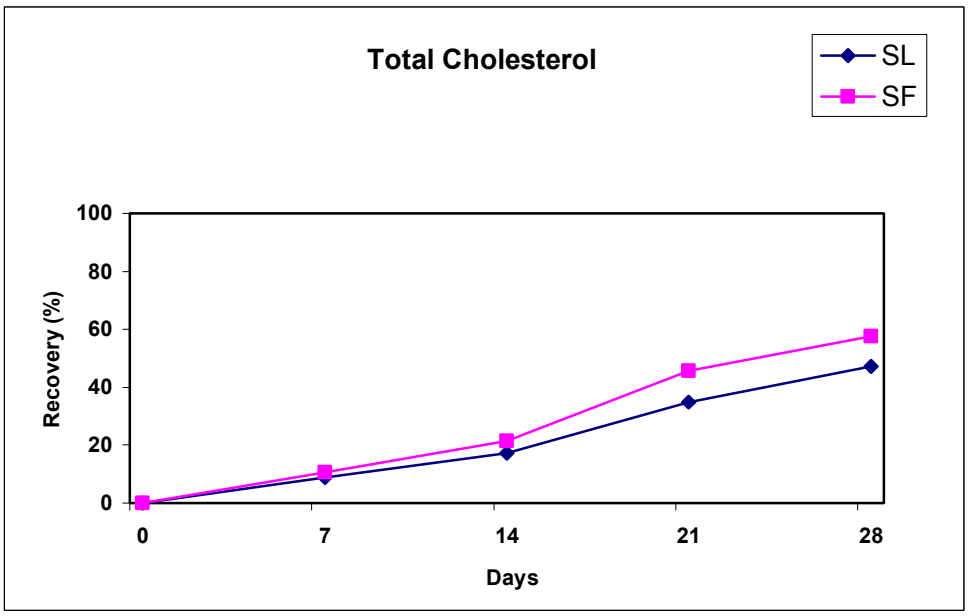

Fig. 20: Recovery of Total Cholesterol in *S. longiceps* and *S. fimbriata* extract treated groups

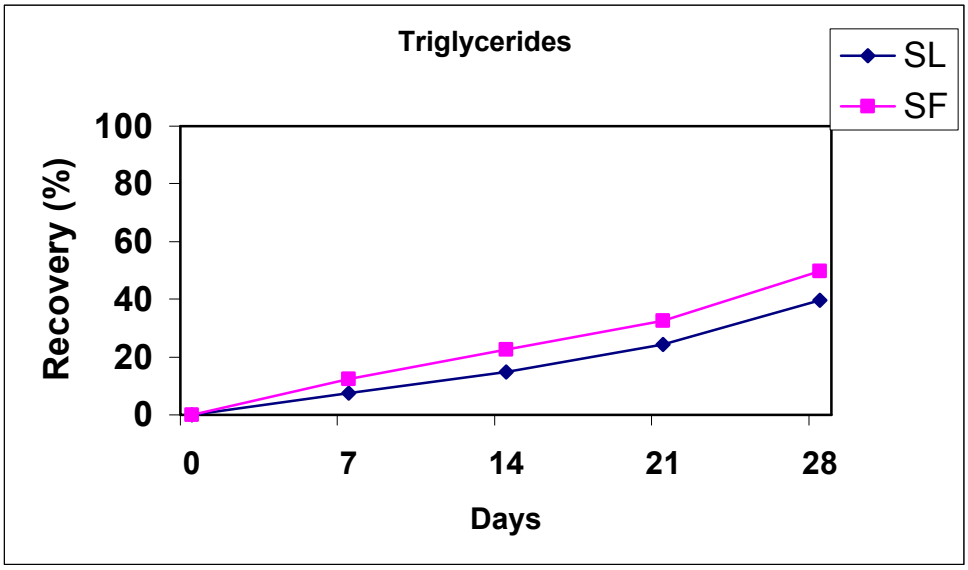

Fig. 21: Recovery of Triglycerides in *S. longiceps* and *S. fimbriata* extract treated groups

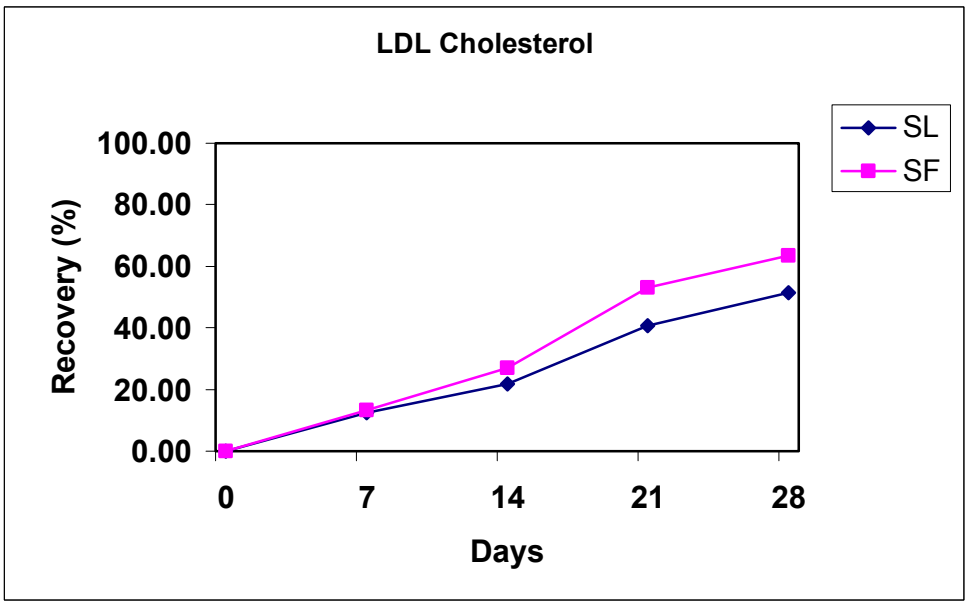

Fig. 22: Recovery of LDL Cholesterol in *S. longiceps* and *S. fimbriata* extract treated groups

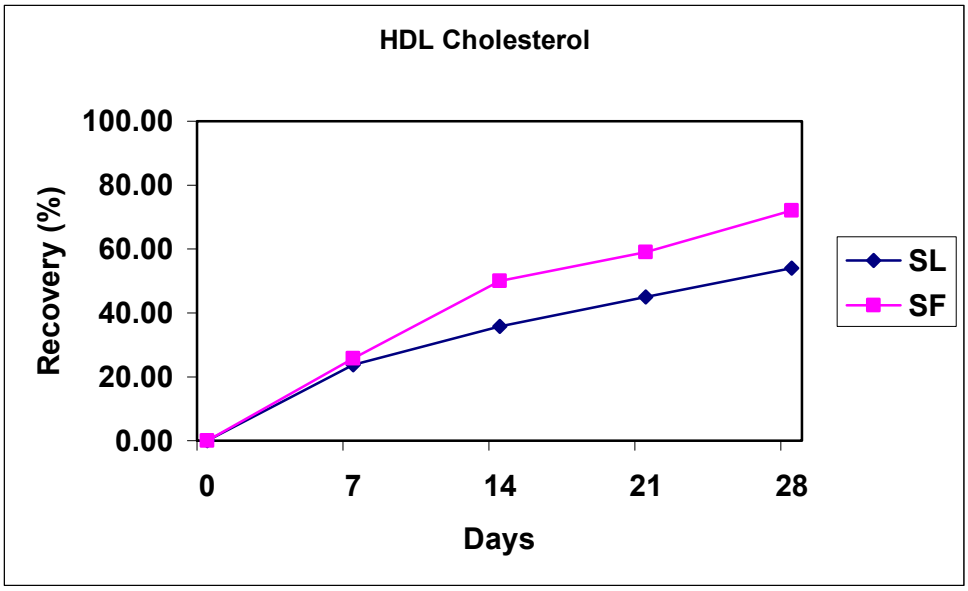

Fig. 23: Recovery of HDL Cholesterol in *S. longiceps* and *S. fimbriata* extract treated groups

5.3.3 Effects of PUFA Extracts on Urea and Creatinine

Urea levels tripled in alloxan-induced mice while Creatinine levels shot up 12 times after inducing alloxan into the mice. The levels remained unchanged through out the experiment. Urea levels in sets administered with fish extracts showed a small but statistically significant ($P < 0.01$) improvement (Fig. 15).

However, creatinine levels improved significantly right from the first collection after drug administration (7^{th} day) but further recovery was slow (Figs. 24, 25) and did not show signs of reaching full normalcy.

Sets administered with *S. fimbriata* extract recovered marginally better as compared to that of *S. longiceps* extract. In summary, it can be

concluded that there is an impact on renal parameters, specifically creatinine, of diabetic subjects due to the administration of these fish extracts.

Table 11 summarises the recovery (%) of various parameters which showed a significant variation.

Table 11: Recovery in bio-chemical parameters in *S. longiceps* extract and *S. fimbriata* extract treated groups

Parameters	Recovery (%) after 28 days	
	S. fimbriata	***S. longiceps***
Total Cholesterol	58	47
LDL Cholesterol	63	51
HDL Cholesterol	72	54
Triglycerides	50	40
Creatinine	25	19

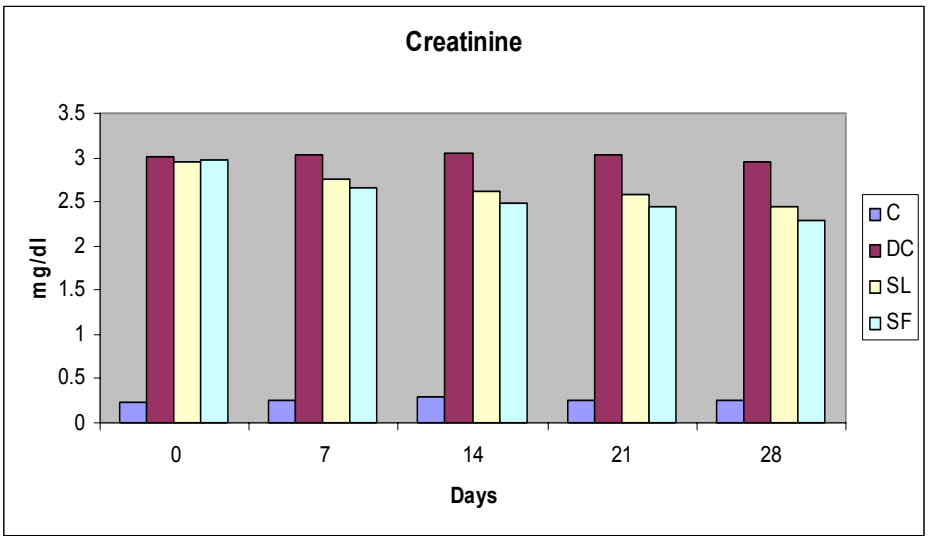

Fig. 24: Creatinine Variation in the four experimental groups

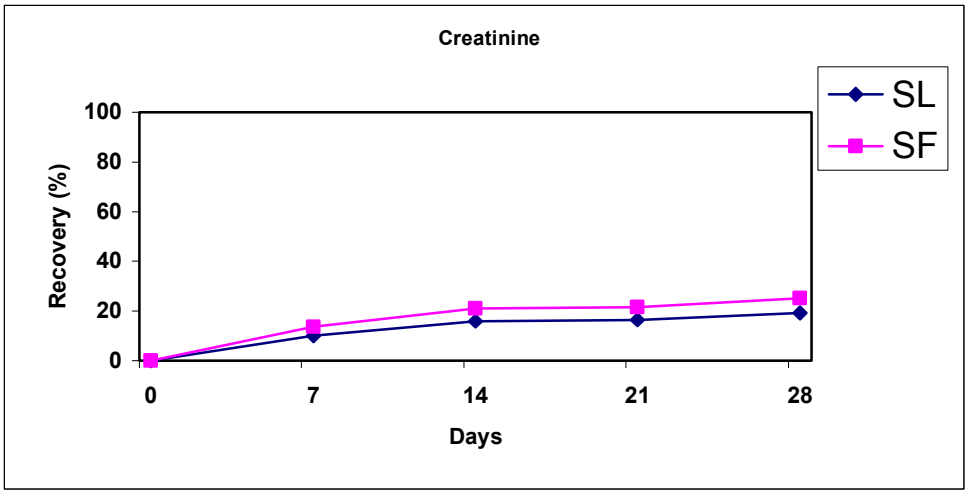

Fig. 25: Recovery of Creatinine in *S. longiceps* and *S. fimbriata* extract treated groups

5.3.4 GC Analysis

The PUFA extracts were analyzed by GC to identify the fatty acids present in the extract. The major compounds identified were unsaturated fatty acids ranging from C20 to C24 with a preponderance of C22:6 (DHA) and C20:5 (EPA) PUFA. GC analyses of the PUFA from the fish *S. fimbriata* showed a DHA content of 65.82% and an EPA content of 24.02%, an EPA-DHA Ratio of 3:8. The GC analyses of the PUFA from the fish *S. longiceps* gave a much lower DHA figure 32.52% while an EPA content of 55.54%, an EPA-DHA Ratio of 3:2.

5.4 Discussion

The purpose of this experiment was to determine effects of two sardine oil extracts on the diabetic condition of mice. PUFA extracts used are rich in Omega-3 fatty acids but with different ratio of DHA and EPA as clear from GC analysis.

5.4.1 Hypoglycemic Effect

Omega-3 fattyacids and fish oils are not known to affect the blood glucose levels in animals or humans. In reviews on dietary and pharmaceutical applications of Omega-3 fattyacids and fish oils (Simopoulos 1991, Kris-Etherton *et al.* 2002), there has been no major studies cited that favourably increased blood glucose levels after the administration of fish oils or Omega-3 fattyacids. In contrast, there is a reported moderate worsening of glycemia noticeable in patients with impaired glucose tolerance and diabetes with levels > 3g/day of Omega-3 fattyacids (Kris-Etherton *et al.* 2002). It is generally accepted that the application of fish oils and Omega-3 fattyacids in anti-diabetic pharmacology is mainly in arresting the associated disorders like hypercholesterolemia and hypertriglyceridemia (Simopoulos 1991). This is very much in accordance with the results of current study on mice subjects where a 28 day administration of two fish oil extracts with differing ratios of EPA and DHA did not significantly decrease the blood glucose levels with recovery percentage being a mere 2-3%.

However, in a 60-day study on low-dose streptozotocin-induced diabetic mice subjects, a decrease in blood glucose level was recorded when fed with an Omega-3 enriched diet (Linn *et al.* 1989). Rubin, D (1991) extracted substantially pure free fattyacids by urea complexing from sardine oil claimed to have found this method to be more effective (52% recovery) in treating diabetes in humans as compared to the fish oil in its natural form (12%). It is unclear on why scattered studies like the above reported a hypoglycemic effect of fish oils and Omega-3 fattyacids. However, there are

several studies on plant extracts and ά-linoleic acid that have a positive hypoglycemic effect (Konrad *et al.* 2001). Hence the lack of hypoglycemic effects for Omega-3 fattyacids may perhaps restricted only to EPA and DHA. However, a more recent study reported that colon-specific delivery of DHA and EPA on mice subjects observed substantial insulin release and subsequent glucose reduction (Morishita *et al.* 2008).

5.4.2 Hypolipidemic Effects

It is well known that in uncontrolled diabetes mellitus, there will be an increase in total cholesterol, triglycerides and LDL cholesterol associated with decrease in HDL cholesterol (Arvind *et al*2002). This was in accordance with the start of the experiment in current study when alloxan induced mice tested high levels of total cholesterol, triglycerides and LDL cholesterol while HDL cholesterol decreased significantly. Patients with diabetes are at increased risk of Coronary Heart Disease (CHD) and to a clustering of risk factors for CHD, including excess weight, hypertension, dyslipidemia, and unfavorable hemostatic changes. Though there has been discordant views on the effect of Omega-3 fattyacids on CHD, evidence of Omega-3 enriched diet showing a positive correlation to reduce CHD is more overwhelming than scattered evidence of no or negative correlation (Kris-Etherton *et al*2002). Dietary Omega-3 fattyacids have been shown to be effective in reducing triglycerides and increasing HDL Cholesterol in patients with diabetes (Simopoulos 1991, De Caterina *et al*2007, Landgraf-Leurs *et al*1990). n-3 PUFA (EPA and DHA in excess of 65%) administered on myocardial rats significantly improved the cholesterol and triglyceride levels specifically increasing the levels of HDL Cholesterol and decreasing

the levels of LDL Cholesterol (Anandan *et al*2007). In the current study, Triglycerides, LDL and Total Cholesterol decreased markedly during the 28 day course of the experiment in both PUFA extracts. Levels of HDL Cholesterol showed a sustained improvement and levels went up to 50-60% of normalcy in 28 days. These results are in accordance with similar experiments with extracts or diets rich in Omega-3 fattyacids; both in animals and in humans.

There are several studies which evidenced DHA to be a comparatively stronger hypocholesterolemic n-3 fattyacid as compared to EPA. Childs *et al.* 1990, in their experiments on normal lipidemic men with three different concentrations of EPA and DHA, concluded that LDL and total Cholesterol were significantly lower in DHA rich diets but did not get affected by diets rich in EPA rich. However, level of triglycerides decreased significantly in all diets. They also concluded that HDL concentrations are better maintained with oil rich in DHA than EPA. Invivo mice studies have also reported specifically that DHA reduced total cholesterol significantly as compared to EPA. However, these studies also established that EPA reduces triglycerides better than DHA (Ikeda *et al.* 1993, Kobatake *et al.* 1984, Willumsen *et al.* 1993). In the current experiment, extracts from *S. fimbriata* fared better over the extracts from *S. longiceps* in both total cholesterol and triglycerides. *S. fimbriata* is DHA rich and this could clearly explain the effect on total cholesterol. However, the higher response to triglycerides for the same extract cannot be explained directly in terms of the relative concentrations of these n-3 fatty acids. Perhaps, the ratio of DHA and EPA in the extract also has a role to play in the recovery of triglycerides in diabetes induced mice. However, a hypotriglyceridimic effect for DHA was

shown in healthy human subjects (Nelson *et al.*1997) and in patients with combined hyperlipidemia (Davidson *et al.*1997). Another study reported a slightly better triglyceride lowering effect in humans for DHA than EPA (Grimsgaard *et al.*1997). In mildly hyperlipidemic men, it was also found that triglycerides levels come down better with DHA than EPA (Mori *et al.* 2000).

Earlier study indicated that fish oil from *Sardinella longiceps* demonstrate a pronounced hypocholesterolemic effect but it was not clear whether the effect was due to EPA or DHA (Sen *et al.*1977). Since there has been no similar studies on DHA rich *S. fimbriata* till date, this current study gains importance as extracts from *S. fimbriata* seems more potent as a hypercholesterolemic agent and result tallies well with earstwhile studies that proved a similar effect for DHA.

5.4.3 Effects on Renal Functioning

Diabetes is associated with several renal disorders and abnormal levels of serum urea and serum creatinine (Simopoulos 1991). The diabetic hyperglycemia induced by alloxan produce elevation in plasma levels of urea and creatinine in animals, which are considered significant markers of renal dysfunction. Action of chemically induced alloxan on animals is not specific to pancreas but also affects organs like kidney (Sabu & Kuttan 2002). A 30 week study on streptozotocin-induced diabetic mice demonstrated that n-3 fatty acids are superior to n-6 fatty acids in renal functioning by controlling urine albumin excretion, glomerulosclerosis and tubulointerstitial fibrosis (Garman *et al.* 2009). It has also been shown that fish oils prolong the survival in mice that develop *lupus nephritis* (Kelley

et al. 1985). Studies reported that Omega-3 fattyacids improve renal functioning in patients who undergo heart and kidney transplants (Holm *et al.* 2001, van der Heide *et al.*1993, Stoof *et al.*1989, Urakaze *et al.*1989). Urakaze *et al.*(1989) reviews several studies done on the effect of omega-3 fatty acids on human subjects with renal disease by assessing serum creatinine among other factors and concludes that two studies reported a statistically significant improvement in serum creatinine when treated with fish oil. The current study on diabetic mice reports a recovery of 15-20% in serum creatinine over a period of 28 days, recovery peaking within 7 days and remaining more or less steady. This reduction suggests potential utility of these fish extracts in diabetes associated complications. It is also established that normal subjects do not show any change in renal function even when given pharmacologic doses of fish oil, which is encouraging from the safety standpoint (Dosing *et al.* 1987).

Moreover, it also known that the beneficial effects on renal function is partly dependent on an increase in EPA and DHA (Holm *et al.* 2001). The mechanism involved is unknown, but experimental studies have shown that omega-3 fatty acids may increase thromboxane A3 formation, coinciding with a fall in thromboxane A2 and a significant increase in total prostacyclin levels (*von Schacky et al.*1985). It is also not clear whether EPA or DHA has a greater effect. In the present study, DHA rich *S. fimbriata* showed a marginally better recovery as against the EPA rich *S. longiceps* perhaps indicating that DHA has a greater role in maintaining creatinine levels and hence renal functioning.

5.4 Conclusion

In conclusion, widely available marine fishes like sardines serve as a rich source of DHA and EPA and is an excellent nutritional source for human subjects having hyperlipidemia and renal disorders associated with diabetes. Though there is no significant positive influence on the blood sugar levels, the positive influence on associated disorders of these compounds creates an opportunity to be used as a supplement to the main drug. Hence these natural sources have the potential to be an excellent source of pharmaceuticals that target these disorders. Fish oil extracts from *Sardinella fimbriata* have higher concentrations of DHA than EPA and hence seem to have greater hypolipidemic and renal effects.

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Chapter 6

Anti-cancer studies of PUFA extracts from *Sardinella longiceps* and *Sardinella fimbriata*: Breast and Prostate cancer

Contents

6.1 Introduction

Human epidemiologic studies have shown that dietary intake of fish oil may protect against the development of certain cancers including breast, colon, and prostate (Hursting *et al.* 1990, Sasaki *et al.* 1993, Caygill *et al.* 1996, Gago-Dominguez *et al.* 2003). Reported benefits of n-3 PUFA dietary supplements given before or during cancer therapy include reversing tumor cell drug resistance (Das *et al.* 1998); reducing the gastrointestinal, hematological, or cardiac side effects of various chemotherapeutic treatments (Hardman *et al.* 1999, Germain *et al.* 1999, Shao *et al.* 1997); decreasing cancer cachexia (Karmali *et al.* 1996, Tisdale *et al.* 1993, Barbet *et al.* 1999); and protecting from alopecia (Takahata *et al.* 1999). The results of *in vitro* studies have shown that a small amount of either EPA or of DHA added to cell culture medium can cause tumour cell death but not kill

cultured normal cells (de Vries *et al.* 1992, Begin *et al.* 1986, Price & Tisdale 1998, de Salis and Meckling-Gill 1995). Thus, it is thought that one or both of these n-3 fattyacids is responsible for the beneficial effects of fish oil against tumor growth.

6.1.1 Breast Cancer

In 2012, 1.5 million people worldwide will be told they have breast cancer. Nearly 1 in 4 women with cancer in the world have breast cancer, and half of them live in developed countries. According to an Indian health news report, one in 22 women in India is likely to suffer from breast cancer during her lifetime, while the figure is definitely more in the US with one in eight being a victim. Hence, biomedical research and application of well known and readily available sources of anti-cancerous compounds for creation of breast cancer drugs is an urgent worldwide need.

There is growing interest in the use of n-3 PUFAs, like EPA and DHA, as an agent against the growth of breast cancer to retard the growth of tumorigenic cells or xenografts (Rose 1997, Bougnoux 1999, Das 1999). The addition of fish oil to the diet of nude mice bearing human tumor xenografts increased the efficacy of the cancer chemotherapy drugs including edelfosine against MDA-MB 231 human breast cancer tumors (Hardman *et al.* 1997), irinotecan (CPT-11) against MCF-7 human breast tumors (Hardman *et al.* 1999), epirubicin against rat mammary tumors (Germain *et al.* 1999), and cyclophosphamide (Shao *et al.* 1997) or mitomycin against MX-1 human mammary tumors (Shao *et al.* 1995). Marine fatty acids, particularly the long-chain EPA and DHA, have been

consistently shown to inhibit the proliferation of breast cancer cell lines in vitro and to reduce the risk and progression of these tumours in animal experiments (Rose and Connolly 1999, Rose and Connolly 2000). Results suggested that PUFA of the linoleic group (n-6 PUFA) stimulate mammary tumor development (Welsch 1994, Bartsch *et al.* 1999), whereas PUFA of the Linolenic group (n-3 PUFA) and especially those from marine origin (EPA and DHA) inhibit tumor growth in mice (Connolly *et al*. 1999).

6.1.2 Prostate Cancer

Prostate Cancer is the most common non-skin cancer in the world, affecting 1 in 6 men in the United States. A non-smoking man is more likely to develop prostate cancer than he is to develop colon, bladder, melanoma, lymphoma and kidney cancers combined. In fact, a man is 35% more likely to be diagnosed with prostate cancer than a woman is to be diagnosed with breast cancer. In 2009, more than 192,000 men have been diagnosed with prostate cancer, and more than 27,000 men died from the disease. (http://www.prostatecancerfoundation.org). In the late 80s and early 90s great attention was given to screening asymptomatic men by measuring concentration of prostate specific antigen (PSA), which eventually led to a significant increase in the detection of clinically insignificant tumours. Though the exact pathogenesis is not clear, epidemiological evidence supports a relationship between prostate cancer and serum levels of testosterone (Ross *et al*. 1992). Other risk factors include advanced age, family history, African-American ethnicity, poor diet and cadmium exposure (Pienta and Esper 1993). The frequency of prostate cancer increases exponentially with advanced age and the natural progression to

prostate cancer tends to be more aggressive in younger men and those with a family history of the disease. Hence, it is imperative that more research is expended in finding excellent sources of naturally available anti-cancerous compounds which can aid drugs against prostate cancer.

Experimental studies show that prostate tumor growth is inhibited by long chain omega-3 PUFA such as EPA and DHA (Connolly JM 1997, Rose 1997, Rose and Connolly 1991, Karmali *et al.* 1987). There is epidemiological support for a protective influence of omega-3 fattyacids against prostate cancer (Godley 1996, Lanier *et al.* 1996). Consumption of oily fish and other foods rich in omega-3 fattyacids may help prevent the spread of carcinoma prostate (Brown *et al* 2006). The omega-3 fattyacids interfere with functions of omega-6, which cancer cells may use as a source of energy and prevent them from spreading beyond the prostate. Both EPA and DHA can inhibit the biological activity of eicosanoids and androgens (Faust *et al.*, 1989, Zaccheo *et al.* 1998, Liang and Liao 1992), which are both known to have a stimulating effect on prostate cancer cell growth (Rose 1997, Ghosh and Myers 1997). In animal models and in human prostate cancer cell lines, EPA and DHA are known to suppress cell growth (Conolly *et al.* 1997). The concentrations of EPA and DHA are high in fish oils and they consistently inhibit tumor cell growth in animal models and in cell lines from human prostate tumors (Rose and Connolly 1999).

Most cancer studies on PUFA or fish oil has not distinguished EPA and DHA separately and most studies believed that both have similar biochemical effects. Some studies reported a similar, but slightly subdued

effect, of DHA as compared to EPA and hypothesized that this could be partly due to retro-conversion to EPA (Price and Tisdale 1998).

The objective of this study was to determine the cytotoxicity on breast cancer cells (MCF-7) of PUFA extracts from two different species of sardines, viz. *S. fimbriata* and *S. longiceps*, found in the same area with in their ranges. Comparison of their respective PUFA profile with the degree of cytotoxicity is also attempted.

6.2 Materials and Methods

6.2.1 Extract Preparation and Determination of PUFA Composition

Freshly caught samples of the fishes were subjected to the procedure documented in Chapter 2 to obtain a mixture of substantially pure PUFA. The composition of PUFAs in the above mixture was directly analysed by Gas Chromatography (GC) adopting the fattyacid methyl ester (FAME) method mentioned in Chapter 3 (3.3) and individual fattyacids were expressed as a percentage of total fatty acids.

6.2.2 Cell Culture

Cancer cell lines MCF-7 and DU-145 were obtained from National Centre for Cell Science (NCCS), Pune, India and cell culture supplies were purchased from HiMedia Laboratories (Mumbai, India). Cells were maintained at 37°C under 5% CO2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml

penicillin and 100 U/ml streptomycin. The culture medium was changed every 2 days and the cells were sub-cultured every 6 days.

6.2.3 Assessment of Cell Viability

Cell viability was determined using MTT (3- (4, 5- dimethylthiazol-2yl)-2, 5- diphenyltetrazolium bromide) assay as described in Mosmann (1983). In order to detect the cytotoxicity of the fish oil extracts, both cell lines were treated with the extract at different concentrations for a specified incubation time. The intensity of formazan, reduced product of MTT after reaction with active mitochondria of live cells, was determined by measuring the absorbance in a 96 well microplate reader (Bio-Tek, Powerwave XS, USA). Results were expressed as percentage inhibition considering absorbance control cells as 100% viable.

MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT to form dark blue impermeable formazan crystals (Mosmann 1983). Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals. The number of surviving cells is directly proportional to the level of the formazan product created. The detailed procedure is as follows.

Seeded MCF-7 (breast cancer) and DU-145 (prostate cancer) cell lines at a density of 5000 cells/well (100µl each) in a 96 well microplate and grown in a 5% CO_2 incubator at 37º C. When cells have adhered and are about 50% confluent, serially diluted concentrations of the cytotoxin (100µl

each) is added. Control wells were devoid of toxin and replaced with 100µl of DMEM.

Stock Concentration:

S. fimbriata: 0.02g/ml of DMSO

S. longiceps: 0.02g/ml of DMSO

Working Concentrations:

100, 200, 400, 600, 800, 1000 µg/ml of 10% DMEM (Dulbecco's Modified Eagles Medium) in triplicates.

After 72 hrs incubation in CO_2 incubator (37º C), aspirated out the 200µl of solution without disturbing the cells and 100µl of MTT is added at a concentration of 1mg/ml of 10% DMEM. This is incubated in dark for 2 hrs in CO_2 incubator at 37º C. 100µl lysis buffer is added to dissolve the formazan crystals and incubated in dark for another 4 hrs. The plate is read on ELISA reader at a wavelength of 570 nm to measure optical density (OD). Calculated percentage of cytotoxicity as per the below formula

Cytotoxicity = 100 – treated OD/control OD x 100.

6.2.4 Statistical Analysis

The obtained results were analyzed using 1-way ANOVA (Zar *et al.* 1984) against the control followed by Fisher's LSD test and $p<0.01$ was considered as significant.

6.3 Results

The respective cytotoxicity profiles of the two species for MCF-7 are illustrated in Figure 26 and values indicated in Table 12 and for DU-145 are

illustrated in Figure 27 and Table 13. Statistically the means for each of the concentrations were found to be significant with $p < 0.01$ (See Appendix).

Table 13 tabulates the IC50 values of the two extracts against both cell lines, along with peak values and corresponding concentrations.

Table 12: Activity of PUFA extracts of *S. longiceps* and *S. fimbriata* on MCF-7 at different concentrations

Conc.	*Sardinella longiceps*		*Sardinella fimbriata*	
	OD Mean±SD	Cytotoxicity	OD Mean±SD	Cytotoxicity
0	2.77±0.07	0.00	2.77±0.07	0.00
100	1.06±0.06	61.50	2.38±0.03	14.02
200	0.38±0.01	86.20	1.18±0.03	57.21
400	0.41±0.03	85.19	0.38±0.03	86.24
600	0.44±0.02	83.79	0.38±0.01	86.20
800	0.54±0.03	80.47	0.4±0.01	85.24
1000	0.58±0.03	78.92	0.38±0.01	85.94

Table 13: Activity of PUFA extracts of *S. longiceps* and *S. fimbriata* on DU-145 at different concentrations

Conc.	*Sardinella longiceps*		*Sardinella fimbriata*	
	OD Mean±SD	Cytotoxicity	OD Mean±SD	Cytotoxicity
0	2.18±0.08	0.00	2.18±0.08	0.00
100	0.25±0.08	88.11	0.54±0.55	74.83
200	0.22±0.01	89.73	0.2±0.04	90.41
400	0.25±0	88.35	0.21±0	90.18
600	0.32±0.04	85.09	0.22±0.01	89.93
800	0.35±0.01	83.80	0.23±0.03	89.13
1000	0.37±0.01	82.83	0.33±0.11	84.52

It can be seen that the peak effects for both species is around 86% for MCF-7 and around 90% for DU-145. For extracts from *S. longiceps*, the peak effect happens at a concentration of 200µg/ml for both MCF-7 and

DU-145. At 200µg/ml,; extracts from *S. fimbriata* show peak activity for DU-145 while a concentration of 400µg/ml is required for the same extract to show peak effect for MCF-7.

Table 14: Summary of the cytotoxic activity of PUFA extracts on MCF-7 and DU-145 cell lines

Parameters	***S. fimbriata***	***S. longiceps***
IC50 (MCF-7)	180.01 µg/ml	81.17 µg/ml
IC50 (DU-145)	67.64 µg/ml	53.07 µg/ml
Peak Cytotoxicity (MCF-7)	86.28 %	86.30 %
Peak Cytotoxicity (DU-145)	90.41%	89.73%
Concentration at Peak (MCF-7)	400 µg/ml	200 µg/ml
Concentration at Peak (DU-145)	200 µg/ml	200 µg/ml
% EPA	32.52%	55.54%
% DHA	65.82%	24.02%
EPA:DHA Ratio	3:8	3:2

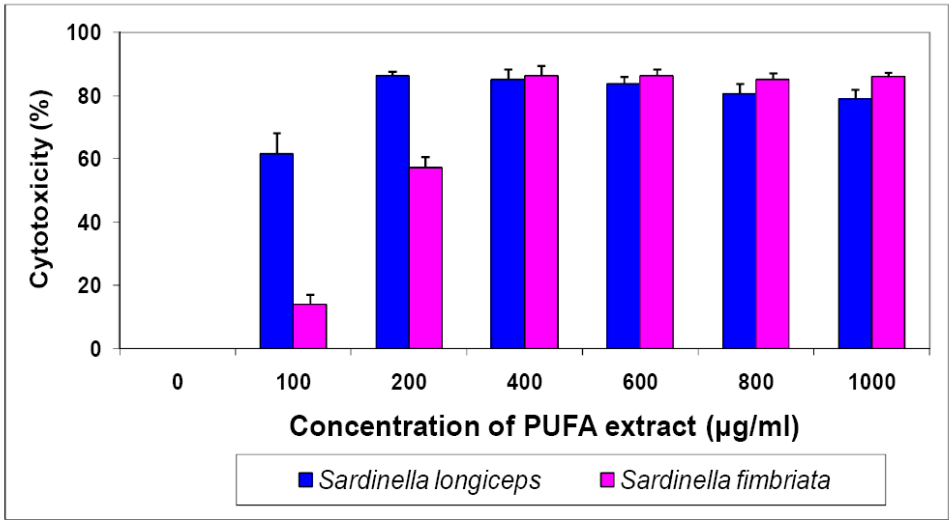

Fig. 26: Cytotoxic Activity of PUFA extracts from *S. longiceps* and *S. fimbriata* on MCF-7 cell line

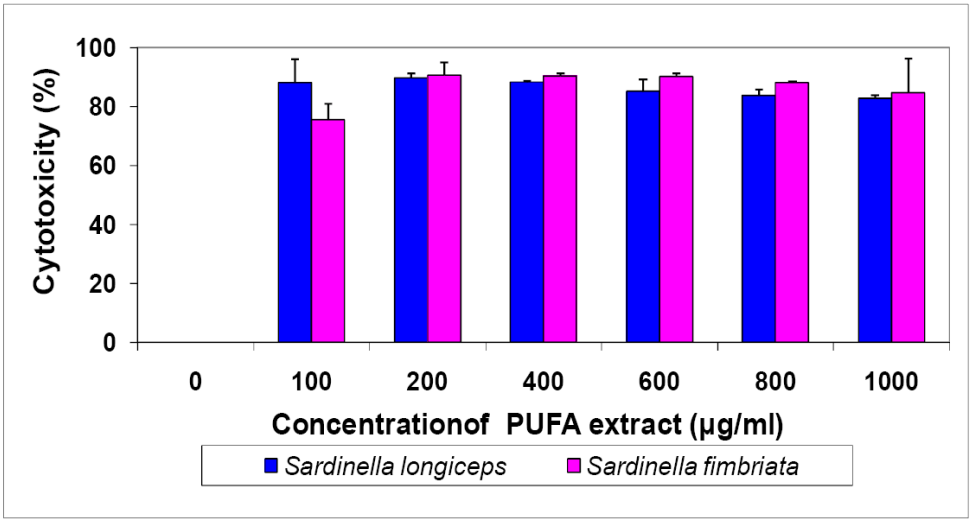

Fig. 17: Cytotoxic activity of PUFA extracts from *S. longiceps* and *S. fimbriata* on DU-145 cell line

The PUFA extracts were analyzed by GC to identify the fatty acids present in the extract. The major compounds identified were unsaturated fatty acids ranging from C20 to C24 with a preponderance of C20:5 (EPA) and C22:6 (DHA) PUFA. GC analyses of the PUFA from the fish *S. longiceps* showed an EPA content of 55.54% and a DHA presence of 32.52%. The GC analyses of the PUFA from the fish *S. fimbriata* gave a much lower EPA content of 24.02% but a correspondingly higher DHA value of 65.82%. To correlate with the concentrations of EPA and DHA in both the extracts, values obtained from Gas Chromatography are also included in Table 14.

6.4 Discussion

The results showed that both the fish oil extracts have a great degree of cytotoxic effects on MCF-7 and DU-145 cells of the order of 86-90% at

the peak – activity itself being dose dependent at lower concentrations. The IC_{50} value of sardine oil emulsion on human breast cancer cell line MCF-7 was as high as 1000 µg/ml (Ueda *et al.* 2007), while it is evident that substantially pure PUFA from our experiments have a much stronger cytotoxic effect which is an order less than prior studies – 180 µg/ml for *S. fimbriata* and 81 µg/ml for *S. longiceps*. It can also be concluded that the EPA-rich *S. longiceps* extracts have a greater cytotoxic effect on MCF-7 cells at lower concentrations as compared to the DHA-rich *S. fimbriata* as evident from the IC_{50} values. This is also true to lesser extent for DU-145 cells too.

Though there are no established studies proving EPA to be more toxic against MCF-7 and DU-145 as compared to DHA, it was believed until recently that the action of DHA is mostly by retro- converting to EPA. Hence, the action of DHA on the annihilation of cancer cells could be delayed until the conversion occurs (Price and Tisdale 1998). However, recent evidences indicate that the action of both EPA and DHA could be very different and differs with different cell lines.

Inhibition of cell growth can be accomplished by either a decrease in cell proliferation or an increase in apoptosis or both. Apoptosis is therefore an important cellular mechanism for growth regulation. In humans, the production of Arachidonic acid (AA) from linoleic acid is suppressed by n-3 fatty acids (Hague and Christoffersen 1984). Suppression of AA production by n-3 fatty acids also suppresses the production of AA-derived eicosanoids. Cyclooxygenase (COX) and lipoxygenase (LOX) act on 20-carbon fatty acids to produce cell-signaling molecules. COX activity on AA or EPA

produces prostaglandins or thromboxanes; LOX activity on AA or EPA produces the leukotrienes. The 2-series prostaglandins produced from AA tend to be pro-inflammatory and pro-proliferative in most tissues. The 3-series prostaglandins produced from EPA tend to be less promotional for inflammation and proliferation; thus, EPA-derived prostaglandins are less favourable for the development and the growth of cancer cells. COX has 2 isozymes: COX 1 and COX 2. COX 1 is constitutively produced by most cell types, and COX 2 is produced as part of the inflammatory response. Incorporation of n-3 fatty acids has been shown to suppress the production of COX 2 (Singh *et al.* 1999, Obata *et al.* 1999) and can reduce the inflammatory response (Needleman *et al.* 1979) by changing the types of eicosanoids that are produced.

Several mechanisms have been proposed for suppression of tumor cell growth by n-3 fattyacids, and new mechanisms are frequently reported as additional knowledge is gained of the regulation of gene expression by fatty acids. It is likely that suppression of tumor cell growth by n-3 fatty acids is due to the combination of these mechanisms rather than to a single, unique activity that is the sole mechanism of action (Hardman 2004). The formation of cytostatic and cytotoxic compounds after peroxidation of long chain PUFAs have been proposed as the primary mechanism for the activity of n-3 PUFAs against cancers (Gonzalez *et al.* 1991, Gonzalez 1995, Das 1990). Other mechanisms proposed include the alteration in prostaglandin synthesis (Rose *et al*. 1994), alteration in gene transcription (Jump & Clarke 1999), suppression of n-6 fattyacid transport (Sauer *et al.* 2000), and modulation of anti oxidant enzymes(AOE) and of apoptosis (Fernandez *et al.* 1996).

It was reported earlier that while omega-3 fattyacids could selectively inhibit tumor cell proliferation, they were not cytotoxic in normal cells (Begin *et al.* 1986, Hardman *et al.* 1997). It is also reported that the anticancer effect of DHA and EPA in different human cancer cell lines (such as MCF-7, MDA-MB-231 and MDA-MB-435s) was also markedly different (Kang *et al.* 2010). Several studies have suggested that the anticancer property of DHA is attributable to its ability to induce apoptosis (Chamras *et al.* 2002, Sun *et al.* 2008, Calviello *et al.* 1999, Connolly *et al.* 1999, Siddiqui *et al.* 2001). It was also reported that treatment of HL-60 cells with EPA results in caspases 3, 6, 8 and 9 activation, bid cleavage, and cytochorme c release (Arita *et al.* 2001). It is speculated that omega-3 FAs may preferentially increase Reactive Oxygen Species (ROS) accumulation in the plasma membrane lipid rafts where the assembly of the death-inducing signaling complex (DISC) and the subsequent activation of caspase 8 takes place (Gajate *et al.* 2009).

Androgens play an important role in proliferation, differentiation, maintenance and function of the prostate (Lee 1996). Evidence shows that androgens are also involved in the development and progression of prostate cancer (Ross *et al.* 1992). The androgen receptor (AR), is a ligand dependent transcription factor belonging to the nuclear steroid hormone receptor super family (Evans 1988), and is the essential mediator for androgen action. In addition to its physiological functions, the AR plays a critical role in the development of prostate cancer. The DU-145 cell line is a well established androgen responsive prostate cancer cell line. DU-145 cells express the AR and a number of androgen inducible genes such a prostate specific antigen (PSA) and hK2 (Horoszewicz *et al.* 1983, Montgomery

et al. 1992). DHA decreases androgen simulated DU-145 cell growth. Furthermore, androgen induction of five androgen regulated genes were significantly repressed by DHA at steady state MRA levels. Similarly EPA was able to reduce both the translational and transcriptional levels of PSA and hK2 genes. Hence, it is suggested EPA and DHA treatment inhibit androgen action including cell growth response (Chung *et al.* 2001)

Fattyacids may modulate prostate carcinogenesis through numerous processes, such as modification of membrane phospholipid composition (Stubbs & Smith 1984), alteration of cell signaling and receptor activity (Sebokova *et al.* 1988, Kubota *et al.* 1998, Novak *et al.* 2003), lipid peroxidation (North *et al.* 1994), cyclooxygenase inhibition (Ringbom *et al.* 2001), cytokine production (Jolly *et al.* 1997), and interference with androgen activity (Liang & Liao 1992).. Another possibility is that EPA and DHA have numerous anti-inflammatory properties that have been linked with decreased cancer risk (Healy *et al.* 2000)

It may be noted that in both extracts, the cytotoxicity marginally decreases after attaining the peak value at a specific concentration – though this phenomenon is unknown among normal drugs, it is displayed by several natural compounds (Scheim 2009)

6.5 Conclusion

In conclusion, widely available marine fishes like sardines serve as a rich source of DHA and EPA that are known to reduce the risk of breast and prostate cancer. These fish oils also have the ability to control the proliferation of cancer cells and hence are potential drug sources for

oncologic pharmaceuticals. It is also clear that the extracts from *S. longiceps* which have a higher content of EPA than DHA, and also more widely available in comparision, seems to possess greater annihilation power as compared to *S. fimbriata*. Mechanism of action of these two compounds is still subject to further study and can vary between cancer cell lines. Such an in-depth study probably will give more insights into the right combination of these fatty acids to be applied for faster impact on different cancer cell lines. This will also mould how a pharmaceutical industry will convert these widely available raw materials into drugs which can be applied for different kinds of cancer conditions.

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Chapter 7
Summary and Conclusion

Contents

As evident in this study, important pharmacological and nutritional properties of the two sardine oil extracts are strongly congruent with their respective fattyacid profile – more specifically their PUFA profile. As mentioned earlier, major constituents of the PUFA from these sardines are EPA and DHA. Though both extracts studied were nearly similar in their responses to the pharmaceutical experiments, there were also distinct variations in their responses to these experiments. This was found to have been induced by their inherent differences in the EPA-DHA ratio (Table 15). DHA rich *S. fimbriata* extracts were found to be more potent against all bacteria tested while the EPA-rich *S. longiceps* extracts were more toxic against both MCF-7 and DU-145 cancer cell lines used in this study. Extracts from *S. fimbriata* fared significantly better in controlling hypercholesterolemia, hypertriglyceridemia and renal side-effects of diabetes in mice. All these evidence from the current study are supported by earlier works in this domain and emphasize the importance of seasonality of fattyacid profile in these sardines. The quantity of PUFA from these sardines peak during December but the quality of EPA in *S. longiceps* and quality of DHA in *S. fimbriata* is are the lowest during this season. A viable

round-the-year pharmaceutical industry based on sardine raw materials needs to take into account these variations in the EPA and DHA in these sardines.

7.1 Recommendations for Dietary Intake

A sardine rich diet is accepted to have high health benefits. In a country like India suffering from malnutrition, much of its populace below poverty line, a sardine-rich diet is a valuable counter measure to reduce effects of nutrient deficiency. Historically, the coasts of Kerala have survived many a famine due to the abundance of cheap nutrient food from the seas in the form of sardines. As found from this study, both species of sardines are rich in EPA and DHA which form the essential ω3 fatty acids and hence it is highly preferred diet for patients who are suffering from hypercholesterolemia, coronary heart disease or undergoing treatment for cancer.

Winter happens to be the time when PUFA accumulates in great amounts in the sardines as found during our study (Chapter 2). Hence, preferentially higher intake of sardines during summer months is required to maintain the constant amount of EPA and DHA in our diet. A recommended intake of 140-175 g / week (4-5 sardines) in winter and about 700 g / week (20 sardines) during peak summer would provide a PUFA of 650mg/day.

Table 15: Summary of Studies on the two Sardine Extracts

	Sardinella longiceps	***Sardinella fimbriata***
Seasonal Fatty Acid Profile	Peak: 160mg/g (in Dec) Peak in PUFA: 46 % (in Dec) Rich in EPA round the year EPA concentration less in Dec.	Peak: 90mg/g (in Dec) Peak in PUFA: 58% (in Dec) Rich in DHA round the year DHA concentration less in Dec.
Feeding	Mainly phytoplankton, juveniles also on zooplankton.	Mainly zooplankton, juveniles also on phytoplankton
EPA-DHA Ratio in PUFA extracts	3:2	3:8
Bactericidal Effects (MIC)	*Staphylococcus aureus* : 50% *Pseudomonas aeruginosa* : 20%	*Staphylococcus aureus* : 50% *Enterococcus feacalis* : 20% *Escherichia coli* : 50% *Pseudomonas aeruginosa* : 10%
Anti-diabetic Effects	Noticeable hypocholesterolemic, hypotriglyceridimic & renal effects. Total Cholesterol Recovery:47% LDL Cholesterol Recovery:51% HDL Cholesterol Recovery:54% Triglycerides Recovery:40% Creatinine Recovery:19%	Noticeable hypocholesterolemic, hypotriglyceridimic & renal effects. Total Cholesterol Recovery:58% LDL Cholesterol Recovery:63% HDL Cholesterol Recovery:72% Triglycerides Recovery:50% Creatinine Recovery:25%
Breast Cancer (MCF-7)	IC_{50}: 81µg/ml; Peak Toxicity: 86%	IC_{50}: 180µg/ml; Peak Toxicity: 86%
Prostate Cancer (DU-145)	IC_{50}: 53µg/ml; Peak Toxicity: 90%	IC_{50}: 67 µg/ml; Peak Toxicity : 89%
Annual Landing	1, 76,128 metric tonnes.	46,801 metric tonnes

7.2 Recommendations for Nutrition Industry

Sardines are excellent raw material for enriching indigenous food products to ensure the proliferation of marine fatty acids into our population. In a country like India with a large vegetarian population, it is very important that synthesized food with enriched EPA and DHA is pushed into the market for ensuring sufficient balance of nutrients. Western countries have been quick to realize the importance of the same and EPA or DHA enriched food products targeting general population or specific sections of the population like children, pregnant or lactating mothers, diabetic patients etc have become quite common place. In India, we are slowly catching up with this technology and ω3-enriched food products are still in its infancy. Effort should be expended to experiment PUFA enriched food products like milk, eggs, health supplement drinks etc based on Sardine oil. Proliferation of such an industry in the coastal zone would provide employment benefits to innumerable people.

Similar to the much popular cod liver oil capsules, there is a vast scope for a locally made sardine oil capsules which can be a good supplement for marine ω3-fattyacids among vegetarian population. Similarly, this is also the age when food materials targeting diseased population are also becoming popular. These fattyacids can also be incorporated into such food items targeting patients undergoing treatment or recovering from cancer, cardio-vascular disorders or diabetes.

7.3 Recommendations for Pharmaceutical Industry

Inclusion of ω3-fattyacids in specific drugs have been experimented and found to be effective. However, for mass production – there is a growing need for obtaining the raw materials like EPA and DHA from cheap natural sources. The abundance of both sardines in the west coast of India opens up an avenue for sardine-based pharmaceutical industry targeted in producing drugs which are ω3-enriched or to produce large quantities of substantially pure PUFA to be used by the industry elsewhere – particularly for drugs combating coronary heart disease and cancer. Such an industry has to understand clearly the cyclical nature of the availability of PUFA in the sardines (Chapter 2) and have to tailor their process and manage the production during all times of the year without impacting the quality of the deliverables.

The ability of PUFA to combat certain bacteria including gram-negative ones (Chapter 3) also opens up frontiers to attempt drugs based on sardines to combat these multi drug resistant strains which are so prevalent in India. Bacteria like MRSA has become so potent that it is becoming more and more resistant to existing drugs and hence newer compounds to arrest these killer bacteria are urgently required. All these industries open up the possibility for further employment of skilled and unskilled labour in the coasts of India and there by bolstering the economy.

7.4 Future Directions

The current work is probably the first investigation of its kind attempting to compare two widely known sardines in the west coast and to

suggest the potential of an investment in a nutritional and pharmaceutical industry based on sardines. However, there are more specific problems which need addressing and they are extremely vital for the establishment of such an industry.

Design and customization of a massive PUFA extraction process with cost-effective and readily available reagents is the first task at hand. Such a methodology should have a high yield year-round and should not contain impurities which are considered harmful for human consumption. Only with such a methodology, we can hope to produce PUFA in great amounts to support a viable supplementary drug or nutrition industry. Secondly, there has not been any evidence on human targets of the effectiveness of sardine-based drugs in India. Hence, specific clinical trials should be conducted to check the effectiveness of sardine-based drugs and sardine-based food items on general population at large and specific disease groups like cancer patients, heart patients etc. A more specific fishery study on the availability of sardines year round in relation to the sustenance of such an industry considering the fluctuations in PUFA in both sardines should also be considered.

With growing evidence on the health-value of ω3-fattyacids supported by this kind of clinical research work on the local population, would pave the way for better utilization of our sardine fishery resource in the coasts of India.

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Appendix

Statistical Analysis of Studies on Anti-Bacterial Activity

Notes: 1-way ANOVA followed by LSD test was done against the control for the values obtained for the extracts from both the species against the four microbial strains that showed a positive response. Comparisons against control is shown for all concentrations and significant (termed as $p < 0.01$) means indicated.

Activity of *Sardinella longiceps* extract on *Enterococcus feacalis* at various concentrations

N	28				
Value by Conc	**N**	**Mean**	**SE**	**Pooled SE**	**SD**
0	4	10.3	0.85	0.52	1.7
5	4	10.3	0.25	0.52	0.5
10	4	10.3	0.48	0.52	1.0
20	4	10.3	0.48	0.52	1.0
30	4	10.5	0.29	0.52	0.6
50	4	10.8	0.63	0.52	1.3
80	4	11.0	0.41	0.52	0.8
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**p**
Conc	2.2	6	0.4	0.34	0.9074
Residual	22.8	21	1.1		
Total	25.0	27			
LSD					
Contrast	**Difference**	**99% CI**			
5 v 0	0.0	-2.1	to 2.1		
10 v 0	0.0	-2.1	to 2.1		
20 v 0	0.0	-2.1	to 2.1		
30 v 0	0.3	-1.8	to 2.3		
50 v 0	0.5	-1.6	to 2.6		
80 v 0	0.8	-1.3	to 2.8		

Activity of *Sardinella fimbriata* extract on *Enterococcus feacalis* at various concentrations

N	28				
Value by Conc	**n**	**Mean**	**SE**	**Pooled SE**	**SD**
0	4	10.0	0.58	0.72	1.2
5	4	10.3	0.85	0.72	1.7
10	4	10.3	0.48	0.72	1.0
20	4	13.3	0.48	0.72	1.0
30	4	15.0	0.41	0.72	0.8
50	4	21.5	1.19	0.72	2.4
80	4	24.0	0.71	0.72	1.4
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**p**
Conc	785.4	6	130.9	63.56	<0.01
Residual	43.3	21	2.1		
Total	828.7	27			
LSD					
Contrast	**Difference**	**99% CI**			
5 v 0	0.3	-2.6	to 3.1		
10 v 0	0.3	-2.6	to 3.1		
20 v 0	3.3	0.4	to 6.1	(significant)	
30 v 0	5.0	2.1	to 7.9	(significant)	
50 v 0	11.5	8.6	to 14.4	(significant)	
80 v 0	14.0	11.1	to 16.9	(significant)	

Activity of *Sardinella longiceps* extract on *Escherichia coli* at various concentrations

N	28				
Value by Conc	**n**	**Mean**	**SE**	**Pooled SE**	**SD**
0	4	13.8	1.25	0.74	2.5
5	4	14.0	0.82	0.74	1.6
10	4	14.0	0.41	0.74	0.8
20	4	14.0	0.41	0.74	0.8
30	4	14.0	0.71	0.74	1.4
50	4	15.0	0.71	0.74	1.4
80	4	14.3	0.48	0.74	1.0
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**p**
Conc	3.9	6	0.7	0.30	0.9288
Residual	45.5	21	2.2		
Total	49.4	27			
LSD					
Contrast	**Difference**	**99% CI**			
5 v 0	0.3	-2.7	to 3.2		
10 v 0	0.3	-2.7	to 3.2		
20 v 0	0.3	-2.7	to 3.2		
30 v 0	0.3	-2.7	to 3.2		
50 v 0	1.3	-1.7	to 4.2		
80 v 0	0.5	-2.4	to 3.4		

Activity of *Sardinella fimbriata* extract on *Escherichia coli* at various concentrations

N	28				
Value by Conc	**n**	**Mean**	**SE**	**Pooled SE**	**SD**
0	4	13.5	1.04	0.63	2.1
5	4	13.5	0.65	0.63	1.3
10	4	13.8	0.85	0.63	1.7
20	4	13.8	0.25	0.63	0.5
30	4	14.5	0.29	0.63	0.6
50	4	16.3	0.48	0.63	1.0
80	4	18.0	0.41	0.63	0.8
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**p**
Conc	72.0	6	12.0	7.58	0.01
Residual	33.3	21	1.6		
Total	105.3	27			
LSD					
Contrast	**Difference**	**99% CI**			
5 v 0	0.0	-2.5	to 2.5		
10 v 0	0.3	-2.3	to 2.8		
20 v 0	0.3	-2.3	to 2.8		
30 v 0	1.0	-1.5	to 3.5		
50 v 0	2.8	0.2	to 5.3	(significant)	
80 v 0	4.5	2.0	to 7.0	(significant)	

Activity of *Sardinella longiceps* extract on *Staphylococcus aureus* at various concentrations

N	28				
Value by Conc	**n**	**Mean**	**SE**	**Pooled SE**	**SD**
0	4	13.8	0.25	0.53	0.5
5	4	13.8	0.63	0.53	1.3
10	4	14.0	0.41	0.53	0.8
20	4	14.0	0.41	0.53	0.8
30	4	14.3	0.85	0.53	1.7
50	4	16.3	0.48	0.53	1.0
80	4	19.3	0.48	0.53	1.0
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**p**
Conc	101.2	6	16.9	14.92	<0.01
Residual	23.8	21	1.1		
Total	125.0	27			
LSD					
Contrast	**Difference**	**99% CI**			
5 v 0	0.0	-2.1	to 2.1		
10 v 0	0.3	-1.9	to 2.4		
20 v 0	0.3	-1.9	to 2.4		
30 v 0	0.5	-1.6	to 2.6		
50 v 0	2.5	0.4	to 4.6	(significant)	
80 v 0	5.5	3.4	to 7.6	(significant)	

Activity of *Sardinella fimbriata* extract on *Staphylococcus aureus* at various concentrations

N	28				
Value by Conc	**n**	**Mean**	**SE**	**Pooled SE**	**SD**
0	4	13.8	0.25	0.81	0.5
5	4	13.8	1.11	0.81	2.2
10	4	13.8	0.85	0.81	1.7
20	4	14.3	0.25	0.81	0.5
30	4	14.3	1.11	0.81	2.2
50	4	18.0	0.41	0.81	0.8
80	4	21.8	1.03	0.81	2.1
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**p**
Conc	229.9	6	38.3	14.77	<0.01
Residual	54.5	21	2.6		
Total	284.4	27			
LSD					
Contrast	**Difference**	**99% CI**			
5 v 0	0.0	-3.2	to 3.2		
10 v 0	0.0	-3.2	to 3.2		
20 v 0	0.5	-2.7	to 3.7		
30 v 0	0.5	-2.7	to 3.7		
50 v 0	4.3	1.0	to 7.5	(significant)	
80 v 0	8.0	4.8	to 11.2	(significant)	

Activity of *Sardinella longiceps* extract on *Pseudomonas aeruginosa* at various concentrations

N	28				
Value by Conc	**n**	**Mean**	**SE**	**Pooled SE**	**SD**
0	4	12.5	0.65	0.44	1.3
5	4	13.3	0.48	0.44	1.0
10	4	13.0	0.41	0.44	0.8
20	4	15.0	0.00	0.44	0.0
30	4	18.0	0.41	0.44	0.8
50	4	20.0	0.41	0.44	0.8
80	4	21.3	0.48	0.44	1.0
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**p**
Conc	308.9	6	51.5	65.53	<0.01
Residual	16.5	21	0.8		
Total	325.4	27			
LSD					
Contrast	**Difference**	**99% CI**			
5 v 0	0.8	-1.0	to 2.5		
10 v 0	0.5	-1.3	to 2.3		
20 v 0	2.5	0.7	to 4.3	(significant)	
30 v 0	5.5	3.7	to 7.3	(significant)	
50 v 0	7.5	5.7	to 9.3	(significant)	
80 v 0	8.8	7.0	to 10.5	(significant)	

Activity of *Sardinella fimbriata* extract on *Pseudomonas aeruginosa* at various concentrations

N	28				
Value by Conc	**n**	**Mean**	**SE**	**Pooled SE**	**SD**
0	4	12.5	0.29	1.01	0.6
5	4	13.3	0.75	1.01	1.5
10	4	23.0	1.78	1.01	3.6
20	4	24.0	1.08	1.01	2.2
30	4	24.8	1.11	1.01	2.2
50	4	26.3	0.85	1.01	1.7
80	4	29.0	0.41	1.01	0.8
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**p**
Conc	984.9	6	164.1	40.43	<0.01
Residual	85.3	21	4.1		
Total	1070.1	27			
LSD					
Contrast	**Difference**	**99% CI**			
5 v 0	0.8	-3.3	to 4.8		
10 v 0	10.5	6.5	to 14.5	(significant)	
20 v 0	11.5	7.5	to 15.5	(significant)	
30 v 0	12.3	8.2	to 16.3	(significant)	
50 v 0	13.8	9.7	to 17.8	(significant)	
80 v 0	16.5	12.5	to 20.5	(significant)	

Statistical Analysis of Studies on Anti-Diabetic Effects

Notes: 1-way pair-wise ANOVA followed by Tukey's test was used for all the biochemical parameters and only the statistics between the four groups after the 28th day of the experiment is demonstrated.

Glucose Variation in all the groups after the experiment

N	20				
Value by Set	**N**	**Mean**	**SE**	**Pooled SE**	**SD**
C	5	80.6	0.24	0.39	0.5
DC	5	320.0	0.45	0.39	1.0
SF	5	310.4	0.40	0.39	0.9
SL	5	311.0	0.45	0.39	1.0
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**P**
Set	204222.6	3	68074.2	87837.68	<0.01
Residual	12.4	16	0.8		
Total	204235.0	19			
Tukey					
Contrast	**Difference**	**99% CI**			
C v DC	-239.4	-241.4	to -237.4	(significant)	
C v SF	-229.8	-231.8	to -227.8	(significant)	
C v SL	-230.4	-232.4	to -228.4	(significant)	
DC v SF	9.6	7.6	to 11.6	(significant)	
DC v SL	9.0	7.0	to 11.0	(significant)	
SF v SL	-0.6	-2.6	to 1.4		

Total Cholesterol Variation in all the groups after the experiment

N	20				
Value by Set	**N**	**Mean**	**SE**	**Pooled SE**	**SD**
C	5	71.2	0.20	0.73	0.4
DC	5	180.8	0.37	0.73	0.8
SF	5	117.6	1.12	0.73	2.5
SL	5	129.0	0.84	0.73	1.9
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**P**
Set	30391.8	3	10130.6	3787.13	<0.01
Residual	42.8	16	2.7		
Total	30434.6	19			
Tukey					
Contrast	**Difference**	**99% CI**			
C v DC	-109.6	-113.4	to -105.8	(significant)	
C v SF	-46.4	-50.2	to -42.6	(significant)	
C v SL	-57.8	-61.6	to -54.0	(significant)	
DC v SF	63.2	59.4	to 67.0	(significant)	
DC v SL	51.8	48.0	to 55.6	(significant)	
SF v SL	-11.4	-15.2	to -7.6	(significant)	

Triglycerides Variation in all the groups after the experiment

N	20				
Value by Set	**N**	**Mean**	**SE**	**Pooled SE**	**SD**
C	5	81.0	0.45	0.37	1.0
DC	5	250.8	0.37	0.37	0.8
SF	5	166.4	0.24	0.37	0.5
SL	5	183.6	0.40	0.37	0.9
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**P**
Set	73233.8	3	24411.3	34873.21	<0.01
Residual	11.2	16	0.7		
Total	73245.0	19			
Tukey					
Contrast	**Difference**	**99% CI**			
C v DC	-169.8	-171.7	to -167.9	(significant)	
C v SF	-85.4	-87.3	to -83.5	(significant)	
C v SL	-102.6	-104.5	to -100.7	(significant)	
DC v SF	84.4	82.5	to 86.3	(significant)	
DC v SL	67.2	65.3	to 69.1	(significant)	
SF v SL	-17.2	-19.1	to -15.3	(significant)	

HDL Cholesterol Variation in all the groups after the experiment

N	20				
Value by Set	**N**	**Mean**	**SE**	**Pooled SE**	**SD**
C	5	39.6	0.24	0.31	0.5
DC	5	19.6	0.24	0.31	0.5
SF	5	34.0	0.45	0.31	1.0
SL	5	30.4	0.24	0.31	0.5
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**p**
Set	1066.2	3	355.4	748.21	<0.01
Residual	7.6	16	0.5		
Total	1073.8	19			
Tukey					
Contrast	**Difference**	**99% CI**			
C v DC	20.0	18.4	to 21.6	(significant)	
C v SF	5.6	4.0	to 7.2	(significant)	
C v SL	9.2	7.6	to 10.8	(significant)	
DC v SF	-14.4	-16.0	to -12.8	(significant)	
DC v SL	-10.8	-12.4	to -9.2	(significant)	
SF v SL	3.6	2.0	to 5.2	(significant)	

LDL Cholesterol Variation in all the groups after the experiment

N	20				
Value by Set	**N**	**Mean**	**SE**	**Pooled SE**	**SD**
C	5	15.40	0.329	0.529	0.73
DC	5	111.04	0.426	0.529	0.95
SF	5	50.32	0.726	0.529	1.62
SL	5	61.88	0.550	0.529	1.23
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**p**
Set	23455.08	3	7818.36	5592.53	<0.01
Residual	22.37	16	1.40		
Total	23477.45	19			
Tukey					
Contrast	**Difference**	**99% CI**			
C v DC	-95.64	-98.39	to -92.89	(significant)	
C v SF	-34.92	-37.67	to -32.17	(significant)	
C v SL	-46.48	-49.23	to -43.73	(significant)	
DC v SF	60.72	57.97	to 63.47	(significant)	
DC v SL	49.16	46.41	to 51.91	(significant)	
SF v SL	-11.56	-14.31	to -8.81	(significant)	

Urea Variation in all the groups after the experiment

N	20				
Value by Set	**N**	**Mean**	**SE**	**Pooled SE**	**SD**
C	5	39.2	0.20	0.30	0.4
DC	5	128.0	0.45	0.30	1.0
SF	5	120.4	0.24	0.30	0.5
SL	5	122.4	0.24	0.30	0.5
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**p**
Set	26867.8	3	8955.9	19902.07	<0.01
Residual	7.2	16	0.5		
Total	26875.0	19			
Tukey					
Contrast	**Difference**	**99% CI**			
C v DC	-88.8	-90.4	to -87.2	(significant)	
C v SF	-81.2	-82.8	to -79.6	(significant)	
C v SL	-83.2	-84.8	to -81.6	(significant)	
DC v SF	7.6	6.0	to 9.2	(significant)	
DC v SL	5.6	4.0	to 7.2	(significant)	
SF v SL	-2.0	-3.6	to -0.4	(significant)	

Creatinine Variation in all the groups after the experiment

N	20				
Value by Set	**N**	**Mean**	**SE**	**Pooled SE**	**SD**
C	5	0.26	0.024	0.028	0.05
DC	5	2.96	0.024	0.028	0.05
SF	5	2.28	0.037	0.028	0.08
SL	5	2.44	0.024	0.028	0.05
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**p**
Set	21.10	3	7.03	1758.46	<0.01
Residual	0.06	16	0.00		
Total	21.17	19			
Tukey					
Contrast	**Difference**	**99% CI**			
C v DC	-2.70	-2.85	to -2.55	(significant)	
C v SF	-2.02	-2.17	to -1.87	(significant)	
C v SL	-2.18	-2.33	to -2.03	(significant)	
DC v SF	0.68	0.53	to 0.83	(significant)	
DC v SL	0.52	0.37	to 0.67	(significant)	
SF v SL	-0.16	-0.31	to -0.01	(significant)	

Statistical Analysis of Studies on Anti-Cancer Properties

Notes: 1-way ANOVA followed by LSD test was done against the control for the values obtained for the extracts from both the species against the both cell lines. Comparisons against control is shown for all concentrations and significant (termed as $p< 0.01$) means indicated.

Cytotoxic Activity of *Sardinella longiceps* extract on Breast Cancer (MCF-7) Cell lines at various concentrations

N	21				
Value by Conc	**n**	**Mean**	**SE**	**Pooled SE**	**SD**
0	3	2.7707	0.04452	0.02568	0.0771
100	3	1.0667	0.03788	0.02568	0.0656
200	3	0.3823	0.00841	0.02568	0.0146
400	3	0.4103	0.01770	0.02568	0.0307
600	3	0.4490	0.01311	0.02568	0.0227
800	3	0.5410	0.01823	0.02568	0.0316
1000	3	0.5840	0.01762	0.02568	0.0305
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**p**
Conc	13.3972	6	2.2329	1128.74	<0.01
Residual	0.0277	14	0.0020		
Total	13.4249	20			
LSD					
Contrast	**Difference**	**99% CI**			
100 v 0	-1.7040	-1.8121	to -1.5959	(significant)	
200 v 0	-2.3883	-2.4964	to -2.2802	(significant)	
400 v 0	-2.3603	-2.4684	to -2.2522	(significant)	
600 v 0	-2.3217	-2.4298	to -2.2136	(significant)	
800 v 0	-2.2297	-2.3378	to -2.1216	(significant)	
1000 v 0	-2.1867	-2.2948	to -2.0786	(significant)	

Cytotoxic activity of *Sardinella fimbriata* extract on Breast Cancer (MCF-7) Cell lines at various concentrations

N	21				
Value by Conc	**n**	**Mean**	**SE**	**Pooled SE**	**SD**
0	3	2.7707	0.04452	0.02194	0.0771
100	3	2.3823	0.01828	0.02194	0.0317
200	3	1.1857	0.01964	0.02194	0.0340
400	3	0.3813	0.01832	0.02194	0.0317
600	3	0.3823	0.01135	0.02194	0.0197
800	3	0.4090	0.01115	0.02194	0.0193
1000	3	0.3897	0.00884	0.02194	0.0153
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**p**
Conc	19.3520	6	3.2253	2234.27	<0.01
Residual	0.0202	14	0.0014		
Total	19.3722	20			
LSD					
Contrast	**Difference**	**99% CI**			
100 v 0	-0.3883	-0.4807	to -0.2960	(significant)	
200 v 0	-1.5850	-1.6773	to -1.4927	(significant)	
400 v 0	-2.3893	-2.4817	to -2.2970	(significant)	
600 v 0	-2.3883	-2.4807	to -2.2960	(significant)	
800 v 0	-2.3617	-2.4540	to -2.2693	(significant)	
1000 v 0	-2.3810	-2.4733	to -2.2887	(significant)	

Cytotoxic Activity of *Sardinella longiceps* extract on Prostate Cancer (DU-145) Cell lines at various concentrations

N	21				
Value by Conc	**n**	**Mean**	**SE**	**Pooled SE**	**SD**
0	3	2.1837	0.05140	0.02866	0.0890
100	3	0.2597	0.04732	0.02866	0.0820
200	3	0.2243	0.00987	0.02866	0.0171
400	3	0.2543	0.00318	0.02866	0.0055
600	3	0.3257	0.02425	0.02866	0.0420
800	3	0.3537	0.01146	0.02866	0.0199
1000	3	0.3750	0.00656	0.02866	0.0114
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**p**
Conc	9.1916	6	1.5319	621.54	<0.01
Residual	0.0345	14	0.0025		
Total	9.2261	20			
LSD					
Contrast	**Difference**	**99% CI**			
100 v 0	-1.9240	-2.0447	to -1.8033	(significant)	
200 v 0	-1.9593	-2.0800	to -1.8387	(significant)	
400 v 0	-1.9293	-2.0500	to -1.8087	(significant)	
600 v 0	-1.8580	-1.9787	to -1.7373	(significant)	
800 v 0	-1.8300	-1.9507	to -1.7093	(significant)	
1000 v 0	-1.8087	-1.9293	to -1.6880	(significant)	

Cytotoxic activity of *Sardinella fimbriata* extract on Prostate Cancer (DU-145) Cell lines at various concentrations

N	21				
Value by Conc	**n**	**Mean**	**SE**	**Pooled SE**	**SD**
0	3	2.1353	0.00567	0.12600	0.0098
100	3	0.5363	0.32533	0.12600	0.5635
200	3	0.2060	0.02600	0.12600	0.0450
400	3	0.2107	0.00567	0.12600	0.0098
600	3	0.2157	0.00767	0.12600	0.0133
800	3	0.2567	0.00267	0.12600	0.0046
1000	3	0.3320	0.06700	0.12600	0.1160
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**p**
Conc	8.9761	6	1.4960	31.41	<0.01
Residual	0.6668	14	0.0476		
Total	9.6429	20			
LSD					
Contrast	**Difference**	**99% CI**			
100 v 0	-1.5990	-2.1295	to -1.0685	(significant)	
200 v 0	-1.9293	-2.4598	to -1.3989	(significant)	
400 v 0	-1.9247	-2.4551	to -1.3942	(significant)	
600 v 0	-1.9197	-2.4501	to -1.3892	(significant)	
800 v 0	-1.8787	-2.4091	to -1.3482	(significant)	
1000 v 0	-1.8033	-2.3338	to -1.2729	(significant)	

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