Developing delivery systems for Omega-3 rich fish oil stabilized by chitosan-antioxidant conjugate: Formulation, stability and bioactivity evaluation

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By

Vishnu. K.V.
(Reg. No. 5044)

Under the Supervision of

Dr. Suseela Mathew
Principal Scientist & Head
Biochemistry & Nutrition Division

Biochemistry & Nutrition Division
ICAR-Central Institute of Fisheries Technology
Matsyapuri PO, Cochin-682029

October 2017

Declaration

I, Vishnu. K. V do hereby declare that the thesis entitled "**Developing delivery systems for Omega-3 rich fish oil stabilized by Chitosan-antioxidant conjugate: Formulation, stability and bioactivity evaluation"** is a genuine record of bonafide research carried out by me under the supervision of Dr. Suseela Mathew, Head and Principal Scientist, Biochemistry & Nutrition Division, Central Institute of Fisheries Technology, Cochin and has not previously formed the basis of award of any degree, diploma, associateship, fellowship or any other similar titles of this or any other university or Institution

Vishnu K.V.
(Reg. No.5044)

Cochin,
October -2017

Telephone : 0484-2412300
Fax : 0091-484-2668212

www.cift.res.in
E-mail : aris.cift@gmail.com
cift@ciftmail.org

भाकृअनुप - केन्द्रीय मात्स्यिकी प्रौद्योगिकी संस्थान
ICAR - CENTRAL INSTITUTE OF FISHERIES TECHNOLOGY
सिफ्ट जंक्शन, विल्लिंडन आईलंड, मत्स्यपुरी पी.ओ., कोचिन, - 682 029, केरल, भारत।
CIFT Junction, Willingdon Island, Matsyapuri P.O., Cochin, - 682 029, Kerala, India.
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This is to certify that the thesis entitled "**Developing delivery systems for Omega-3 rich fish oil stabilized by Chitosan-antioxidant conjugate: Formulation, stability and bioactivity evaluation**" embodies the original work done by Vishnu. K. V, under my guidance and supervision. He has incorporated all the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and by the doctoral committee. I further certify that no part of this thesis has previously been formed the basis of award of any degree, diploma, associateship, fellowship or any other similar titles of this or in any other university or Institution

Cochin,
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Dr. Suseela Mathew
Principal Scientist & Head
Biochemistry & Nutrition Division

Telephone : 0484-2412300
Fax : 0091-484-2668212

www.cift.res.in
E-mail : aris.cift@gmail.com
cift@ciftmail.org

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Dr. K. K. Asha
Senior Scientist
Biochemistry & Nutrition Division

Dr. Niladri Sekhar Chatterjee
Scientist
Biochemistry & Nutrition Division

Cochin-29
October- 2017

Dedicated to

My parents & my brothers...
The reason of what I am today
Thanks for your great support and continuous care....

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ABBREVIATIONS

%	Percentage
7-AAD	7-Aminoactinomycin D
ACP	Acid phosphatase
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AO	Acridine orange
AOAC	Association of Analytical Communities
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BF3 /MeOH	Boron trifluoride-methanol solution
CAT	Catalase
cDNA	Complementary DNA
CE	Cholesteryl ester
cm	Centimeter
CO_2	Carbon dioxide
COX	Cyclooxygenase
CPCSEA	Committee for the Purpose of Control And Supervision of Experiments on Animals
CS	Chitosan
CSLM	Confocal layer scanning microscopy
D_2O	Deuterium oxide
DAG	Diacyl glycerol
DCFDA	Dichlorodihydrofluorescien diacetate
DHA	Docosahexaenoic acid
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
EB	Ethidium bromide
EDTA	Ethylenediaminetetraacetic acid
EE	Encapsulation efficiency
ELISA	Enzyme-linked immunosorbent assay

EPA	Eicosapentaenoic acid
ESI	Electron spray ionization
FAME	Fatty acid methyl ester
FAO	Food and Agriculture Organization
FBS	Fetal bovine serum
FDA	Food and Drug administration
FID	Flame ionization detector
FORV	Fishery Oceanographic Research Vessel
FTIR	Fourier-transform infrared spectroscopy
g	Gram
GA	Gum arabic
GC-MS	Gas Chromatography- Mass spectrometry
GLP	Glucose like peptide
GPR	G-protein coupled receptor
GPx	Glutathione peroxidase
GSH	Glutathione
GST	Glutathione s transferase
h	Hour
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HDL	High density lipoprotein
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
IAEC	Institute animal ethical committee
IL-1	Interleukin-1
IP	Induction Point
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
Kg	Kilo gram
KI	Potassium iodide
LC	Loading capacity
LC-PUFA	Long chain polyunsaturated fatty acids
LDL	Low density lipoprotein
LE	Loading efficiency
M	Molar
m	meter
MD	Maltodextrin

mEq	Milli equivalent
MHz	Mega Hertz
min	Minute
mL	Milli litre
mm	Milli meter
MMP	Mitochondrial membrane potential
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	Normal
$Na_2S_2O_3$	Sodium thiosulfate
Nf-Kb	Nuclear factor kappa b
NMR	Nuclear maganetic resonance
NSAIDs	Nonsteroidal anti-inflammatory drug
^{o} C	degree celsius
OD	Optical density
PBS	Phosphate-buffered saline
PC	Phsphatidyl choline
PDI	Photoionization detector
PG	Prostaglandin
PV	Peroxide Value
PY	Percentage yield
RLP	Remnant lipoproteins
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	rotation per minute
RT	Room temperature
RT-PCR	Reverse Transcriptase polymerase chain reaction
SD	Standard deviation
SEM	Scanning electron microscopy
SGR	Specific growth rate
SM	Sphingomyeline
SO	Surface oil
SOD	Superoxide dismutase
SO-M	Sardine oil loaded microparticles
TAG	Triacyl glycerol
TBARS	Thiobarbituric acid reactive substances
TEM	Transmission electron microscopy

TG	Triglycerides
TLC	Thinlayer chromatography
TO	Total oil
Tris	Hydroxymethyl aminomethane
TV	Tapped volume
TXA2	Thromboxane A2
U/mg	Unit per milligram
UV–Vis	Ultraviolet- Visible
v/v	Volume per volume
Va-g-Ch	Vanillic acid grafted chitosan
VLDL	Very density lipoprotein
w/v	Weight per volume
WHO	World Health Organization
WPI	Whey protein isolate
XRD	X-ray power diffraction
µ g	Micro gram
µ moles	Micro moles
µl	Micro litre

Chapter 1

Introduction & Review of Literature

1.1 General Introduction
1.2 Significance of the study
1.3 Objectives of the study
1.4 Review of Literature

1.1 General Introduction

Long-chain omega-3 polyunsaturated fatty acids (LC-PUFA) have been reported to have extensive nutritional and health benefits. Some among the notable health benefits associated with the consumption of foods rich in LC-PUFA such as EPA and DHA include: potential use in treatment of psoriasis, bowel diseases, prevention of mental illnesses, several types of cancer, rheumatoid arthritis, cardiovascular diseases, diabetes, asthma, developmental coordination disorders, movement disorders, obesity, weak bones and preventing weight loss (Uauy and Valenzuela, 2000; Riediger *et al.,* 2009). This has fostered the consumption of LC-PUFA globally. Fish oil remains as one of the major dietary sources of LC-PUFA. Because of its rich content of health beneficial compounds such as polyunsaturated fatty acids, viz, Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA), fish oils are often categorized under functional foods. However, the PUFA composition may vary among different species of fishes and hence a detailed study has to be carried out in investigating the lipid profiling. In the Indian scenario, mostly sardine and sharks are being employed for oil extraction and production of oil capsules. But as of now, there are no scientific reports available on the quality of commercial fish oils and capsules. Albert *et al* (2015) have studied the quality of fish oil supplements in New Zealand and reported that they are highly oxidized and do not meet label content of n-3 PUFA. This shows that there is a pressing need to analyze the quality of commercially

available fish oil supplements to ensure how safe they are from the point of consumption. With the subsequent increase in awareness of the health benefits associated with the consumption of fish oil, there has been a growing demand for fish oil and fish oil fortified foods. However due to its inherent oxidative instability nature, its direct incorporation into foods has become a major concern to the food industry.

Apart from this, if included directly in a food product, it will undergo oxidation and may affect the sensory acceptance of the final product. Hence, certain intervention strategies have to be adopted to preserve the integrity of these unstable compounds and to increase the overall sensory acceptance of food.

Microencapsulation is one of the promising technologies to protect fish oil from oxidation and to make it more oxidatively stable by forming an impermeable barrier around it. Moreover, the technology helps in masking the off-flavor of fish oil and converts it into a free flowing powder for better handling and storage. As of now, there are several technologies available for the microencapsulation of sensitive compounds such as freeze drying, spray drying, coacervation (simple and complex), in situ polymerization, fluidized bed drying etc. Among these, the most commonly used microencapsulation technology is spray drying which is economical, flexible, efficient and can produce microcapsule with low water activities (Ashady, 1993). Another important factor that plays a crucial role in governing the success of microencapsulation process is the appropriate selection of wall material.

The judicious selection of the wall material can have profound influence on the physico-chemical properties of the microparticle. Some of the most commonly used wall materials include gum arabic, maltodextrin, whey protein, guar gum, sodium caseinate, gelatin, chitosan, sodium alginate etc. Chitosan, the second abundant biopolymer on earth, is having wide applications in food as well as the pharmaceutical industries owing to its inherent properties such as antioxidant, antimicrobial, biodegradable, nontoxic and emulsifying nature. Of late, researchers are working on the chemical modification of chitosan to improve its physico chemical properties for broadening its application in food industries. Vanillic acid,

the oxidized form of vanillin, is found to have diverse pharmacological activities and is being used in many traditional medicinal formulations. It is also considered as safe and is licensed as a food additive (FAO/WHO Expert Committee on Food Additives, JECFA no. 959). Till date, grafting of vanillic acid with amino functionality of chitosan has not been reported and it is clear that these chitosan derivatives will open up new applications in innovative functional food and nutrient delivery. Whatever be the wall material used or technologies adopted for encapsulation, the microcapsule prepared should be subjected to various in-vitro and in-vivo assays to ensure whether proper encapsulation has been achieved. Similarly, bioavailability, oxidative stability studies have to be carried out to study the positive and negative implications of the encapsulation process. In the present study, an attempt has been carried out to optimize the microencapsulation of omega 3 rich fish oil and PUFA using emulsification and multiple emulsification process with various biopolymers to study the physico-chemical characteristics of microparticles and their health benefits. The cardio protective role of developed microparticles was evaluated on cardiomyoblast cell lines. The enhancement of immune and metabolic responses in albino rats if any by feeding the encapsulated fish oil microparticles was assessed.

1.2 Significance of the study

There is an increasing body of evidence of the positive impact of several marine lipids in human health. Marine lipids which are rich in ω-3 polyunsaturated fatty acids, have been shown to improve blood lipid profiles, improve overall immune status, exert anti-inflammatory and cardioprotective effects. However, the high instability of these compounds to oxidative deterioration along with its hydrophobicity nature has a drastic impact in pharmacokinetics. Thus, the bioavailability of these compounds may be affected, resulting in their inability to reach the target sites at effective concentrations. In this regard, development of stable micro/nanoparticles can offer a wide range of solutions that can prevent the degradation of targeted molecules and thereby increasing their absorption, uptake and bioavailability.

1.3 Objectives of the study

The present study aims to:

1. Comprehensive lipid profiling and bioactivity screening of sardine oil (*Sardinella longiceps*) and Bramble shark liver oil (*Echinorhinus brucus*): semi-targeted lipidomics; in vivo, in vitro bioassays.
2. To develop sardine oil loaded vanillic acid grafted chitosan microparticles by employing microencapsulation technology using polysaccharide-phenolic acid complex as wall material and their characterization.
3. To evaluate the cardio protective activity of sardine oil loaded vanillic acid grafted chitosan microparticles on cardiomyoblast cell lines (H9c2)
4. To evaluate the effect of sardine oil loaded vanillic acid grafted chitosan microparticles on improvement of metabolic and immune responses in experimental rats.
5. To develop PUFA loaded microparticles by co-encapsulation of betalain through water in oil in water (w/o/w) multiple emulsification with chitosan-whey protein emulsifier conjugate as wall material.

1.4 Review of Literature

1.4.1 Marine Lipids - Physiological significance

Lipids provide the densest form of energy in marine ecosystems. Among lipids, certain essential fatty acids and sterols are considered to be important determinants of ecosystem health and stability. The importance of marine long-chain omega-3 polyunsaturated fatty acids (LC-PUFA) in the diet, especially eicosapentaenoic (EPA, C20:5 n3) and docosahexaenoic acid (DHA, C22:6 n3), has been well established by researchers around the globe. Omega-3 fats are long chain polyunsaturated fats containing methylene-separated double bonds starting from the third carbon atom counted from the methyl-terminus (Kralovec *et al*., 2012). These fatty acids are required by humans, but cannot be synthesized endogenously and hence considered as essential fatty acids. Therefore, the requirements for these fatty acids must be obtained from the diet. The two

important omega-3 fatty acids -EPA and DHA play a key role in: (1) cell membrane formation, integrity, and functions; (2) functioning of brain, retina, liver, kidney, adrenal glands, and gonads; and (3) local hormone production for the regulation of blood pressure and immune and inflammatory responses. Lot of researches have been carried out worldwide to study the importance of omega-3 fatty acids and their role in human health such as : the potential use of n−3 PUFA in psoriasis (Zulfakar *et al.*, 2007), on bowel diseases (Razack & Seidner, 2007), on treatment and prevention of mental illnesses (Stillwell & Wassall, 2003), on the prevention of several types of cancer (MacLean *et al.*, 2006), effect on rheumatoid arthritis (Ariza- Ariza *et al.*, 1998) and to protect from cardiovascular diseases (Das, 2008). Structures of n-3 and n-6 fatty acids are given in Fig: 1

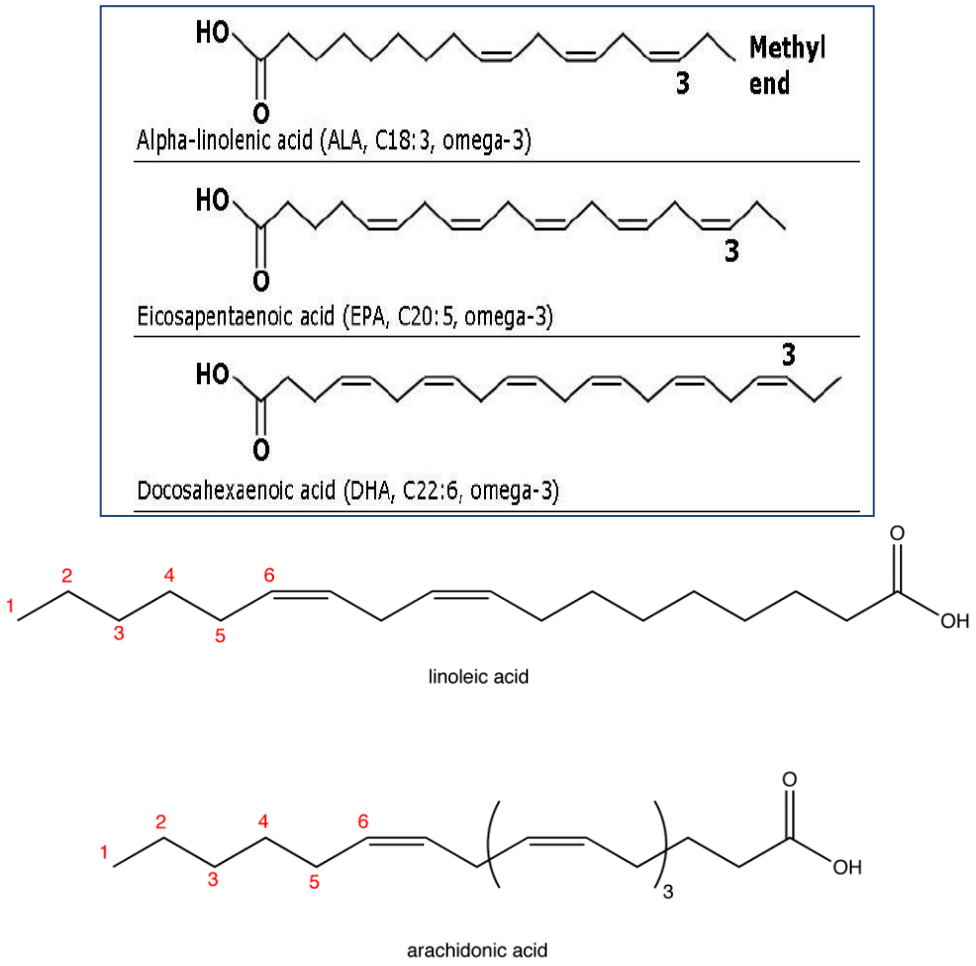

Fig. 1.1 Structures of n-3 and n-6 fatty acids

1.4.2 PUFA and its biomedical applications

Dietary fatty acids are considered as primary energy source in humans. Apart from being a major dietary source, it is also reported to have extensive nutritional and health benefits, especially, n-3 polyunsaturated fatty acids. Since these omega-3 fatty acids are essential for humans and cannot be synthesized endogenously, they must be obtained from the diet. There are several sources recognized for n-3 polyunsaturated fatty acids such as olive oil, rice bran, fish oil etc. Among these, fish oil is being widely recognized as an excellent dietary source of n-3 polyunsaturated fatty acids, especially, EPA and DHA. Fatty fishes like sardine and mackerel are considered as better sources of n-3 fatty acids like EPA and DHA (Gunstone, 1996). Researchers have shown that fish oil supplementation is highly beneficial as it is having many health attributes such as in the prevention of coronary heart disease, rheumatoid arthritis, hypertension, Crohn's disease, Type 2 diabetes, etc. (Simopoulos, 1999; Tur *et al.,* 2012). However, the fatty acid profile of fishes might vary according to species, the environment in which it grows, season, diet, stage of sexual maturity and sex also.

1.4.2.1. Role in inflammation

Inflammation, which is body's response to infection and cellular injuries, is mainly manifested by the production of several inflammatory mediators such as cytokines, reactive oxygen species, expression of adhesion molecules and arachidonic acid derived eicosanoids. However, studies have shown that the increased consumption of n-3 polyunsaturated fatty acids inhibits the arachidonic acid metabolism by competing with arachidonic acid for the enzymes for eicosanoid production. This process results in an increased production of n-3 derived eicosanoids which is having anti-inflammatory effects. Apart from the production of anti-inflammatory eicosanoids, some studies have also reported the production of certain mediator compounds of EPA and DHA which is also having anti-inflammatory actions. For instance, E-series resolvins and D-series resolvins, docosatrienes and neuroprotectins formed from EPA and DHA respectively is reported to have anti-inflammatory properties. Bouwens *et al* (2009) have studied

the effect of fish oil supplementation in inducing anti-inflammatory gene expression profiles in human blood mononuclear cells. The study has reported that the supplementation of EPA and DHA resulted in a decreased expression of genes which are mainly involved in inflammatory- and atherogenic related pathways.

1.4.2.2 Role in prevention of cardiovascular diseases

Several studies have reported the association of fish oil consumption and reduction in the risk of cardiovascular diseases. The relationship between weekly fish consumption and the reduced risk factors of cardiovascular diseases such as obesity, hypertension, and glycohemoglobin has been reported by Kris-Etherton, 2003 and Burr *et al.,* 1989). They have studied the effect of n-3 supplementation (either fish oil capsules of fatty fish twice in a week) on patients with a recent myocardial infarction for a period of 2 years. They have observed 29% reduction in total mortality and in deaths from coronary heart diseases in the group administered with an increased intake of n-3 PUFA. Taking into consideration the cardioprotective effects of fish oil, American Heart Association recommended that adults should eat fish at least two times per week (Krauss *et al.,* 2000; Kris-Etherton, 2003). Mechanism of cardioprotection as illustrated by Krauss *et al.,* 2000 is given in Fig.1.2

1.4.2.3 Role in prevention of thrombosis

The antithrombotic effect of fish oil was first reported in an epidemiological study of Greenland Eskimo by Dyerberg & Bang (1979) and Dyerberg (1986), suggesting the relation between a low incidence of heart diseases and seafood consumption. It was later found that the consumption of fish resulted in increased levels of tissue plasminogen activator (TPA) and decreased concentrations of plasminogen activator inhibitor. One of the possible mechanisms of anti-thrombotic effect of omega-3 fatty acids is that it inhibits platelet TXA2 (Thromboxane A2) synthesis and acts as antagonists of the pro-aggregatory TXA2/PG H2 (Prostaglandin H2) receptor in human platelets in vitro. Decreased concentration of TG (Triglyceride) and RLP (Remnant lipoprotein) favours cardioprotection.

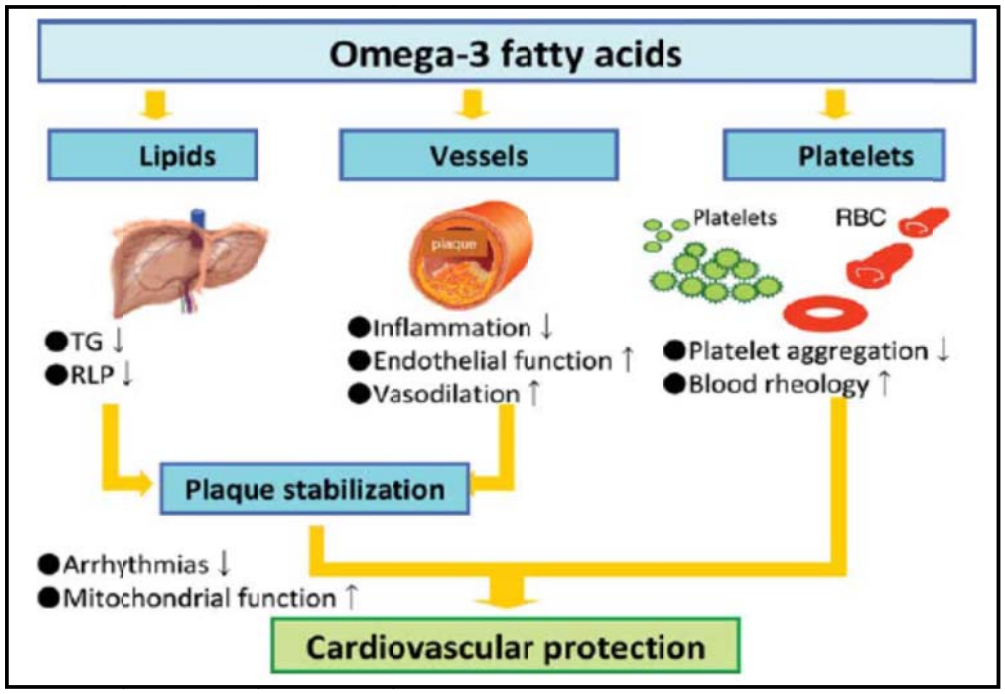

Fig.1.2. Role of omega-3 fatty acids in cardioprotection (Krauss *et al.*, 2000)

1.4.2.4 Role in prevention of Rheumatoid arthritis

Kremer (2000) have studied the effect of fish oil supplementation on rheumatoid arthritis and found that patients taking dietary supplements of fish oil exhibit significant improvements. Fish oil consumption resulted in a significant decrease in levels of IL-1 (Interleukin) β from baseline. Even, some patients who take fish oil on a daily basis were able to discontinue the non-steroidal drugs, some patients who take fish oil were able to discontinue NSAIDs (Nonsteroidal anti-inflammatory drugs) without experiencing a disease flare. Caughey *et al.*, 2010 studied the combined effect of fish oil and paracetamol on the anti-inflammatory effect in patients with rheumatoid arthritis and found out that there has been a significant suppression of COX-2 (Cyclooxygenase) generated prostaglandin PG E2 synthesis.

1.4.2.5 Role in the treatment of ulcerative colitis

Ulcerative colitis is a disease condition which is characterized by the influx and accumulation of neutrophils in the colonic mucosa. The presence of

leukotriene B4, a potent chemotactic factor, was observed in high levels in inflamed colonic mucosa and was reported to have a pivotal role in the accumulation of neutrophils in the affected region. Hence, treatments which will reduce the synthesis of leukotriene B4 will be beneficial in controlling the incidence of ulcerative colitis. Diets containing high levels of n-3 fatty acids, such as eicosapentaenoic acid and docosahexaenoic acid, are known to modify leukotriene production. Eicosapentaenoic acid levels in cell membranes rise with an increase in eicosapentaenoic acid derived lipoxygenase products, such as leukotriene B56, which has markedly reduced chemotactic potency compared with leukotriene B4. In addition, synthesis of lipoxygenase products derived from arachidonic acid is reduced as a result of diminished substrate (John *et al.,* 2010)

1.4.2.6 Glucose lowering capacity

Diabetes mellitus (DM) is a chronic disease in which the blood glucose level is too high because of the insulin deficiency, decreased ability to use insulin, or both. The World Health Organization (WHO) has estimated that 347 million people worldwide have DM (WHO, 2015). The hypoglycemic and antidiabetic effect of n-3 PUFAs was well explained by Iwase *et al.,* 2015. According to Iwase *et al* (2015), the insulin-sensitizing effect caused by n-3 PUFAs, which is based on GLP-1 (Glucagone like peptide) secretion mediated by GPR120 (G protein-coupled receptor). Targeted delivery of n-3 PUFAs to the colon is essential for the control of blood glucose level by n-3 PUFAs. The insulin-sensitizing effect of n-3 PUFAs mediated by sterol regulatory element-binding proteins and peroxisome proliferator-activated receptors will alter the lipid metabolism, suppress inflammation and can thereby ameliorate insulin resistance.

1.4.2.7 Effect on Lipid profile

Many clinical trials and studies have shown that consumption of n-3 PUFAs significantly changes the serum lipid profile. There is a relationship exists between n-3 PUFA consumption and decreased circulating plasma triacylglycerol (TG) concentrations (Park & Harris, 2003). Long chain omega-3 fatty acids (FAs) significantly alter the plasma triglyceride (TG) levels. At the pharmaceutical dose,

3.4 g/day, it reduces plasma triglycerides by about 25-50% after one month of treatment, after the treatment it results in the decreased hepatic very low density lipoprotein (VLDL-TG) production and increase the VLDL clearance. Numerous studies have been shown to contribute to the TG overproduction, but a key component is an increase in the availability of fatty acids in the liver (Shearer *et al.,* 2012).

1.4.2.8 Effect on Antioxidant system

Reactive oxygen species (ROS) are produced as a result of aerobic respiration and substrate oxidation. It includes hydroxyl radicals (•OH), superoxide anions ($O2•^{2}$) and hydrogen peroxide (H_2O_2). Low levels of ROS are required for the normal metabolic processes because it involves in many biochemical processes, including intracellular messaging in the cell differentiation, apoptosis (Ghosh & Myers, 1998), immunity (Yin *et al.*, 1995), and defense against micro-organisms (Bae *et al.,* 1997; Lee *et al.,* 1998). The high doses of ROS result in oxidative stress and it can cause severe metabolic malfunctions and damage to our macromolecules (Chopra &Wallace, 1998; Wojtaszek, 1997). The naturally occurring antioxidants will protect the cells from oxidation (Silva *et al.,* 1998; Meyer *et al.,* 1996). The enzymatic and non-enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), glutathione (GSH), beta-carotene, and vitamin A. (Scott *et al.,* 1989). Studies show that feeding of some natural antioxidant compounds in rats results in the decreased activity of metabolic enzymes like aspartate amino transferase, alanine aminotransferase, alkaline phosphatase and acid phosphatase and antioxidant enzymes like superoxide dismutase, catalase and glutathione S-transferase (Tejpal *et al.,* 2017). According to Kesavulu *et al.,* 2001 omega-3 fatty acid can change the antioxidant enzyme levels in stressed condition and an increased activity of enzymes was observed.

1.4.3 Extraction and Characterization of fish oil

The oil extraction is mainly performed using chloroform and methanol. Both polar and non-polar lipids can be isolated by using this solvent system. The procedure involves the homogenization of tissue with a mixture of chloroform and methanol in such proportions that a miscible system is formed with the water in the tissue. Two layers were formed as a result of adding water. The chloroform layer contains all the lipids and the methanolic layer contains all the non-lipids. By isolating the chloroform fraction, it is easy to extract the lipid. This method has been applied to both fish muscle and liver (Folch *et al.,* 1957).

1.4.3.1 Characterization of fish oil lipids

GC-MS (Gas chromatography- Mass spectrometry) has been widely used for the selective analysis of lipids in the oil sample. Prior derivatization of the carboxylic acids to an ester form is necessary for the separation on chromatographic columns. Normally the identification and quantification of fatty acids were done with the help of fatty acid methyl ester external standard. The selective ion monitoring with simultaneous full scan properties are facilitated by modern mass spectrometers. It allows the perfect selective quantitation of specific peaks (Moore *et al.*, 2007). Lipidomics is one of the emerging fields for determining the structures, functions and dynamic changes of lipids present in the tissues. Mass spectrometry (MS) is the most important technology for lipid analysis.

Shotgun lipidomics analysis using mass spectroscopy has been extensively used to characterize and document the total lipid profile. Mass spectroscopy with aid of bioinformatics tools for mass spectrum are employed to identify and interpret authentic lipid profiling which has got wide range of attention in recent times (Han & Gross, 2005). Previous reports are available regarding the total lipid profiling using triple quadrupole mass spectroscopy in short time by using positive and negative ion mode acquisitions. Identification and quantification of total lipids using lipid identification bioinformatics tools provide authentic data (Simons *et al.*, 2012).

1.4.4 Recommended intake of DHA and EPA

The emphasis on the importance of omega-3 fatty acids has led to a substantial increase in the available number of fish oil capsule supplements as well as various food products enriched with omega-3 fatty acids in the markets. But there are certain dietary guidelines that specify the daily intake of EPA and DHA per person. FDA (Food and drug administration) 2000 stated that the daily intake of EPA and DHA should not exceed 3.0 g per person per day in the form of fish oil, from food and dietary supplements. An average intake of 0.2 g of omega-3 LC PUFA (EPA plus DHA) per person per day has been recommended by The European Academy of Nutritional Sciences (EANS), as well as UK dietary guidelines (Ruxton, 2004). But, the International Society for the Study of Fatty Acids and Lipids (ISSFAL) recommends an adequate intake of omega-3 LC PUFA to be 0.65 g of DHA plus EPA per person per day (as minimum 0.22 g of each). The American Heart Association recommends adults to eat fish (in particular fatty fish) at least two times per week (Krauss *et al.*, 2000).

Though the consumption of omega-3 fatty acids has been recommended by many of the health organizations, there should be an appropriate balance between the omega-6 to omega-3 fatty acids in the diet. It is also recommended that the ratio of omega-6 PUFA to omega-3 PUFA should not exceed 4:1 in order to optimize the bioavailability, metabolism, and incorporation into membrane phospholipids (Garg *et al*., 1988, Volker & Garg, 1996). But this ratio has been now escalated to about 10:1 because of the elevated consumption of vegetable fats and oils rich in omega-6 PUFA (Sanders, 2000). An alternative to maintain this balance is to increase the consumption of omega-3 rich foods such as fish oil. The Joint FAO/WHO Expert Consultation on Fats and Oils in Human Nutrition recommended that individuals with linoleic to linolenic acid ratio in excess of 10:1 should be encouraged to consume foods rich in n-3 PUFA, such as green leafy vegetables, legumes, fish, and other seafood (Nishida & Uauy, 2009).

1.4.5 Limitations with fish oil addition

Though the consumption of fish oil is beneficial for human health, there are certain limitations associated with the direct addition of fish oil into food. Fish oil, which is rich in unsaturated omega-3 fatty acids are highly susceptible to oxidation, resulting in the formation of toxic hydroperoxides and other byproducts giving off- flavors and shorter shelf-life to products (Nawar, 1996). Hence a direct addition of fish oil is not advisable as it decreases the safety of fortified foods. Moreover, the high temperatures and air during processing and further storage can also contribute to the rapid deterioration of these fatty acids and might decrease the sensory acceptance of food. Microencapsulation is an effective strategy that can be employed to protect fish oil against oxidation. This will allow the manufacturers to handle and incorporate oil in food products (Kolanowski *et al*., 1999; Klinkesorn *et al*., 2005). It transforms oil into a powder, where the small droplets of oil are surrounded by a dry matrix of coating materials (Heinzelmann, 2000). The microencapsulation process can also help to mask undesirable fishy odors and flavors in the final product. A number of companies, including BASF, Roche, Clover, and Ocean Nutrition, manufacture and sell microencapsulated fish oil powders for use in food products. The technology makes fish oil fortification in instant foods, as well as in food products easier.

1.4.6 Encapsulation

Encapsulation is a rapidly expanding technology that involves the entrapment of an active compound within another polymeric substance. The active compound that will be entrapped is usually referred to as the core material and the polymeric substance surrounding it is known as the wall material or the shell material. The technology finds its applications in many areas such as pharmaceutical, nutraceutical and even in cosmetic industries (Sanguansri *et al.,* 2013).

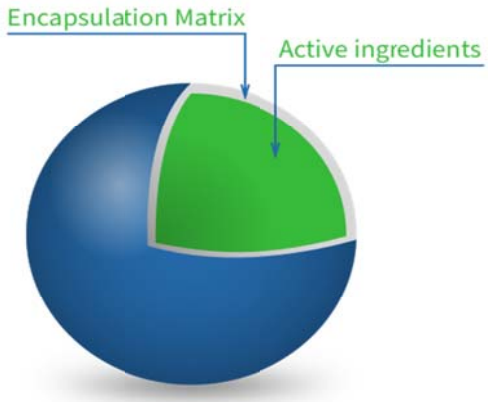

Fig.1.3. Structure of a encapsulated microparticle

The important benefits associated with the encapsulation of bioactive compounds are as follows:

- For better handling of the bioactive components eg. it transforms the oil into a powder, where the small droplets of oil are surrounded by a dry matrix of proteins and/or carbohydrate coating materials (Heinzelmann, 2000; Keogh *et al.,* 2001).
- Protection of active components from factors that can cause oxidation and hence a prolonged shelf life.
- The microencapsulation process can also help to mask undesirable fishy odors and flavors in the final product.
- For the controlled release of active components, especially in the case of drug delivery systems.
- For enhanced bioavailability and efficacy

According to their morphology the microcapsules can be categorized into mononuclear, polynuclear and matrix type. The mononuclear microcapsules contain the wall material layer around the core, polynuclear microcapsules will be having number of cores enclosed within the wall material. In matrix type, there will be a homogenous distribution of the core material in the wall material. Since

the wall material has an important role to play in many aspects such as encapsulation efficiency, stability as well as the protection of the core compound, a proper selection of the wall material is highly essential.

1.4.7 Wall materials for encapsulation

The selection of wall materials plays a crucial role in the success of encapsulation. The wall materials are to be selected in accordance with the properties of the core molecule to be encapsulated and the purpose of microencapsulation. The wall material selection depends on number of factors such as solubility, molecular weight, glass transition temperature, diffusibility, film forming and emulsifying properties etc. The main role of wall material is to protect the core material from factors that can affect its oxidative stability and to assist in the controlled release of core material under the desired conditions. Apart from wall material, certain other substances such as emulsifiers, plasticizers or defoaming agents, are sometimes included in the formulation to improve the characteristics of final products. The cost of wall material also has to be taken into account as the total process has to be economical. Other than this, the encapsulant material can be modified by physical or chemical means to achieve the desired functionality. Hence, it can be stated that the encapsulant material chosen are dependent on a number of factors, including its physical and chemical properties, compatibility with the target food application and influence on the sensory and aesthetic properties of the final food product (Brazel & Peppas, 1999; Gibbs *et al*., 1999).

The most commonly used wall materials include:

- Polysaccharides – chitosan, maltodextrin, sucrose, gum arabic, modified starch, corn syrup solids, agar, alginates, carrageenan, pectin, etc.
- Proteins – Whey protein isolate, skimmed milk powder, gelatin, sodium caseinate
- Materials that are commonly used as microencapsulates for food applications are shown in table.1.1

Table.1.1 Common materials used as microencapsulate in food application (Madene *et al.*, 2006)

Encapsulant materials		
Carbohydrates	**Proteins**	**Lipids and Waxes**
Native starches	Sodium caseinate	Vegetable fats and oils
Modified starches	Whey proteins	Hydrogenated fats
Resistant starches	Isolated whey proteins	Palm stearin
Maltodextrins	Soy proteins	Camauba wax
Gum acacia	Gelatins	Bees wax
Alginates	Zein	Shellac
Pectins	Albumin	Polyethylene glycol
Carrageenan		
Chitosan		

Though a wide variety of substances are being used as wall materials, gum arabic (GA) still remains as the most commonly used wall material for microencapsulation of fish oil owing to its superior properties. But the fluctuations in its availability along with the higher price have fostered researchers in identifying newer wall materials that are relatively cheaper, but of superior quality. Common wall materials used for the microencapsulation of fish oil are shown in table. 1.2. Maltodextrin (MD), whey protein (WPI), chitosan (CS), gum Arabic, lecithin are the main wall materials used in the fish oil encapsulation.

Table.1.2 Common materials used as wall material for microencapsulation of fish oil

Wall materials	Percentage of wall materials	Encapsulation efficiency	Reference
WPI + CS+ MD **For tuna oil**	CS (0.5, 1, 1.5 % w/w) MD(1%w/w),WPI(10%w/w),	80%	Klaypradit & Huang (2008)
WPI **for fish oil**	WPI (1:2) , SPI (3:1)	WPI – 97% SPI – 93%.	Rusli *et al.*, (2006).
CS + Lecithin **for tuna oil**	CS (0.2% w/w) Lecithin (1%w/w)	87%	Klinkersorn *et al.*, (2005)
WPI + MD	90:10, 50:50, 10:90	45 – 65%	Bae & Lee, (2008)
GA **WPI** **GA + WPI** **For cardamom oil**	100g 100g GA + WPI(1:1) GA + WPI (3:1)	92 % 69.2% 83.3% 74.3%	Cerqueira *et al.*, 2015

Chitosan, a cationic polysaccharide (Fig. 1.4) obtained by deacetylation of chitin, is found to have wide range of bioactivities such as antioxidant, antimicrobial, antifungal, antitumor, antiallergic, immune system activating, anti-hypertensive and cholesterol lowering. It is used in many food and beverage preparations as it is non-toxic, bio-degradable and biocompatible in nature. Owing to its emulsification capacity and film forming ability, it is also being used as an encapsulation agent for many sensitive substances such as hydrophilic and lipophilic drugs, vitamins, astaxanthin, fish oil, curcumin etc. It has also been observed that being a poly cationic polysaccharide, chitosan surrounds oil-in-water (o/w) emulsion droplets and repels pro-oxidant metals in the surrounding environment (Klinkesorn *et al.,* 2005). Interestingly, chitosan is also a bio-functional natural polysaccharide. Many researchers have reported that, dietary supplementation of chitosan may result in health promoting effects such as hypolipidemic and cardio-protective activities (Zhang *et al.,* 2013; Anandan *et al.,* 2015).

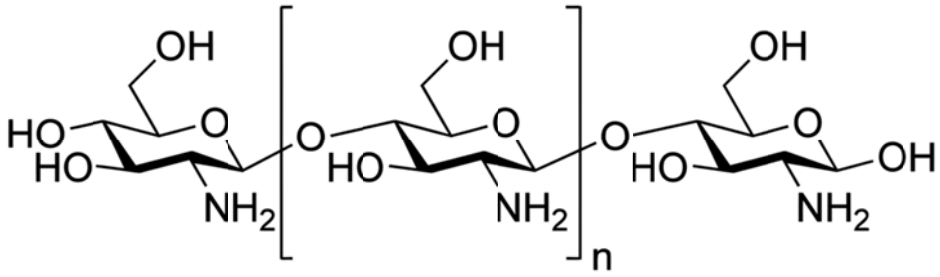

Fig.1.4 Structure of Chitosan

Considering this, grafting of Chitosan with various functional moieties was studied to improve its bioactivity, modulate solubility and emulsifying properties. Particularly, synthesis of antioxidant-chitosan conjugates by grafting of antioxidant molecules onto chitosan has received much attention. Grafting of phenolic acids like gallic acid, caffeic acid and ferulic acid to chitosan have been reported (Cho *et al.,* 2011; Woranuch & Yoksan, 2013). Researchers have opined that the phenolic acid grafted chitosan derivatives could potentially be used as new food additives or even as functional foods (Xie *et al.,* 2014). It has been

reported that phenolic acid grafted chitosan derivatives demonstrate various bio-activities, such as antioxidant, antimicrobial, anti-diabetic etc. (Chatterjee *et al.,* 2015; Liu *et al.,* 2013; Lee *et al.,* 2014). Hence, application of phenolic acid grafted chitosan derivatives for microencapsulation and delivery of bioactive lipids may result in the development of new food additives or novel functional foods. Recently application of gallic acid grafted chitosan in the delivery of bioactive components has been reported (Hu *et al.,* 2015; Hu & Luo, 2016). Chatterjee *et al.,* 2016 reported application of ferulic acid grafted chitosan for microencapsulation and controlled release of thiamine and pyridoxine. Promising potential of phenolic compounds in stabilizing highly unsaturated fish oil has been recorded in recent literature (Vaisali *et al.,* 2016). As of now, there exists no report on the application of phenolic acid grafted chitosan derivatives for microencapsulation and stabilization of highly unsaturated fish oils. Besides being a licensed food additive with pleasant creamy smell (FAO/WHO Expert Committee on Food Additives, JECFA no. 959), vanillic acid has been associated with a variety of pharmacological activities (Gitzinger *et al.,* 2011). Hence it is surmised that the vanillic acid grafted chitosan can be a better encapsulant for fish oil. Apart from being as encapsulant, the consumption of this product can also have numerous health benefits.

Apart from the wall materials used, another important criteria that determines the success of encapsulation is the method of encapsulation. There are a number of encapsulation technologies now being used to attain a better encapsulation process and efficiency. The technologies that are presently being used for fish oil encapsulation are discussed here.

1.4.8 Microencapsulation Technologies

1.4.8.1 Spray drying

One of the most commonly used microencapsulation technologies is spray drying which finds wide range of applications in food and pharmaceutical industries. It is a very economical, flexible, efficient, easy to scale-up technology which produces good quality powder with low water activities that can be easily

stored and transported (Ashady, 1993). The process of spray drying involves the dissolution of wall material and core material resulting in the formation of an emulsion, followed by proper homogenization, pumping of the emulsion, atomization of the emulsion and the subsequent dehydration of the atomized droplets to yield microcapsule. The size of microcapsules formed will depend on the concentration of solids in the dispersion content and accordingly can vary from smaller to larger particles. Apart from this, the viscosity of the emulsion, feed rpm, inlet and outlet temperatures also have an influence on the particle size as well as the oxidative stability of the particles. Many researchers have used spray drying technology for the encapsulation of fish oil using different wall materials such as gelatin, caseinate and maltodextrin; casein and lactose; sodium caseinate and dextrose equivalence; highly branched cyclic dextrin and sodium caseinate; methylcellulose and hydroxypropyl methylcellulose; sodium caseinate, glucose, glucose syrup; n-octenylsuccinate, derivatized starch/glucose syrup or terhalose, gum arabic; sugar beet pectin and glucose syrup , corn syrup solids etc. (Lin *et al.*, 1995; Keogh *et al.,* 2001; Hogan *et al.,* 2003; Kagami *et al.,* 2003; Kolanowski *et al.,* 2004; Augustin *et al.,* 2006; Drusch *et al.,* 2006; Kolanowski *et al.,* 2006)

1.4.8.2 Freeze drying

Freeze drying is widely accepted as one of the best methods for production of superior quality dried products (Calvo *et al*., 2011). Though spray drying is the most widely accepted technology for encapsulation, a lower oxidative stability of spray dried products has also been reported. Because of the low temperature employed in freeze drying process and removal of about 97-98% moisture content, the technology is often reported to produce good quality products than spray drying (Minemoto *et al.,* 2001). The process of freeze drying involves three processes, freezing at a lower temperature of −90 and −40 °C, followed by primary and secondary drying under low pressure. But one limitation of this technology is that it is an expensive process requiring high energy consumption and processing time. Certain researchers have reported spray drying as a better

process than freeze drying for fish oil encapsulation (Chen *et al.,* 2013). There are reports saying that freeze drying produces microencapsulated powder with porous, irregular, and flake-like structure that can accelerate the oxidation process (Heinzelmann *et al.,* 2000 and Anwar & Kunz, 2011). Taking into consideration the limitations of both freeze drying and spray drying, it can be concluded that the freeze drying can be employed to encapsulate products that are highly sensitive to heat.

1.4.8.3 Coacervation

Coacervation, also known as phase separation is the separation of two liquid phases in a colloidal solution. The phase which is rich in polymer is known as the coacervate phase and that is devoid of polymer is known as equilibrium solution. There are two kinds of coacervation, simple and complex. In case of simple coacervation, there will be only one polymer whereas in complex coacervation, the interaction between oppositely charged polymers is used (Ke-Gang *et al*., 2005). The biopolymers that are being widely employed in the complex coacervation process are gelatin or whey protein and oppositely charged gum arabic, sodium polyphosphate or carboxy methyl cellulose. This method produces microcapsules that are having better and controlled release activities along with heat resistant properties (Jun-xia *et al.,* 2011). The microcapsules thus produced are collected by centrifugation or filtration and further dried by either spray or fluidized bed drying (Carvalho *et al.,* 2015). The size of microcapsules produced depends on a number of factors such as temperature, stirring speed, viscosity and pH (Carvalho *et al.,* 2015). One limitation with this technology is that the coacervates produced are stable over a narrow range of pH and ionic strength. Fish oil has been encapsulated by complex coacervation using hydroxypropyl methylcellulose with maltodextrin; sugar beet pectin and glucose syrup; whey protein and gum arabic (Wu *et al.,* 2005; Drusch, 2007).

1.4.8.4 Extrusion

Extrusion process involves mixing of the molten wall material with the core material which is then allowed to pass through a nozzle under high pressure to produce microcapsules of higher density and less porosity (Serfert *et al.,* 2009). But the technology is more expensive than spray drying and moreover, the generation of high shear forces during the extrusion process affects the stability of the microcapsule (Gouin, 2004). The extrusion microencapsulation includes 3 processes such as centrifugal extrusion (co extrusion), melt injection and melt-extrusion. Centrifugal extrusion, also known as co-extrusion is another extrusion technology that is commonly used for microencapsulation which can produce microcapsules in the size range of is 500–1000µm. Since the particle size of the extruded powder is more, it can provide the particular mouth feel. Melt injection process involves dispersion of the core material in a matrix containing starch, anti-oxidants, sugars, emulsifiers and water at about 130°C, extruded thorough a die or filter into a bath filled with organic solvent such as isopropanol which solidifies the sugar matrix. The microcapsules thus formed are collected by filtration or centrifugation (Valentinotti *et al.,* 2005). The melt-extrusion process and melt-injection is almost similar, where melt-injection is a vertical screw less process with surface-washed particles, while the melt extrusion is a horizontal screw process with particles that are not surface-washed.

1.4.8.5 In situ polymerization

In situ polymerization is commonly used for the preparation of microcapsules and functional fibers. The process doesn't include any reactants in the core material and polymerization occurs in the continuous phase itself. By adjusting pH and temperature, the wall material precipitates and distributes evenly over the surfaces of core material. Particles produced by this technology are found to have better encapsulation efficiency, good chemical, thermal and storage stability and controlled release.

1.4.8.6 Inclusion complexation

In this method, a cyclic oligosaccharide cyclodextrin is used as a main encapsulant which can form complexes with fish oil and thereby increasing its oxidative stability. The formation of an inclusion complexation was observed by mixing fish oil and gamma cyclodextrin in the presence of nitrogen at 45 °C for 24 h and the resultant product contained fish oil of 15–40% (Schmid *et al.,* 2001). Similarly, Choi *et al* (2010) reported that fish oil encapsulated in beta cylodextrin had 84.1% encapsulation efficiency.

1.4.8.7 Liposome entrapment

Liposomes are microscopic, spherical lipid bilayers that can enclose a number of aqueous compartments. The most commonly used encapsulating agent in this method is phospholipids and they are bio compatible and biodegradable substances (Kim & Baianu, 1991). The formation of a lipid bilayer is mainly attributed to the amphiphilic nature of phospholipids. These liposomes can be used to encapsulate omega-3 fatty acids by dissolving them in phospholipid before the addition of water. This mixture of phospholipid, omega-3 oil and water is then sonicated to form encapsulated products and oil encapsulated in liposomes is said to have better oxidative stability (Kubo *et al.,* 2003). But the limitation of this technology is its high cost and low stability.

1.4.8.8 Fluidized bed drying

Fluidized bed drying is the method that is restricted mainly to the encapsulation of solid core materials where a coating is applied on the powder particles (Rumpler & Jacob, 1998). Hence this method cannot be used for the direct encapsulation of fish oil, instead it can be considered as a secondary method to provide an additional coating on the already microencapsulated fish oil for better oxidative stability and physicochemical properties. In one of the patented technologies for double encapsulation of fish oil, corn starch was used for coating the already spray dried powder (Skelbaek & Andersen, 2002). In another method,

molten hydrogenated palm wax (30% w/w) was used to coat over the already spray dried fish oil powder (Ponginebbi & Puglisi, 2006).

1.4.9 Other emerging methods for the encapsulation of fish oil

1.4.9.1 Multiple emulsification and spray drying

Multiple emulsions, often known as double emulsions or emulsions of emulsions are one of the novel carrier systems where both w/o and o/w emulsion exists simultaneously in a single system. Surfactants, both lipophilic and hydrophilic are used for stabilizing these two emulsions respectively. There are two major types of multiple emulsions such as the water-oil-water (w/o/w) and oil-water-oil (o/w/o) double emulsions. Among these, the most commonly used and the most stable systems are the w/o/w type. Water-in-oil-in-water emulsions consist of water particles (w1) dispersed inside fat globules (o), which are further dispersed in turn in a continuous aqueous phase (w2). Multiple emulsions offer some advantages for food applications such as producing low calorie and reduced fat products, masking flavors, prevent oxidation, improving sensory characteristics of foods, controlled release of and protecting labile ingredients (McClements *et al.,* 2007).

Though multiple emulsions have so many advantages over the single emulsion sytems, they are more difficult to be prepared than simple emulsions and have a strong tendency to release entrapped compounds in an uncontrolled manner (Garti, 1997). Another disadvantage is the scarcity of suitable food-grade emulsifiers and stabilizers for preparation of double emulsions. Hence the success of double emulsion often depends on the adequate selection of surfactants for stabilization and their use in appropriate amounts. Some of the methods followed for synthesis of double emulsions are membrane emulsification and emulsification followed by spray drying. Multiple emulsifications followed by spray drying are most commonly preferred because that process is highly economical.

1.4.9.2 Electrospraying for ultrathin coating

The electro spraying method was used to microencapsulate DHA using zein prolamine and the nano particles thus formed were found to have increased induction period and hence a better oxidative stability. Moreover, the method doesn't seem to alter the textural characteristics of the product (Sergio *et al.,* 2010).

1.4.9.3 Spray granulation and fluid bed film coating

In this method, a combination of two technologies, spray granulation and fluid bed coating is used for encapsulation (Anwar *et al.,* 2010).

1.4.9.4 Encapsulation using ultrasonic atomizer

Ultrasonic atomizers use ultrasonic energy for atomization of emulsion and produces smaller particles of uniform size distribution. This is quite different from the conventional pressure spray nozzles where there is no control over the size of particles produced (Topp & Eisenklam, 1972; Bittner & Kissel, 1999). Fish oil encapsulated using ultrasonic atomizer gives small particle size and better emulsion stability (Klaypradit & Huang, 2008).

1.4.10 Characterisation of encapsulated microparticles

Microencapsulated oil produced by different encapsulation technologies using a wide variety of wall materials has to be characterized for studying its physicochemical properties and its oxidative stability. The important parameters that have to be taken into account for its characterization are encapsulation efficiency, loading capacity, percentage yield, particle size, bulk density, tapped density, moisture content, hygroscopicity, oxidative stability and other indices such as SEM (Scanning electron microscopy), TEM (Transmission electron microscopy), XRD (X-ray powder diffraction) etc. These indices are discussed in detail below: (Kumar *et al.,* 2017)

The encapsulation efficiency (EE) is the ratio of oil entrapped inside the wall material to the initial concentration used. For better encapsulation efficiency

the content of surface oil, which is the oil that is not entrapped inside the wall material, should be as low as possible. This is because of the fact that the surface oil content remains unprotected and can easily undergo oxidation, resulting in off-flavour generation and poor oxidative stability. For safe storage and better oxidative stability, ideally the surface oil should be less than 0.1% (w/w).

Encapsulation efficiency = (TO-SO/SO)*100

Where, TO - Total oil content; SO- Surface oil content

Loading capacity is the measure of the amount of oil encapsulated per unit mass of the encapsulation material.

LC = (Mass of encapsulated oil / Mass of wall material)*100

The percentage yield (PY) is a measure of the encapsulation process to produce the encapsulated powders.

PY = (Mass of loaded encapsulation system/ Mass of oil and wall material used)*100

The size of microcapsule has an important role in the oxidative stability of the product and its size range should be of uniform nature in order to maintain the product consistency. The size of the microcapsules can be measured using laser scattering or particle size imaging using microscopy. For the detailed study of the morphology of microcapsules, high resolution imaging using electron microscopy or confocal laser scanning microscope (CSLM) can be used. Bulk density and tapped density are important parameters from economic point of view. These two parameters play an important role in the packaging, storage and transport of powdered products. Based on these two parameters, the flowability and cohesiveness of the encapsulated material can be measured.

The moisture content of the encapsulated powder plays an important role in determining the flowability, stickiness, cohesiveness, hygroscopicity and storage life. The encapsulation method adopted is also found to have a profound influence on the moisture content. The maximum moisture content allowed for dried powders of food industry application was found to be around 3 - 4 %.

Hygroscopicity is the capacity of food to contain occluded moisture and this can be affected by inherent product composition and the concentration of the carrier material used. This also plays an important role in the product reconstitution since it can lead to caking and thereby reducing dispersibility (Ferrari *et al.,* 2011).

The main application of encapsulation technology is to protect the bioactive material against oxidation by providing an oxygen barrier in the form of wall materials. Currently, a number of substances are being used as the wall material for encapsulation depending upon the nature of substance to be encapsulated and the type of encapsulation technology used. Oxidative stability of the encapsulated powder is measured by storing the microcapsules under a set of temperatures and relative humidity for a definite period and quantifying the type and amount of oxidative products formed. There are different methods employed for the measurement of oxidative stability of the encapsulated powders. Peroxide value, TBARS (Thiobarbituric acid reactive substances), acid value, propanal and accelerated rancimat test are some of the commonly used tests to assess the oxidative stability of the product. Apart from this, many other characterizations are being used for the encapsulated product such as Scanning electron microscopy measurements for determining the surface morphology, FTIR (Fourier-transform infrared spectroscopy), zeta potential, water activity, colour, true density, wettability, solubility for complete analysis (Klinkesorn *et al.,* 2005).

1.4.11 In-vitro digestibility of encapsulated fish oil

Fish oils especially sardine oil and shark liver oil contains adequate amount of n-3 polyunsaturated fatty acids mainly EPA (Eicosapentaenoic acid, C20:5 n-3) and DHA (Docosahexaenoic acid, C22:6 n-3) (Klinkesorn *et al.,* 2004). Health benefits of these polyunsaturated fatty acids were previously reported (Harris, 2004). Because of their health benefits, fish oils are having very much demand as functional food ingredients. But utilization of fish oil is limited due to the presence of highly unsaturated fatty acids. Nowadays encapsulation technologies are used to protect fish oil for preserving its integrity (Kagami *et al.,* 2003). Various biopolymers are used as wall material for the preparation of fish

oil microparticles. Chitosan is one of the effective wall materials for the preparation of fish oil microparticles (Klaypradit & Huang, 2008; Klinkesorn *et al.,* 2005). Chitosan is the deacetylated form of chitin, which is the structural component of crustacean, insects and mollusks. Biological and physicochemical properties of chitosan are well established (Kim & Thomas, 2007; Shahidi *et al.,* 1999). In humans, the absorption of fat mainly occurs in the small intestine (Mu & Hoy, 2004). The ingested fats are emulsified due to the action of mouth and stomach. After leaving the stomach, the emulsified lipids enter the small intestine and are digested by pancreatic lipase in the presence of bile salts and colipase (Brockman, 2000). Characteristics of fish oil microparticles and the nature of coating materials used in the preparation of microspheres may play an important role in determining the in-vitro digestibility (Armand *et al.,* 1999). By using chitosan as wall material, fish oil can be protected from oxidation that can extend the shelf life of n-3 fatty acids in foods (Klinkesorn, 2005). A wall material may be highly effective at protecting fish oil from oxidation within a food product during storage, but if it does not release fish oil within the gastrointestinal tract, then it would not be a good delivery system (Klinkersorn, & McClements 2009). Some researchers suggest that human digestive enzymes cannot digest chitosan (Rodriguez & Albertengo, 2005) and some others have reported that chitosan can be partially degraded by some kind of enzymes like cellulase, hemicellulase, pectinase and lipase. According to Klinkersorn & McClements (2009) chitosan coated fish oil microparticles can be easily degraded by lipase under simulated gastrointestinal conditions. Hence chitosan coated lipid microspheres can be used as a better vehicle for the perfect delivery of marine oils.

1.4.12 Doxorubicin induced cardiotoxicity

Doxorubicin, commonly known as Adriamycin or Rubex, is one of the effective anticancer drugs available in the market (Fig.1.5). Chemically, it is an anthracycline compound and consists of a naphthacenequinone group and daunosamine, an amino sugar. It has both hydrophilic and hydrophobic regions and has the capability to bind with plasma proteins and cell membranes.

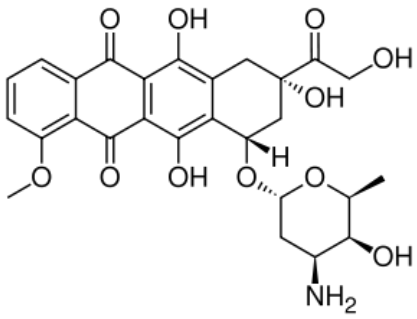

Fig. 1.5 Chemical Structure of Doxorubicin

Many of the intracellular oxidoreductases will reduce the doxorubicin to a semiquinone radical. Peroxidation of this radical leads to the production of reactive oxygen species (Weiss, 1992; Jordon, 2002). Major problem of doxorubicin is its adverse effect against cardiac function, which may limit its use. Doxorubicin cardiomyopathy, once developed, carries a poor prognosis (Jordon, 2002). Studies showed that doxorubicin treatment will increase cell death, ROS generation, and induced apoptotic cell death (Vishnu *et al.,* 2017). Doxorubicin induced cardiotoxicity is an excellent model to elucidate the cardio protection activity of various bioactive molecules. Doxorubicin accumulates in mitochondria and promotes apoptosis and in turn release cytochromes C. Cytochrome C will initiate the apoptotic pathway through caspase activation (Octavia *et al.,* 2012). H9c2 cell line is widely regarded as the best in vitro model to study cardioprotection activity due to its high resemblance to primary cardiomyocytes (Pelloux *et al.* 2006). Doxorubicin can induce cardiac damage through a complex mechanism and comprises series of events. Doxorubicin induced cardiotoxicity causes elevated oxidative stress and malfunctioning of mitochondria. All these events lead to death of cardiomyocetes by apoptosis and/or necrosis (Mukhopadhyay *et al.,* 2007). Doxorubicin causes the weakening of anti-oxidant defense mechanism by triggering the production of huge free radicals which leads to increased density of mitochondria occupancy and elevated aerobic metabolic activities unlike normal cells. This creates adverse atmosphere in cardiomycetes and finally leads to death (Granados- Principal *et al.,* 2014). High ROS generation by doxorubicin accelerates NF-κB production and subsequently activation of apoptotic mediated cardiac damage. Apart from this, high ROS is responsible for

peroxidation of lipids, loss of mitochondrial membrane integrity and activates p53 activity (EI-Bakly *et al.*, 2012: Park *et al.,* 2014).

1.4.13 Cardiomyopathy: Molecular mechanisms

Doxorubicin operates on many levels by different mechanisms including, altering the mitochondrial membrane potential, increased ROS (Reactive oxygen species), increased expression of Nf-kb, increased necrotic pathway etc.

1.4.13.1 Necrosis

Necrosis is often described as an uncontrolled, energy-independent process. Early rupture of the plasma membrane and swelling of cytoplasmic organelles occur during necrosis (Dorn, 2013). Numerous studies showed that necrosis are increased in DOX treated mouse hearts (Ikegami *et al.,* 2007). The correlation between oxidative stress and cardiomyocete death is very well established. The antioxidant compounds or free radical scavengers protect cardiomyocetes from induced necrosis (Ikegami *et al.,* 2007). The increased reactive oxygen species will lead to mitochondrial calcium overloading; mitochondrial swelling and ATP depletion, and hence triggers necrotic cell death (Gustafsson & Gottlieb 2008). Doxorubicin also causes mitochondrial DNA damage and ATP depletion. All these events contribute to necrosis (Wallace, 2003). In addition, reactive oxygen species will oxidise the lipids and that will lead to cardiomyocete necrosis (Casey *et al.,* 2007)

1.4.13.2 Mitochondrial membrane potential and cardiotoxicity

Mitochondria are one of the main organelles that control crucial cellular energy balance. They are the primary site of production of free radicals, and in addition it contains some regulators of apoptosis. So the changes of mitochondria are highly sensitive indicators of cell health and stress. The energy produced during respiration is stored as an electrochemical gradient across the mitochondrial membrane, and this accumulation of energy in healthy cells creates a mitochondrial trans-membrane potential, ($\Delta\Psi m$) that enables the cell to drive the synthesis of ATP. Loss of the mitochondrial inner transmembrane potential is often, but not always, observed to be associated with early stages of apoptosis.

This potential change will make some pores in the membrane and cytochrome C will be released into the cytosol; it will trigger the events in the apoptotic pathway. Depolarization of the inner mitochondrial membrane potential is thus a reliable indicator of mitochondrial dysfunction and cellular health, which has become increasingly important in the study of apoptosis, drug toxicity and multiple disease states (Ichikawa *et al.,* 2014)

1.4.13.3 Reactive oxygen species and NF-kb expression

In the DOX induced cardiotoxicity, the action of DOX is mainly by redox activation of reactive oxygen species (ROS) which can cause myocyte apoptosis (Spallarossa *et al.,* 2005). Some studies show that DOX induced apoptosis occurs in the cardiomyocytes through activation of Nuclear Factor kB (NF-kB). NF-kB is one of the transcription factors in mammalian cells (Wang *et al.,* 2002). Dissociated form of NF-kB will bind to the promoter elements and it activates some selected target genes involved in apoptosis such as Bcl-2 proteins and caspase inhibitors (Karin & Ben-Neriah, 2000). Natural antioxidants could inhibit DOX-induced apoptosis in cardiac cells by inhibiting the ROS production (Han *et al.,* 2008).

Cardiac health promoting role of omega-3 polyunsaturated fatty acids rich oils especially fish oils has been well established since past many decades of research. Previous reports consolidate the adverse action of oxidized omega-3 polyunsaturated fatty acids rich oils rather than its beneficial action. Moreover, a robust scientific data is lacking regarding molecular mechanism behind the cardioprotective activity of omega-3 rich fish oil. Researchers have seldom exploited the technique of encapsulation of fish oil with natural antioxidant in order to produce highly stable PUFA rich fish oil. There is a strong correlation between stable and less oxidized fish oil consumption and cardiac health. So an attempt was made to produce highly stable omega-3 rich fish oil powder by encapsulation with natural antioxidant wall material and investigate the exact molecular mechanism of action involved in cardioprotective activity of fish oil against DOX induced cardiac cell line model.

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Chapter 2

Comprehensive lipid profiling and bioactivity screening of marine lipids: semi-targeted lipidomics; in vivo, in vitro bioassays

2.1 Introduction

2.2 Materials and Methods

2.3 Results and Discussions

2.4 Conclusion

2.1 Introduction

The immense health benefits of marine oil have been studied in the past few decades and were reported as a source of nutrients such as lipid soluble vitamins and essential fatty acids. Because of its therapeutic health benefits such as wound healing property and the stimulation of haematopoiesis, it is being used in traditional medicine in Scandinavian countries (Pugliese *et al.,* 1998). Fish oil has been widely accepted as an excellent dietary source of polyunsaturated n-3 fatty acids such as EPA and DHA. Fish oil has been used for over 40 years as both therapeutic and preventive agent (Colombo *et al.,* 1997). The main constituents in deep sea shark liver oil are fatty acids, alkylglycerols, squalene, vitamins and long chain PUFA. Oil of ray species such as *Dasyatis brevis* and *Gymnura marmorata* contains comparatively high amount of PUFA, n-3 PUFA being dominant among fatty acids (Navarro-Garcia *et al.,* 2000; Le Nechet *et al.,* 2007).

Taking into consideration the health benefits of these fish oils, many health organizations have recommended the consumption of fish oil either directly or in the form of capsules. Nowadays there is increasing consumer awareness on

authenticity of fish and fisheries products. Globally, there is increasing consumer awareness on traceability and authenticity of fish and fisheries products. Fish oil, rich in the health promoting poly unsaturated fatty acids (PUFA) is in high demand in health food markets. The price and nutritional value of fish oils are determined by factors such as fish species used, processing method and storage. The differences in price between such oils may lead to mislabeling and adulteration.

Beneficial health effects of these compounds are well demonstrated and include prevention of number of diseases such as coronary heart diseases, cancer, arthritis, bacterial, viral and fungal infections, allergic reactions, autoimmune disorders, adjuvant therapy and treatment of neoplastic disorders and as immune booster in infectious diseases (Von Schacky & Harris, 2007; Pugliese *et al.*, 1998). Generally shark liver oils are rich in bio-active compounds but they remain unexplored with regard to their anti-ulcer and anti-inflammatory activities. Bramble shark (*Echinorhinus brucus*) is one of the species of deep sea sharks in the family Echinorhinidae. This rarely encountered shark is rich in liver oil and its liver oil is highly valued in countries like South Africa as medicine, whereas in India it is used to coat canoes to discourage wood boring beetles. Sardine oil (*Sardinella longiceps*) is rich in numerous nutrients that have been found to support cardiovascular health. They are one of the most concentrated sources of the omega-3 fatty acids EPA and DHA, which have been found to reduce the ulcer and inflammatory symptoms. There is no evidence of *in vivo* animal model to support the above reports.

The present study is focused on qualitative shotgun lipid profile of shark liver oil and sardine oil using electron spray ionization mass spectrometry to compare both the shark and sardine oils. The anti-ulcer and anti-inflammatory activities of the oils were studied using a rat model.

2.2 Materials and Methods

2.2.1 Materials

FAME (Fatty acid methyl ester) external standard mixtures were obtained from Sigma-Aldrich (USA). All other chemicals and solvents used in the study were of analytical grade.

2.2.2 Sample collection and oil extraction

Samples (*Sardinella longiceps*) were collected from the local fish market (Cochin, Kerala, India) and Bramble shark (*Echinorhinus brucus*) (12kg and 5.5ft) was caught during the cruise No.318 (400-600m depth) of the FORV Sagar Sampada between Mangalore and Kochi on the west coast of India. Both the samples were immediately frozen at -20^{o}C and subsequently brought to the laboratory for further analysis. Oil extraction from these two samples was achieved by following (Folch *et al.*, 1957) method, employing a 2:1 mixture of chloroform-methanol to a weighed portion of the liver sample. 20% of water was added to this mixture and the layers were allowed to separate. The aqueous layer was discarded and the solvent chloroform was completely flash evaporated to get the pure oil alone. The oil was stored in amber colored bottles, under nitrogen at -60˚C.

2.2.3 Peroxide value of oil

Peroxide value analysis (PV) of sardine oil and bramble shark liver oil was determined by an iodometric titration as described by Kolanowski *et al.* (2006). Briefly, 15mL of acetic acid-chloroform solution was added into the oil and was stirred for some time. 5mL of saturated potassium iodide (KI) solution was added and the solution was kept in dark for 30 minutes. To this, about 30 mL of distilled water was added and titrated with 0.01N sodium thiosulphate ($Na_2S_2O_3$) until the yellow color of the solution disappeared. The titration was continued after the addition of starch indicator to the solution until the blue colour disappeared. A blank was run simultaneously. PV was calculated as:

$$PV = (S - B) \times N \times 1000/W$$

Where, S is the volume of $Na_2S_2O_3$ added to the sample, B is the volume of $Na_2S_2O_3$ of the blank; N is the normality of $Na_2S_2O_3$ solution, and W is the sample weight (g).

2.2.4 Fatty acid analysis by Gas Chromatography

Fatty acid profile of both sardine oil and bramble shark liver oil were done by Folch *et al.*, (1957) method. A fraction of the lipid extract was saponified with methanolic NaOH followed by methylation in 14% boron trifluoride in methanol (BF3 /MeOH). Methyl esters of the fatty acids thus obtained were separated by gas chromatography (Thermo Trace GC Ultra) equipped with a Perkin Elmer Elite 225® capillary column (30 ×0.25 mm × 0.25 µ) and a flame ionization detector. Identification and quantification were done with the help of FAME external standards.

2.2.5 Lipid profiling of bramble shark liver oil and sardine oil using semi-targeted lipidomics

Five samples each of Sardine (*Sardinella longiceps*) oil and Shark (*Echinorhinus brucus*) liver oil were diluted to a concentration of 80µg/mL in 1:2 (v/v) chloroform, methanol with 5mM ammonium acetate and directly infused in a quadrupole-linear ion trap (SCIEX QTRAP® 4000 system) with electron spray ionization (ESI) ion source. Data was acquired for 23 precursor ion scan and 4 neutral loss scan experiments in positive ionization mode. Similarly, in negative ionization mode data was acquired for 52 precursor ion scan and 5 neutral loss scan experiments.

2.2.6 Evaluation of Anti-inflammatory and Anti-ulcer Activity

2.2.6.1 Animals

Wistar strain albino rats (160-200g) were taken for the experiment. They were housed individually in polyurethane cages under hygienic conditions, maintained at room temperature (28±2˚C) and provided food and water ad libitum for the

survival. Animal experiments were performed as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee (IAEC).

2.2.6.2 Anti-inflammatory activity

Anti-inflammatory activity of both the oils were determined, by the method of Hunskaar *et al.,* (1985) using the formalin- induced rat paw edema test. Percentages of inflammation inhibition was obtained for each group by calculating the difference in mean paw size from control and expressed as percentage. (Owolabi & Omogbai, 2007). Changes in anti-oxidant enzymes were also evaluated **(**Reduced glutathione (GSH) levels by Sedlak & Lindsay, 1968: superoxide dismutase (SOD) activity by Marklund & Marklund, (1974): Catalase (CAT) activity by Aebi, (1984).

2.2.6.3 Anti-ulcer activity

Anti-ulcer activity was determined, by the method of Hara & Okabe, (1985). Male and female wistar rats were kept in standard laboratory conditions randomly assigned to four groups consisting of 6 animals each. Group I (Positive control) received hydrochloric acid-ethanol (0.6%v/v) to induce ulcer while group II acted as negative control and was fed with regular diet only. Group III was pre-treated with sardine oil along with the vehicle (Oil: DMSO = 4:1) at 0.5 g/kg bodyweight prior to induction of ulcer. Groups IV was administrated DMSO alone. Group V was pre-treated with bramble shark liver oil along with the vehicle (Oil: DMSO = 4:1) at 0.5 g/kg body weight prior to induction of ulcer. All the animals were fasted overnight before the induction of ulcer. After 4 h of induction of ulcer, all animal groups underwent surgery as per the procedure of Takeuchi *et al.,* (1976). The stomach was inflated with normal saline, incised, counted the number of lesions and further subjected to histopathology. Gastric juice was taken for determining the p^H. The ulcer index was calculated by adding the total number of ulcers per stomach and the total severity of ulcers per stomach (Hollander *et al*., 1985). The number and severity recorded for ulcer scores were as follows:

0 = normal stomach; 0:5 = red coloration;

1:0 = spot ulcers; 1:5 = hemorrhagic streaks;

2:0 = > 3 but < 5 ulcers; 3:0 = > 5 ulcers

The mean ulcer score for each animal is expressed as the ulcer index.

The percentage protection is expressed as:

$$Percentage\ protection\ =\ 100\ -\ Ut/Uc * 100$$

Where

Ut = the ulcer index of the treated group; and

Uc = the ulcer index of the control group:

2.2.6.4 Preparation of subcellular fractions of stomachs

Stomach tissue samples exposed to HCl and ethanol were taken for the biochemical assays. The damaged parts were weighed and homogenized with 200mM potassium phosphate buffer (pH 6.5). The homogenate was used to measure the reduced glutathione (GSH) levels and then centrifuged at 11,000 rpm for 20min at 4°C. The supernatant was used to establish superoxide dismutase (SOD) and catalase (CAT) activity.

2.2.6.5 Determination of reduced glutathione (GSH) levels

GSH levels in gastric mucosa were determined as described earlier Sedlak & Lindsay, 1968. Tissue homogenate previously prepared were mixed with 12.5% trichloro acetic acid and centrifuged for 4000 rpm, 15 min at 4 °C. The absorbance of supernatant plus Tris buffer (0.4 M, pH 8.9) and 5,5′-dithiobis 2-nitrobenzoicacid (DTNB, 0.01 M) absorbance of the supernatant was measured at 420 nm and expressed as mg GSH/g of tissue.

2.2.6.6 Determination of superoxide dismutase (SOD) activity

The activity of SOD was determined as described previously by Marklund & Marklund (1974). Aliquots of tissue homogenate were mixed with Pyrogallol (1mM) and buffer solution (Tris-HCl 1mM – EDTA 5 mM, pH 8.5). The reaction

was incubated for 20 min, stopped with the addition of 1N HCl and then centrifuged for 4 min at 14,000 rpm. The absorbance of the supernatant was measured at 405 nm. The amount of SOD that inhibited the oxidation of pyrogallol by 50%, relative to the control, was defined as one unit of SOD activity. The enzymatic activity was expressed as U/mg of protein.

2.2.6.7 Determination of catalase (CAT) activity

Catalase activity was measured as described earlier (Aebi, 1984). Samples aliquots of supernatant were mixed with a solution containing 30% H_2O_2, milli-Q water and buffer 5 mM Tris EDTA, pH 8.0. The absorbance was determined by spectrophotometry at 240 nm for 60s. The enzymatic activity was expressed as mmol/ min/mg of protein

2.2.7 Statistical Analysis

Two way analysis of variance was carried out to compare the direct and interaction effect of different treatments. Tukey's test was performed to compare the marginal means and t-test used to compare the interaction means at 5% level of significance. All the statistical analysis was done using SAS 9.3 software.

2.3 Result and Discussion

2.3.1 Peroxide value of extracted oil

Around 500 mL of oil has been obtained from 5 Kg of sardine. In *Echinorhinus brucus* (12Kg) the extracted oil was around 700 mL. Primary oxidation of oil and the measurement of hydroperoxides are determined by peroxide value analysis. The peroxide value of oil sample extracted from *Sardinella longiceps* and *Echinorhinus brucus* was 0.58 meq/Kg and 0.74 meq/Kg respectively. The peroxide value (PV) provides a quantitative measure of hydroperoxide levels. The oxidation of *n*-3 PUFA is the main problem in fish oil and the rate of oxidation of fish oil are influenced by many factors like fatty acid composition, presence of O_2, light, temperature, antioxidant content, and the presence of heavy metals (Shahidi & Zhong, 2010). The initial oxidation products of fish oils are peroxides and hydroperoxides, which decompose into a variety of

radical molecules. These radicals react with PUFA to form additional hydroperoxides. These are strongly linked to the rancid smells and off flavors (Shahidi & Zhong, 2010; Ritter & Budge, 2012). The peroxide value limit of fish oil for consumption should be below 10 mEq/kg (Guillen *et al*., 2002). In this study, peroxide value of *Sardinella longiceps* and *Echinorhinus brucus* was found to be less than 1 mEq/kg. From this result it is clear that there was very little primary oxidation of fish oil during the extraction process and can be used for further studies.

2.3.2 Fatty Acid Analysis of bramble shark liver oil

Fatty acids identified in the saponifiable fraction of shark liver oil in this study is summarized in Table 2.1. Major nutritionally significant fatty acids were observed in the profile and they contribute for bioactivity of corresponding oil. Palmitic acid, myristicacid, and stearic acid are the prominent saturated fatty acids. When unsaturated fatty acids are taken into account, oleic acid, linoleic acid, linolenic acid, arachidonicacid, EPA (Eicosapentaenoic acid) and DHA (Docosahexaenoic acid) contribute to the major portion. This is in accordance with previous results (Garcia *et al.,* 2005). Diet composition and water parameters are two important factors responsible for the content of EPA and DHA in liver oil of sharks. Planktonic crustaceans are important sources of food for sharks and hence EPA and DHA concentration in shark liver oil is affected by the environmental temperature: increased in water temperature could cause a decrease in the EPA and DHA levels in shark liver oil (Malins *et al*., 1965). Lipid content and composition in shark liver oil could be affected by different known factors, such as fishing season, species, location and availability of food. Shark liver oil is regarded as an excellent source of PUFA (Polyunsaturated fatty acids). Two of these acids, eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) have been reported to be important in preventing or reducing heart diseases (Stansby, 1990), inflammatory disorders, as a nutritional supplement for the brain and retina development in babies.

Table 2.1. Fatty acids from liver oil of *Echinorhinus brucus*. Data expressed as a percentage of wet weight. Value represents the mean ± SD.

Fatty Acid	*(%) in terms of total fatty acids*
C14 (Myristic acid)	2.36(±0.40)
C16 (Palmitic acid)	14.79(±0.03)
C16:1 (Palmitoleic acid)	3.51(±0.01)
C17:1 (Heptadecanoic acid)	2.01(±0.06)
C18:0 (Stearic acid)	8.27(±0.20)
C18:1n9 (Oleic acid)	12.13(±0.11)
C18:2n6 (Linoleic acid)	9.24(±0.05)
C18:3n3 (α Linolenic acid)	0.89(±0.09)
C18:3n6 (γ Linolenic acid)	2.23(±0.13)
C20:1 (Eicosenoic acid)	0.55(±0.02)
C20:3n3 (Eicosatrienoic acid)	5.17(±0.10)
C20:5n3 (EPA)	16.27(±0.22)
C22:6n3 (DHA)	18.1(±0.99)
C23:0 (Tricosanoic acid)	1.13(±0.89)

2.3.3 Fatty acid analysis of sardine oil

The mass spectrometry analysis of sardine (*Sardinella longiceps*) fish oil methyl ester was carried out according to AOAC (2000) official method highlighted the presence of 23 compounds. Fatty acid profile of oil extracted from *Sardinella longiceps* contains major nutritionally significant n-3 PUFA (like EPA and DHA) and contributes to the major bioactivity of the oil (Fig. 2.1). The major fatty acids observed were palmitic acid (21.29%), 8, 11, 14-eicosatrienoic acid (16.02%), docosahexenoic acid (14.00%), palmitoleic acid (10.57%). Myristoleic acid is present in very small amount (0.05%) compared to other compounds present in the sardine oil. Fig. 2.2 and Fig. 2.3 shows saturated and monounsaturated fatty acids. From our study, it is understood that the fish oil contains different types of polyunsaturated fatty acids in which eicosapentaenoic acid and docosahexenoic acid are present in high amounts. EPA and DHA mainly

help to reduce inflammation, improve cardiac health, memory and have various other health benefits (Chapkin et *al.,* 2008).

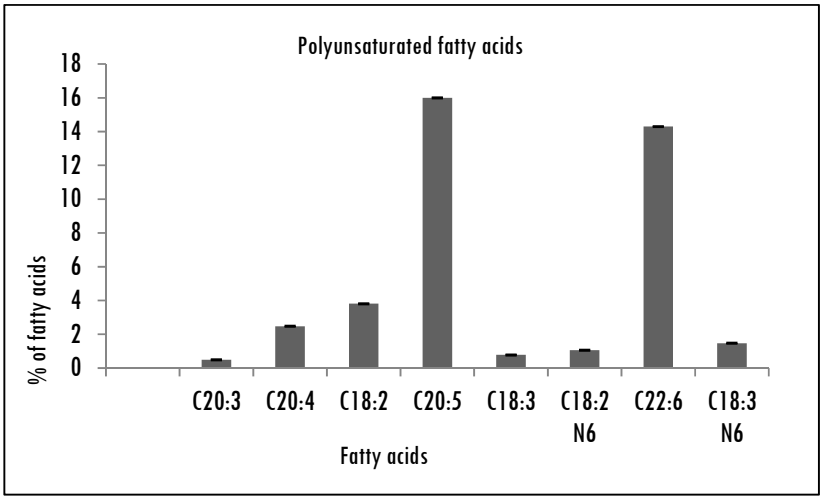

Fig. 2.1 The main polyunsaturated fatty acids from oil of *Sardinella longiceps*. Data expressed as a percentage of wet weight. The value represents the mean ± SD.

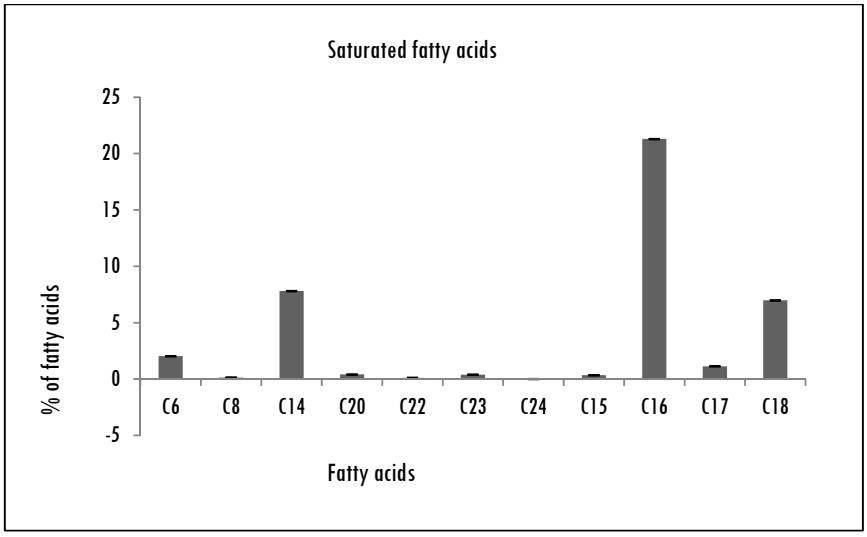

Fig.2.2 Saturated fatty acids from the oil of *Sardinella longiceps*. Data expressed as a percentage of wet weight. The value represents the mean ± SD

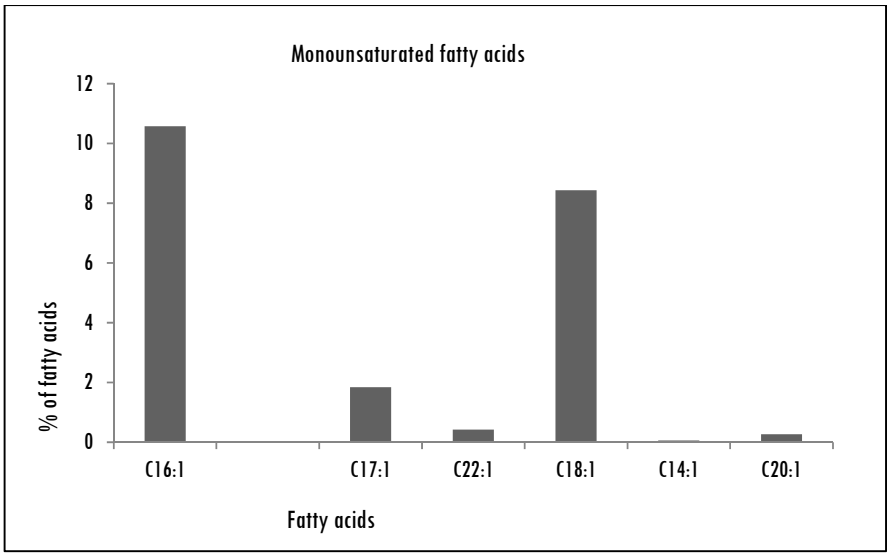

Fig. 2.3. Mono unsaturated fatty acids from oil of *Sardinella longiceps*. Data expressed as a percentage of wet weight. The value represents the mean ± SD

2.3.4 Lipid profiling using semi-targeted lipidomics

Lipids play an important role in cell, tissue, and organ physiology. In the present study we performed qualitative lipid profiling using targeted precursor ion and neutral loss scanning on electron spray ionization for the complete lipid profile of both sardine oil and bramble shark liver oil. Nowadays, tandem mass spectrometry strategies have been proven to be well suited for knowing the detailed characterization of lipids.

Analysis of intact lipids present in the sample was performed without any chemical hydrolysis or derivatization. The work flow of shotgun lipidomics given (Fig 2.4)

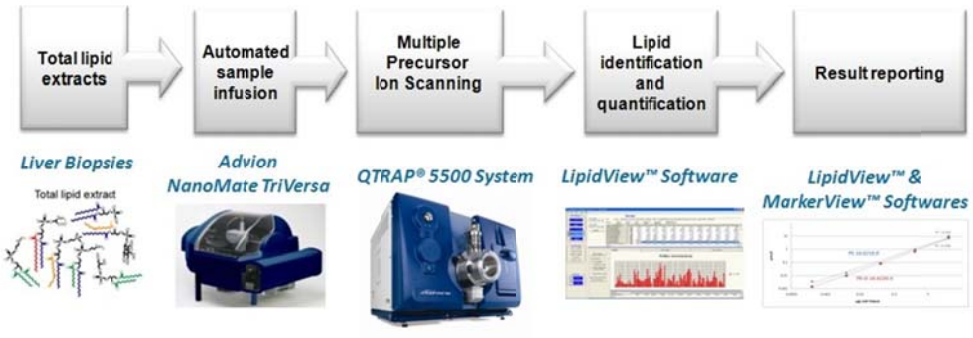

Fig.2.4 Shotgun Lipidomics Work flow. This work flow includes automated sample infusion, multiplexed precursor ion scanning (XPIS) acquisition on the QTRAP®4000 System and automated lipid identification and quantification using LipidView™ Software.

Thus native lipids profile as esters (eg. Cholesteryl ester), phospholipids, triacyl glycerol (TAG) pattern was obtained and documented. In both the species analyzed, major lipids observed includes different cholesteryl esters (palmitate ester, linoleate ester, sterate ester etc), DAG (diacyl glycerols), TAG (triacyl glycerols), phospholipids such as sphingomyeline, phosphatidine choline, fatty acids etc.

Fig 2.5 depicts cholesteryl esters profile of two species studied. Cholesteryl esters content was high in bramble shark oil and the major esters observed were CE-C14 (myristoleate ester), CE-C16 (palmitate ester), CE-C18:1(stearate ester) and CE-C20:5(eicosapentaenoate ester). Cholesteryl esters control transport of fatty acids and lipoprotein and crucial in lipid metabolism (Daniels *et al.*, 2009). Exact types of cholesteryl ester profiling signify the quality of the oil and can be used to generate typical data profile of corresponding oils. Among bramble shark oil and sardine oil studied, many types of CE were noticed and most prominent ester observed was CE-C18:1.

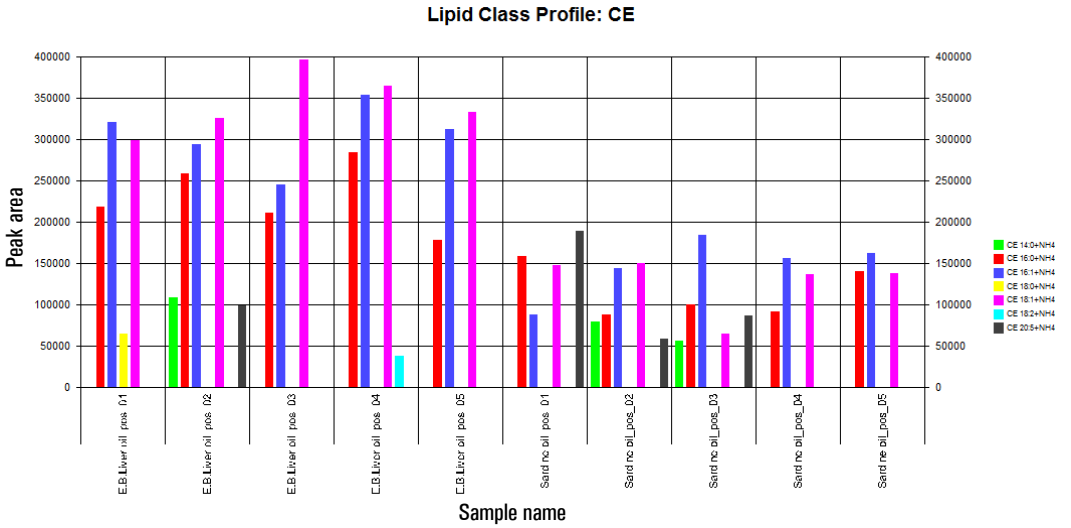

Fig.2.5. Cholestryl ester content of sardine oil and bramble shark liver oil

Fig 2.6 and 2.9 show DAG (Diacyl glycerols) & TAG (Triacyl glycerols) content of bramble shark liver oil and sardine oil. TAG content were below detectable level in sardine oil but observed in bramble shark oil. Major TAGs observed were TAG C30 and C32. In contrast DAG content was observed high in sardine oil compared to shark liver oil. DAG namely, C32:1, C34:1were high in sardine oil whereas C36:2, C36:4 were observed in high quantity in shark liver oil. This result clearly demonstrates the role of DAG and TAG profiling in identifying the authentification of typical oils.

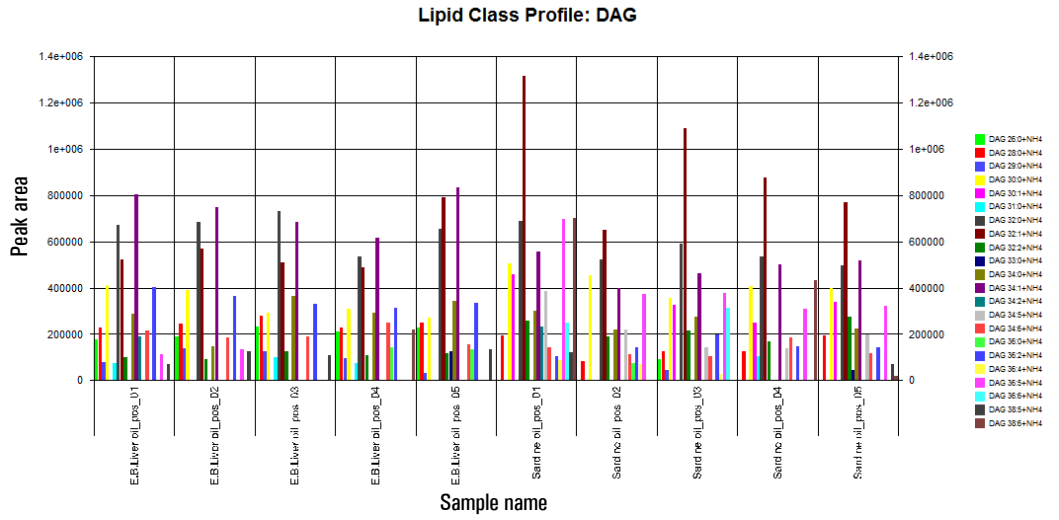

Fig.2.6. Diacyl glycerol content of sardine oil and bramble shark liver oil

Fig 2.7 and 2.8 illustrate phosphatidyl choline (PC) and sphingomyeline content in both the studied oil. PC was not detected in shark liver oil whereas present in sardine oil. In contrast to PC profile, sphingomyeline content was observed high in shark liver oil but almost absent in sardine oil. This result can be used to differentiate sardine oil and shark liver oil based on phospholipids content. No previous reports are available regarding the phospholipids profile of sardine oil and shark liver oil to elucidate their identity based on lipid profile. So our results highlight the significance of profiling of phospholipids to reveal the authenticity of different oils.

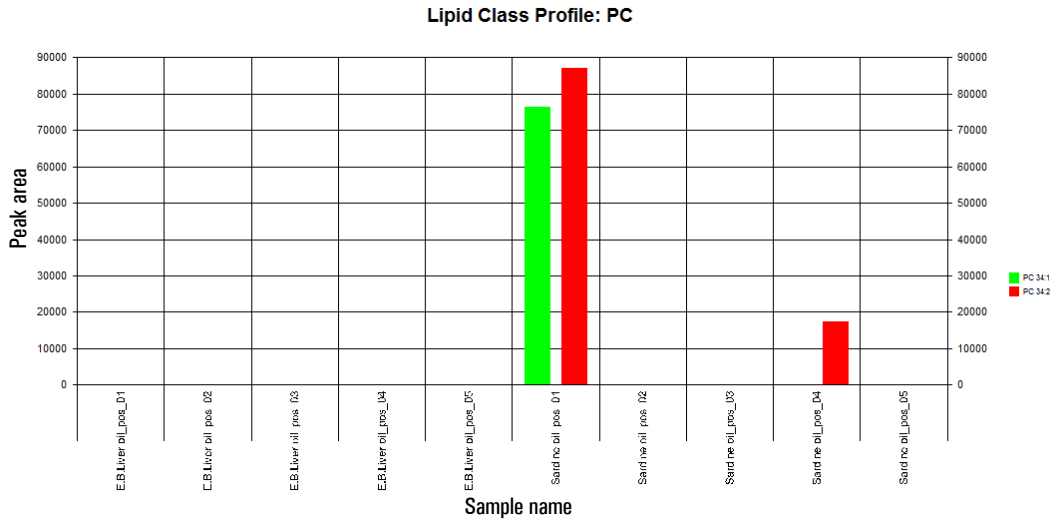

Fig.2.7. Phosphatidyl choline content of sardine oil and bramble shark liver oil

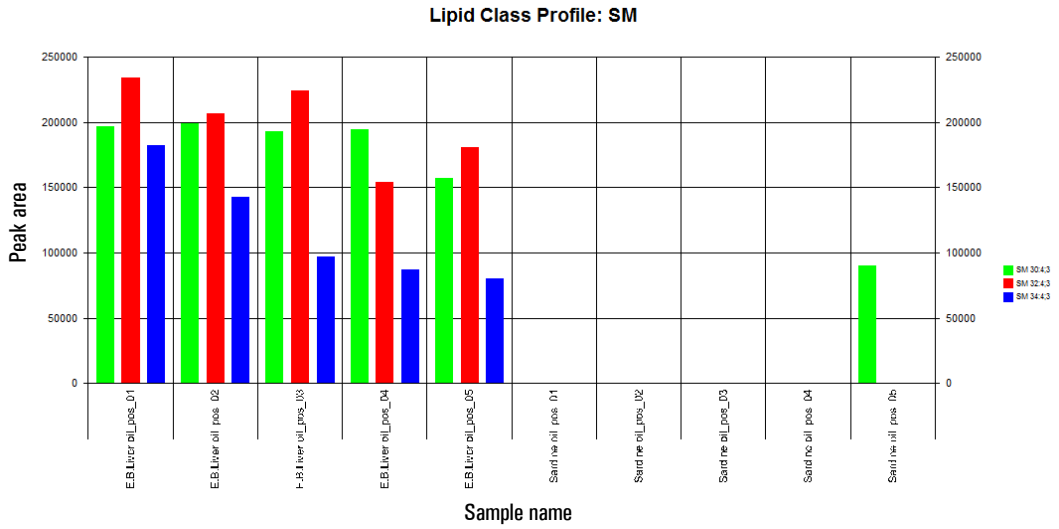

Fig.2.8. Sphingomyelin content of sardine oil and bramble shark liver oil

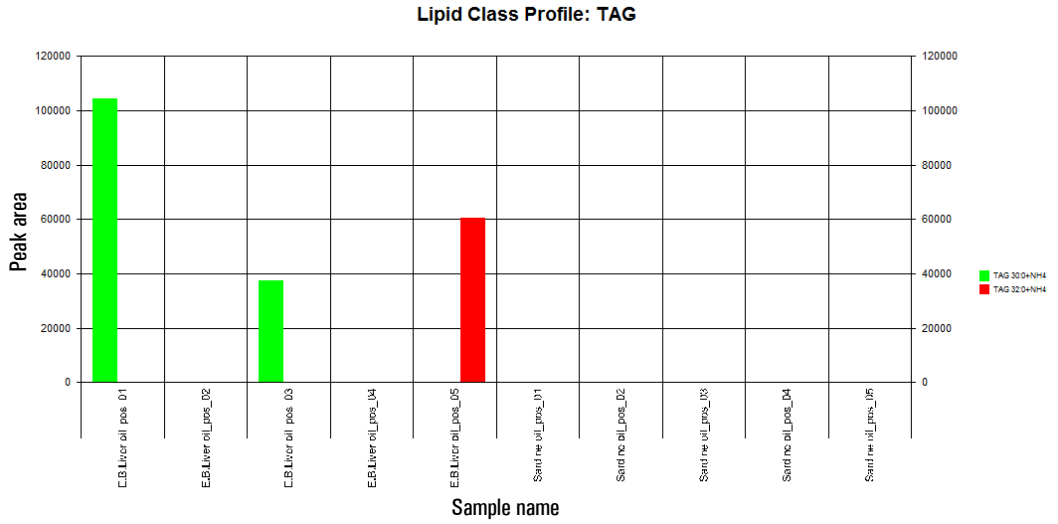

Fig.2.9. Triacyl glycerol content of sardine oil and bramble shark liver oil

2.3.5 Anti-inflammatory effect

Formalin-induced rat paw edema model is an effective method to study the anti-inflammatory effect of compounds (Ruangsang *et al.*, 2010). When formalin is subcutaneously injected into a rat, it forms edema around the injected place, due to increased vascular permeability of the capillary venules in the skin. Substances that antagonize the activity of histamine receptors reduce the area of the edema formed. In this study, the paw size data of control and treated groups of rats is summarized in table 2.2 Results obtained revealed significant anti-inflammatory effect of *E. brucus* liver oil and sardine oil. A steady increase of paw size was observed for control rats and maximum paw size of 6.31±0.33 mm was observed after 3h. Compared to control, steady decrease of paw size was observed for liver oil-treated samples. Paw size was minimum (5.25±0.44mm) for liver oil treated group which is closer to that of ibuprofen treated sample (4.91±0.25 mm). In the case of sardine oil treated group, it is 5.39±0.34. Table 2.3 depicts percentage of inhibition of paw size of treated animals. Significant percentage of inhibition 47.8% was observed for liver oil treated sample and 41.9% for sardine oil treated samples. The above result has similarity with ibuprofen treated sample 48.4 ± 0.21% after 3 hours. The analgesic and anti- inflammatory properties of liver oil of four different sharks, namely *Neohariotta raleighana*, *Centrosymnus*

crepidater, *Apristurus indicus* and *Centrophorus scalpratus*, captured from the Arabian Sea and the Indian Ocean were reported by Mathen *et al* (2008). In comparison to control rats, liver oil and sardine oil treated animals showed significant reduction of paw size which indicates significant anti-inflammatory effect. There was no significant reduction of paw size for control and DMSO treated samples at mentioned time interval. As expected ibuprofen-treated groups was superior among all groups with significant decrease in paw size.

Table.2.2 Anti-inflammatory effect of *E. brucus* liver oil and sardine oil on formalin induced paw edema in rats: Paw size at different intervals. Results are mean±SD of three duplicates of paw size. Values in the row with a different superscript letter (a, b, c) differ significantly ($p<0.05$) with each other.

Paw size (in mm)	Control	Ibuprofen	DMSO	Liver oil	Sardine Oil
Initial	3.15±0.11a	3.28±0.19^{a}	3.47±0.19^{b}	3.60±0.06^{b}	3.51±0.05 ^{b}
First h	6.82±0.19c	5.50±0.27^{a}	6.53±0.11^{b}	5.67±0.32^{a}	5.72±0.24 ^{a}
Second h	6.68±0.22b	5.17±0.22^{a}	6.09±0.17^{b}	5.42±0.43^{a}	5.58±0.22 ^{a}
Third h	6.31±0.33c	4.91±0.25^{a}	6.10±0.41^{b}	5.25±0.44^{a}	5.39±0.34 ^{a}

Table.2.3 Anti-inflammatory effect of shark liver oil and sardine oil on formalin induced paw edema in rats: Percentage of inhibition. Value represents the mean of six animals ±SD. Different superscript (a, b, c, d) in the row indicate significant difference ($p<0.05$) between the control and treatment groups. Marginal means are given in bold (Superscript A, B, C, D and E)

Percentage of Inhibition	Fist Hour	Second Hour	Third Hour	Mean
Ibuprofen	39.5±0.12^{b}	46.5±0.18 ^{a}	48.4±0.21^{a}	**44.66** ^{B}
DMSo	16.6±0.21^{b}	25.8±0.22^{a}	16.8±0.28^{a}	**19.55** ^{D}
Liver oil	43.5±0.11^{b}	48.4±0.10^{a}	47.8±0.11^{a}	**46.30** ^{A}
Sardine oil	41.5±0.09^{b}	45.4±0.13^{a}	41.9±0.10^{a}	**42.91** ^{C}
Control	15.8±0.18^{b}	29.3±0.15^{a}	18.4±0.11^{b}	**21.34** ^{E}
Mean	**31.20** ^{C}	**39.06** ^{A}	**34.60** ^{B}	

2.3.6 Anti-ulcer effect

The anti-ulcer effect of sardine oil and bramble shark liver oil was evaluated by comparing the degree of gastric ulceration in treated versus control animals by histopathology. The administration of ethanol/HCl caused histopathological lesions including degeneration, hemorrhage, and edematous appearance of the gastric tissue. Pretreatment with sardine oil (0.5 g/kg) and bramble shark liver oil (0.5 g/kg) was found to offer significant protection against all such damage to the mucosa. (Fig.2.10).

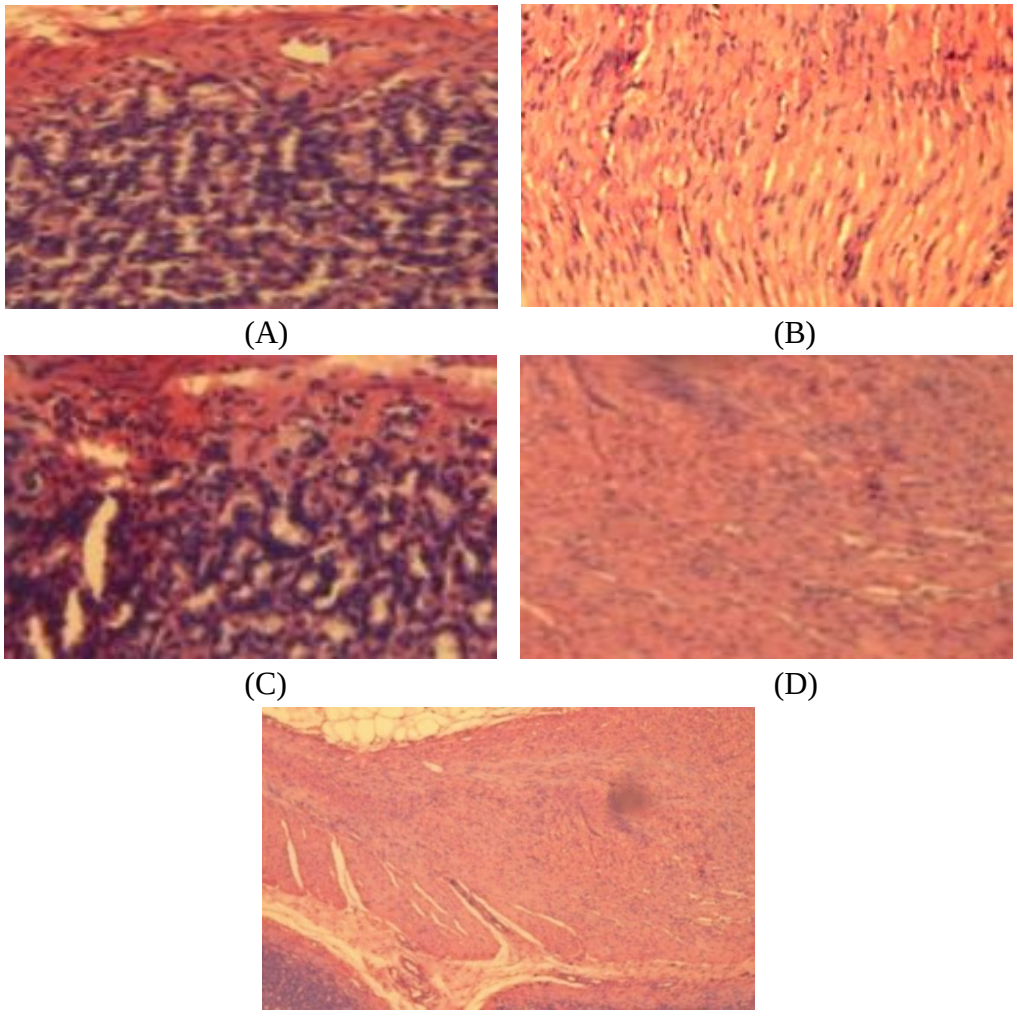

Fig.2.10. Anti - ulcer activity of sardine oil and bramble shark (*E. bucus*) liver oil on gastric mucosa: Histopathological section. A: Positive control (HCl/ethanol induced group), B: Negative control (Normal diet received group), C: DMSO treated group (Vehicle), D: Sardine oil treated group, E: Bramble shark liver oil treated.

Oxidative stress is one of the main factors that plays an important role in the pathogenesis of stomach mucosal injury. HCl/ethanol induced gastric lesions are thought to arise as a result of direct damage of gastric mucosal cells, resulting in the generation of free radicals and hyper oxidation of lipid (Jainu & Devi, 2006). The gastric lesions in the stomachs of ethanol-treated rats were significantly higher when compared to the normal control. Reduction of ulcer lesions (Fig. 2.11) was observed in sardine oil-treated groups and liver oil treated groups. Severe blood vessel damage and tissue damage with 11-17 lesions were observed for positive control. Reduction of tissue and blood vessel damage were noticed for oil-treated samples. The number of lesions has also been reduced to 2-3.

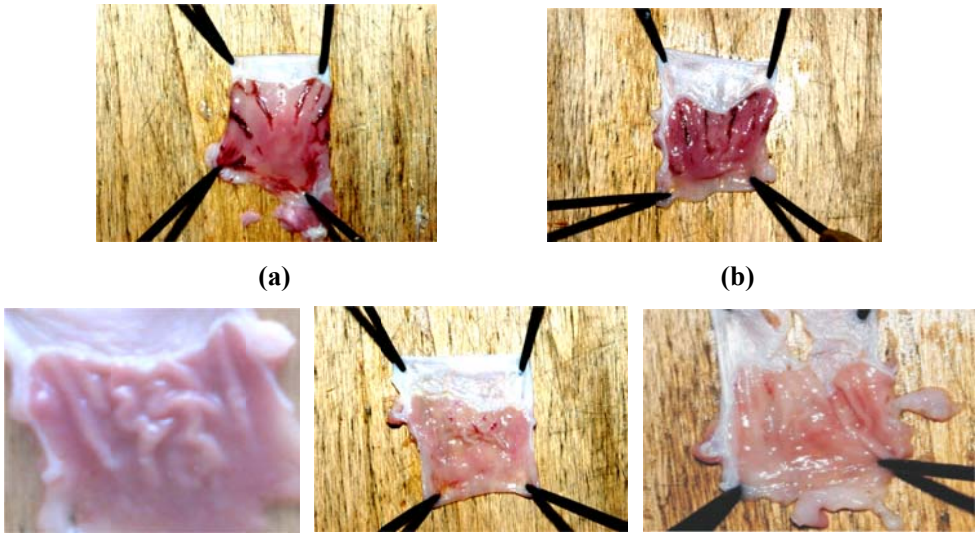

Fig.2.11 Antiulcer activity of sardine oil (*Sardinella longiceps*) and bramble shark liver oil on gastric mucosa: stomach section. A: Positive control (HCl/ethanol induced group), B: DMSO treated group (Vehicle), C: Negative control (Normal diet received group), D: Sardine oil treated group, E: Bramble shark liver oil treated

Oils of different origin were proven as effective anti-ulcer agents. Shark liver oils are reportedly known for their anti-ulcer properties (Vishnu *et al.*, 2015). Bioactive compounds present in oils could be the reason behind their anti-ulcer properties (Peana *et al.*, 2002). The anti-ulcer effect may be due to the presence of bioactive fatty acids and other potent compounds in the fish oil (Al-Harbi *et al.*,

1995). Sardine oil (0.5g/kg) and bramble shark liver oil treatments showed significant protection from ulcerative lesions caused by ethanol and HCl. In the ethanol induced ulceration study, pretreatment with these oils produced a significant decrease in number of ulcer, ulcer score and ulcer index (Table 2.4).

Table.2.4 Effect of sardine oil and shark liver oil on ulcer score, ulcer index and ulcer inhibition in ulcer induced rats. Values are expressed as mean±S.E.M. for six animals in each group.

Treatment	No. of ulcer	Ulcer score	Ulcer index	Ulcer inhibition (%)
Normal Control	0.0±0.0	0.0±0.0	-	-
Ethanol/HCl induced	17±0.5	3±0.8	20±0.8	-
DMSO treated	13±0.3	3±0.6	16±0.7	20
Sardine oil treated	3±0.4	1±0.7	4±0.5	80
Liver oil treated	2±0.5	1.5±0.6	3.5±0.3	82.5

The ulcer inhibition was found to be 80% at a dose of 0.5g/kg sardine oil and 82.5% of inhibition by liver oil treated group (Table. 2.4). The results show that the SOD, CAT, and GSH levels in the ethanol treated groups were significantly lower than that of the control group. Treatment with these oils increased the activity of antioxidant enzymes (Table. 2.5), which in turn demonstrates that both oils are more efficient in reducing oxidative damage, and therefore, has greater potential to promote higher quality gastric healing. Increase in gastric volume, low pH value and acidity are the major parameters in assessing severity of ulcer. Increase in pH and reduced gastric volume is highly correlated with effective anti-ulcer activity. In our results we observed an elevation of pH of 6.08 for sardine oil treated group and 6.12 for shark liver oil treated group, showing decreased acidity, (Table.2.6) which could be an effective indication of protection of sardine oil and bramble shark liver oil against gastric ulcer.

Table.2.5 Effects of sardine oil and bramble shark liver oil treatments on superoxide dismutase (SOD), catalase (CAT) and non-protein sulfhydryl groups (GSH) at ulcer site induced by ethanol and HCl in rats. Results as mean ± SD for six rats of each group.

Group	SOD (U/mg of protein	CAT(mmol/min/mg of protein)	GSH (µg/g of tissue)
Normal Control	1684±104	3018±28	481±34
Ethanol/HCl induced	285±98	265±82	289±48
DMSO treated	435±79	486±47	317±38
Sardine oil treated	1348±211	2897±77	514±28
Liver oil treated	1218±122	2731±68	488±27

Table.2.6 Effects of sardine oil and shark liver oil on gastric secretion, pH of gastric content and total acidicity. Results as mean ± S.E.M

Treatment	Gastric content (mL)	pH of gastric content	Total acidicity(mEq/L)
Normal Control	2.12±0.2	4.02±0.1	44.32±0.2
Ethanol/HCl induced	3.59±0.2	2.26±0.7	74.23±0.3
DMSO treated	2.92±0.7	2.87±0.3	60.33±0.2
Sardine oil treated	1.58±0.5	6.08±0.2	41.38±0.1
Liver oil treated	1.38±0.3	6.12±0.4	40±0.3

2.4 Conclusion

The present study clearly depicts the complete lipidomics, anti-ulcer and anti-inflammatory activities of sardine oil (*Sardinella longiceps*) and bramble shark liver oil (*Echinorhinus brucus*) which are further supported by biochemical and histopathological studies of the stomach wall tissues of differently treated groups of rats. Recently, lipidomics pattern of different oils has received much attention since it can be used to identify typical lipid profile of oil. This technique can also be used to compare the quality of normally available sardine oil and unexploited oils like deep sea shark liver oil. Most importantly, this technique helps to differentiate among the different oils based on their specific lipid profile. In the present study, we could generate typical lipids profile of deep sea shark oils and sardine oils and observed definite changes in the lipid profile. Diseases like

inflammation and ulcer are becoming quite common due changes in the life style habits. Foods rich in bioactive compounds can find importance in treating such diseases since no side effects are reported as of now with their consumption. The anti-ulcer and anti-inflammatory effect of sardine oil and shark liver oil was also proved with the help of animal model and points the importance of marine oils to prevent these diseases. Based on the significant findings of the study, it can be concluded that marine oils are rich in bioactives that have potential to combat diseases such as ulcer and inflammation.

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Chapter 3

Microencapsulation of sardine oil using vanillic acid grafted chitosan as a bio-functional wall material

3.1 Introduction

3.2 Materials and Methods

3.3 Results and Discussions

3.4 Conclusion

3.1 Introduction

Health benefits of long chain polyunsaturated fatty acids (PUFA) containing marine fish oils are well-studied (Comunian & Favaro-Trindade, 2016; Ruxton *et al.*, 2004). Because of the health benefits of PUFA, many food companies and food processors have come forward with novel functional food products, fortified with PUFA rich oils. The high presence of easily oxidizable unsaturated fatty acids makes the usage of fortified PUFA rich oils limited. Microencapsulation has been considered as a promising technique for such fortification of highly unsaturated marine fish oils in food products, as it imparts controlled release behaviour and protects against oxidation during processing, handling and storage (Ghorbanzade *et al.*, 2017; Caceres *et al.*, 2008; Hermida & Gallardo, 2015).

Choice of emulsifier and wall material are important factors among others that determine the success of the microencapsulation process (Encina *et al.*, 2016; Klinkesorn *et al.*, 2004). Chitosan, a versatile amino polysaccharide with excellent film forming and emulsifying properties has often been used as a wall material, and as an emulsifier for microencapsulation of highly unsaturated oils (Shen *et al.*, 2010; Shaw *et al.*, 2007). It has been observed that being a poly cationic

polysaccharide, chitosan surrounds oil-in-water emulsion droplets and repels pro-oxidant metals in the surrounding environment (Klinkesorn *et al.,* 2005). Interestingly, chitosan is also a bio-functional natural polysaccharide. Many researchers have reported that, dietary supplementation of chitosan may result in health promoting effects such as hypolipidemic and cardio-protective activities (Zhang *et al.,* 2013; Anandan *et al.,* 2015).

Grafting of chitosan with various functional moieties has been attempted to improve its bioactivity, modulate solubility and emulsifying properties. Particularly, synthesis of antioxidant-chitosan conjugates by grafting of antioxidant molecules onto chitosan has received much attention. Grafting of phenolic acids like gallic acid, caffeic acid and ferulic acid to chitosan have been reported (Aytekin *et al.,* 2011; Woranuch & Yoksan, 2013). Researchers have opined that the phenolic acid grafted chitosan derivatives could potentially be used as new food additives or even as functional foods (Xie *et al.,* 2014). It has been reported that phenolic acid grafted chitosan derivatives demonstrate various bio-activities, such as antioxidant, antimicrobial, anti-diabetic etc. (Chatterjee *et al.,* 2015; Liu *et al.,* 2013; Lee *et al.,* 2014). Hence, application of phenolic acid grafted chitosan derivatives for microencapsulation and delivery of bioactive lipids may result in the development of new food additives or novel functional foods.

Recently application of gallic acid grafted chitosan in the delivery of bioactive components has been reported (Hu *et al.*, 2015; Hu & Luo, 2016). Previous research also reported application of ferulic acid grafted chitosan for microencapsulation and controlled release of thiamine and pyridoxine (Chatterjee *et al.,* 2016). Promising potential of phenolic compounds in stabilizing highly unsaturated fish oil has been recorded in recent literature (Vaisali *et al.*, 2016). However, to our knowledge, there exist no reports on the application of phenolic acid grafted chitosan derivatives for microencapsulation and stabilization of highly unsaturated fish oils. Besides being a licensed food additive with pleasant creamy smell (FAO/WHO Expert Committee on Food Additives, JECFA no. 959), vanillic acid has been associated with a variety of pharmacological activities

(Gitzinger *et al.,* 2011). In the present study an attempt has been made to encapsulate sardine oil using vanillic acid grafted chitosan (Va-g-Ch) as wall material. We hypothesise that the antioxidant properties of Va-g-Ch will impart enhanced oxidative stability to the encapsulated oil and the microparticles could be a new functional food ingredient.

3.2 Materials and methods

3.2.1 Materials

Sardine oil (*Sardinella longiceps*) was obtained from Arbee agencies Kottayam, Kerala, India and was stored in dark amber coloured-glass bottles at -20° C, until use. Chitosan (MW = 100 kDa, 88 % degree of deacetylation) was prepared in the pilot plant facility of Central Institute of Fisheries Technology (ICAR-CIFT) and the source is shrimp shell, Cochin, Kerala, India. Vanillic acid, Tween 20, folin-ciocalteu reagent, FAME (Fatty acid methyl ester) external standard mixture and ethanol (HPLC grade) were obtained from Sigma-Aldrich (USA). Acetic acid, hydrogen peroxide (H_2O_2) and ascorbic acid were obtained from Merck Millipore (Germany).

3.2.2 Synthesis and characterisation of Va-g-Ch

Vanillic acid grafted chitosan (Va-g-Ch) was synthesized, as previously described (Chatterjee *et al.,* 2015). Briefly, 10 g of chitosan was dissolved in 2 L of 2 % acetic acid solution (v/v) in a 5 L three necked round bottom flask. 20 ml of 1 M H_2O_2 containing 1.08 g of ascorbic acid was added drop wise to the chitosan solution, followed by the addition of 10 g vanillic acid dissolved in100 ml ethanol. The reaction was maintained under nitrogen environment for 24 h at 25°C with constant stirring. The reaction mixture was dialysed against distilled water for 72 h to remove unreacted phenolic acids. The reaction mixture and pure vanillic acid were developed on silica coated TLC plates, to confirm complete removal of free vanillic acid. Finally, the dialysate was freeze dried to yield Va-g-Ch derivative in solid form. A Hitachi U-2910 spectrophotometer (Tokyo) was used to determine an absorbance maximum. FTIR spectra was recorded on a

Thermo Nicolet, Avatar 370 spectrometer (Waltham, USA) as KBr pellets of the derivative, over a spectral range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1}.The derivative was solubilised in D_2O (Heavy water) before recording ^1H NMR spectra (400 MHz, Bruker, Massachusetts, USA).The grafting ratio of vanillic acid on chitosan was estimated using Folin-Ciocalteu reagent procedure (Curcio *et al.,* 2009).

3.2.3 Rheology of Va-g-Ch

Rheological measurements of chitosan and Va-g-Ch were performed on a Brookfield DV-III ultra-programmable rheometer (Middleboro, USA) with a cone and plate geometry (cone diameter = 50 mm, angle = 4°, gap = 0.05 mm) while the temperature was adjusted at 25°C (Zhang *et al.,* 2015). Experimental flow curves were fitted to a powerl aw model as shown in Eq 1

$$\eta = K\gamma^{n-1} \quad (1)$$

where η was the viscosity (Pa·s), γ was the shear rate (s^{-1}), *K* was the consistency index (Pa s^n) and n was the index that provided information about the flow behaviour related to the effect of shear rate.

3.2.4 Preparation of oil in water (o/w) emulsion

Va-g-Ch (0.8 % wt/v) was dissolved in acetate buffer solution (2 mM sodium acetate and 98 mM acetic acid in water, pH 3.0) with overnight stirring on a magnetic stirrer. Sardine o/w emulsion was prepared by blending sardine oil (30 wt % of total solid), Tween 20 (0.25% wt/v) and the Va-g-Ch solution. For emulsification, a high speed homogeniser (Bio-Gen PRO-250, Scientific Inc, USA) at 15,000 rpm for 30 min was used. It was stored in graduated, stoppered test tubes for one week to observe emulsion stability.

3.2.5 Characterization of the o/w emulsion

The microstructure of the matured emulsions was observed on an optical microscope (Leica ICC50 HD) at an objective magnification of 40X while the images were captured by the digital image processing software, Image-pro plus™

(V6). The "size distribution by number" of the emulsion droplets, ζ potential and poly dispersity index (PDI) were measured on a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, England). The emulsions were not diluted before analysis. A polydisperse model was used to analyse the data and presented as size distribution by number. Particle size was expressed as Z average hydrodynamic diameter (d.nm).

3.2.6 Spray-drying of o/w emulsion

The optimized emulsion was up-scaled and spray-dried using a pilot-plant spray dryer (S M Scientech, Kolkata) to yield sardine oil loaded microparticles (SO-M). The inlet and outlet temperature of the spray dryer were 140°C and 77°C respectively. The fish oil powder obtained was immediately transferred into a cold glass jar. The spray dried powder was then used for further characterization studies.

3.2.7 Characterization of SO-M

The moisture content of the SO-M was determined by AOAC method (AOAC, 2000), while an electric hygrometer (Novasina IC–500 AW– LAB, Novasina) recorded the water activity. Bulk density was determined by loosely packing 2 g of the powder in 10 ml graduated cylinder and was calculated by dividing weight of the sample by the bed volume. Colour parameters of the SO-M were measured on a Hunter lab colour meter (Reston, VA, USA). The sample was filled in a 64 mm quartz sample cup to a pre-determined level and measurements of lightness L^* and chromaticity a^*, b^*($+a^*$ = red $-a^*$ = green; $+b^*$ = yellow $-b^*$ = blue) were carried out. These parameters were employed to calculate the cylindrical parameters (Croma, C^*) and Hue angle (H°) according to Eqs. (2) and (3).

$$C^* = \sqrt{a^{*2} + b^{*2}} \quad (2)$$

$$H° = \tan^{-1}(b^*/a^*) \quad (3)$$

For particle size and zeta potential measurement, 50 mg of the micro-particles were re-dispersed by vortexing in 10 ml of acetate buffer (2 mM sodium

acetate and 98 mM acetic acid in water, pH 3.0). Samples were mounted on metal stubs with the help of double backed adhesive tapes, coated with gold in an argon atmosphere before examining them under a JEOL scanning electron microscope (JSM- 6390LV, Tokyo, Japan). X-ray powder diffraction data of the samples were recorded on an X-Ray Diffraction Instrument (Bruker Kappa Apex II, Massachusetts, USA) over a 2θ range from 3 to 40°, using a scan rate of 0.04° min^{-1}.

3.2.8 Determination of encapsulation efficiency and loading efficiency

Encapsulation efficiency (EE) of the process was determined by estimating total oil (TO) and surface oil (SO) in a given weight of the SO-M using Eq 4 (Shen *et al.,* 2010).

$$EE\ (\%) = \frac{(TO-SO)}{TO} \times 100 \qquad (4)$$

The loading efficiency (LE) was determined using Eq 5.

$$LE\ (\%) = \frac{Total\ oil\ content}{Theoretical\ oil\ content} \times 100 \qquad (5)$$

Theoretical oil content is the weight of oil per gram of total solid taken during emulsion preparation. Total oil and surface oil content were determined as described elsewhere with slight modifications (Li *et al.,* 2013). Briefly, spray-dried powder (1 g) was dissolved in 100 ml of 1% acetic acid with continuous overnight stirring on a magnetic stirrer. The released oil was extracted thrice with 50 ml of petroleum ether each time. The oil thus extracted was concentrated using vacuum rotary evaporator and dried at 105°C until constant weight. The weight, determined gravimetrically was noted as total oil content. For determination of surface oil, spray-dried microcapsules (1 g) were suspended in 50 ml of petroleum ether and extracted thrice for 10 min each. The amount of surface oil was similarly determined gravimetrically.

3.2.9 Oxidative stability studies of SO-M

Oxidative stability of SO-M was studied over a period of storage for 4 weeks, stored at room temperature in amber coloured-glass bottles. Total oil was extracted from the powder, before determining Peroxide value (PV) by an iodometric titration method, described in literature (Kolanowski *et al.,* 2007). Changes in secondary oxidation products were determined by measuring the thiobarbituric acid reactive substance (TBARS) value as described in literature (McDonald & Hultin, 1987). Changes in fatty acid composition were studied by fatty acid methyl ester (FAME) analysis using GC–FID as per AOAC official method of analysis (AOAC, 2000). Chromatographic separation was achieved in a Thermo Trace GC Ultra gas chromatograph equipped with a Perkin Elmer Elite 225® capillary column (30 m ×0. 25 mm × 0.25 μ). Identification and quantification were done with the help of FAME external standard mixture. The accelerated rancimat test was also used to determine the oxidative stability of the SO-M. Powder, containing three gram total sardine oil and three gram freshly purchased sardine oil were dispersed separately in equal volume of canola oil. The 743 Rancimat apparatus (Metrohm, Herisau, Switzerland) was operated at 110°C and with 20 ml/hair flow. Induction point (IP) values were automatically detected and registered. Analyses were performed in triplicates.

3.2.10 In vitro release

The in vitro release property of microencapsulated sardine oil was studied using a simulated gastrointestinal model as reported in literature (Goyal *et al.,* 2016).

3.2.11. Statistical Analysis

Two way analysis of variance was carried out to compare the direct and interaction effect of different treatments. Tukey's test was performed to compare the marginal means and t-test used to compare the interaction means at 5% level of significance. All the statistical analysis was done using SAS 9.3 software.

3.3 Results and discussion

3.3.1 Synthesis and characterisation of vanillic acid grafted chitosan

Among different synthetic approaches reported for grafting phenolic acids with chitosan, free radical mediated grafting of phenolic acids is considered as most rapid, economic and ecofriendly (Pasanphan *et al.,* 2010; Yu *et al.,* 2011; Bozic *et al.,* 2012; Curcio *et al.,* 2009). It has been suggested that in acetic acid solution ascorbic acid is present as a di-acid and can further react with H_2O_2 to generate hydroxyl ($OH^{\cdot}$) radical. Then the $OH^{\cdot}$ abstracts hydrogen from amino and hydroxyl group of chitosan resulting in chitosan macro radicals. The corresponding phenolic acid molecules become acceptors of chitosan macro radicals, thus forming chitosan-phenolic acid conjugates (Liu *et al.,* 2013). Reaction pathways involving 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) and/or laccase catalyzed polymerization have been considered disadvantageous due to difficulties in removal of the reactants from reaction media and loss of active phenol hydroxyl groups of the phenolic acid respectively (Liu *et al.,* 2014). The yield of vanillic acid grafted chitosan varied between 58 and 65 %. No spots corresponding to vanillic acid was observed on the developed TLC plates for vanillic acid grafted chitosan, confirming absence of free vanillic acid and successful grafting on chitosan (**Fig. 3.1**).

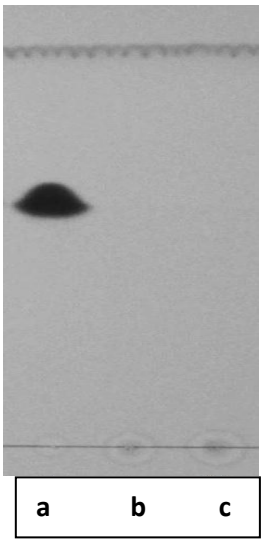

Fig. 3.1. TLC plates showing spots for a) Vanillic acid, and b and c) Va-g-Ch

FTIR spectra of chitosan exhibited major characteristic bands at around 3428 cm^{-1} (OH), 2883 cm^{-1} (C-H stretching), 1650 cm^{-1} (amide I), 1550 cm^{-1} (amide II), 1072 cm^{-1} (COC) and 899 cm^{-1} (pyranose ring) (Fig. 3.2) (Bobu *et al.,* 2011). As compared to chitosan, intensity of amide I (at around 1644 cm^{-1}) and amide II (at around 1549 cm^{-1}) bands in vanillic acid grafted chitosan increased, indicating formation of new amide linkage. It was also observed that integral ratio of the CH stretching band (at around 2933 cm^{-1}, belonging to chitosan and vanillic acid) to the pyranose band (at around 896 cm^{-1}, belonging to chitosan), i.e. I2933/I896 was higher in vanillic acid grafted chitosan as compared to chitosan (Woranuch and Yoksan 2013).

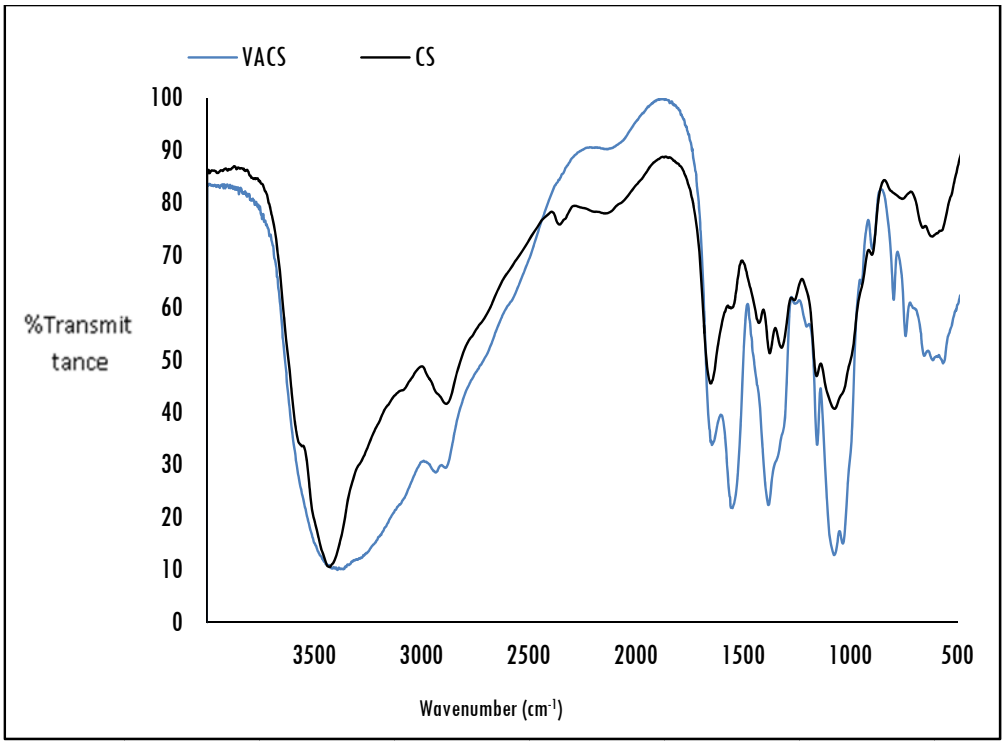

Fig.3.2. FTIR spectra of chitosan and vanillic acid grafted chitosan

UV–vis absorption spectra of vanillic acid grafted chitosan (Fig. 3.3**)** was recorded between 200 and 500 nm. Chitosan in 1 % acetic acid (v/v) showed no absorption peak between 200 and 500 nm. Vanillic acid grafted chitosan showed primary absorption peak at 258 nm which was same as that of vanillic acid.

Similar observations have been made by authors working with phenolic acid grafted chitosan (Woranuch and Yoksan, 2013). Some authors have also recorded a slight red shift in absorption wavelength of the conjugates than that of corresponding phenolic acids (Liu *et al.,* 2014).

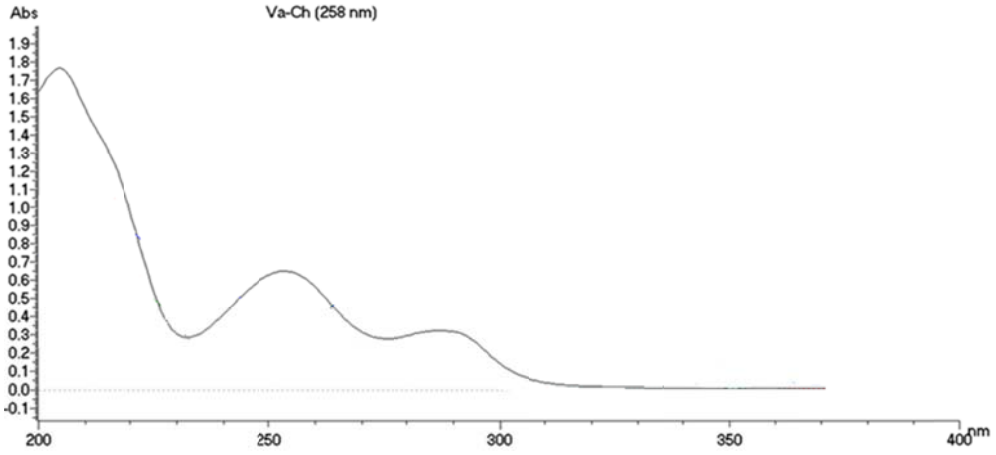

Fig. 3.3. UV-vis spectra of chitosan-vanillic acid conjugate

The proton nuclear magnetic resonance (^{1}H NMR) spectra of chitosan and vanillic acid grafted chitosan have been presented in Fig. 3.4. The ^{1}H NMR assignments of chitosan were as follows: ^{1}H NMR (deuterium [D_2O], 400 MHz) δ =2.00 ppm (NH [CO] CH_3) ;δ = 3 ppm (H-2), δ = 3.2-3.9 ppm (H-3-H-6) and δ = 4 ppm (H-1), δ = 5 ppm (D_2O). The ^{1}H NMR assignments of vanillic acid grafted chitosan were as follows: at δ = 2 ppm (NH [CO] CH_3); δ = 3.5-3.6 ppm (H-2, -OCH_3 substitution in benzene ring); δ = 3.6-3.9 ppm (H-3-H-6); δ = 4 ppm (H-1);δ = 5 ppm (D_2O); δ = 6.9 ppm (methine protons of vanillic acid); δ = 7-7.6 (f, b, methine protons of vanillic acid). This confirms successful grafting of vanillic acid onto chitosan.

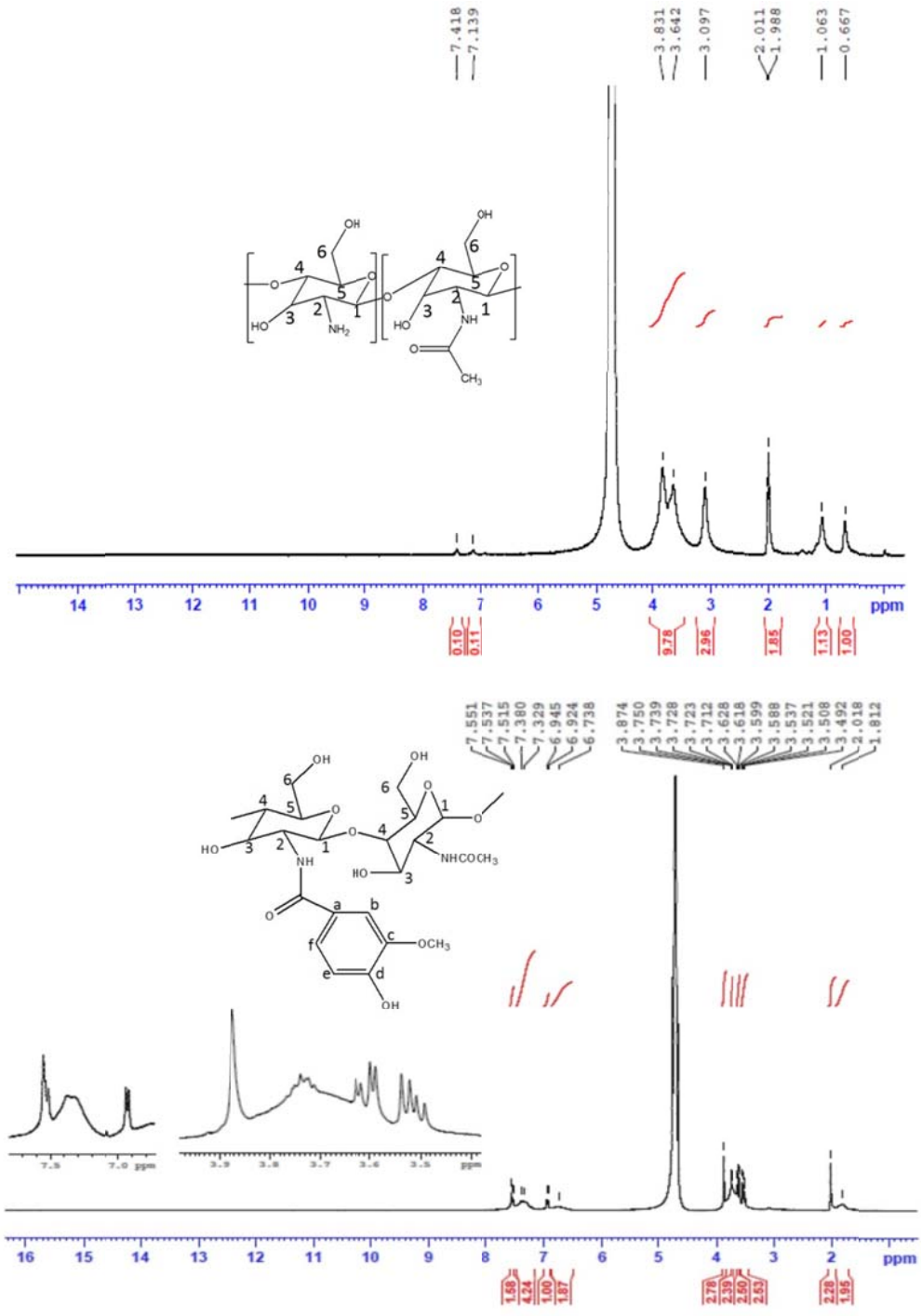

Fig.3.4 [1] H NMR spectra and structure assignment of chitosan and vanillic acid grafted chitosan

Grafting ratio of vanillic acid on chitosan, estimated using Folin-Ciocalteu reagent procedure was found to be 305 mg vanillic acid equivalent/g of polymer. Elsewhere for similar reaction, the grafting ratio of ferulic acid grafted chitosan and gallic acid grafted chitosan were reported to be 66.7 mg ferulic acid equivalent/g of polymer (Liu *et al.,* 2014) and 7 mg gallic acid equivalent/g of polymer (Curcio *et al.,* 2009). Higher grafting ratio in the present study may be due to longer reaction time and inert reaction environment.

3.3.2 Rheological properties of vanillic acid grafted chitosan

Rheology of chitosan and vanillic acid grafted chitosan solutions (0.8 % wt/v) in acetate buffer of pH 3.0 were studied. Shear rate dependence of the viscosity of two fluids has been presented in Fig. 3.5. Chitosan behaved as a typical shear thinning non-newtonian fluid. However viscosity of vanillic acid grafted chitosan decreased significantly and it behaved almost as a Newtonian fluid. Shear stress and shear rate data of both chitosan and vanillic acid grafted chitosan fitted well in the Power law model as indicated by R^2 values of 0.996 and 0.998 respectively. The flow index of chitosan was 0.82 (<1 for shear thinning non-Newtonian fluid) whereas for vanillic acid grafted chitosan the flow index was found to be 1.08 (1 for Newtonian fluid, >1 for shear thickening non-Newtonian fluid). Newtonian behavior of fluids/emulsions has been recorded at low solute/dispersed phase concentration. This intern has been explained by the reduced viscosity at low solute/dispersed phase concentration (Pal, 2000). It is likely that during grafting reaction using H_2O_2-Ascorbic acid reagent pair the long chains of chitosan were broken by the peroxide free radicals, resulting in reduced viscosity. These changes in chain length and reduced viscosity of vanillic acid grafted chitosan may best explain the almost Newtonian behavior. To our knowledge the effect of H_2O_2-Ascorbic acid free radical mediated grafting of phenolic acid on viscosity and molecular weight of the grafted chitosan has not been studied earlier in published literature. The reduced viscosity of vanillic acid grafted chitosan increases its desirability as a wall-material for microencapsulation using spray drying technique. High viscosities of the wall

material often interfere with the atomization process and lead to the formation of elongated and large droplets that adversely affect the drying rate (Gharsallaoui *et al.,* 2007).

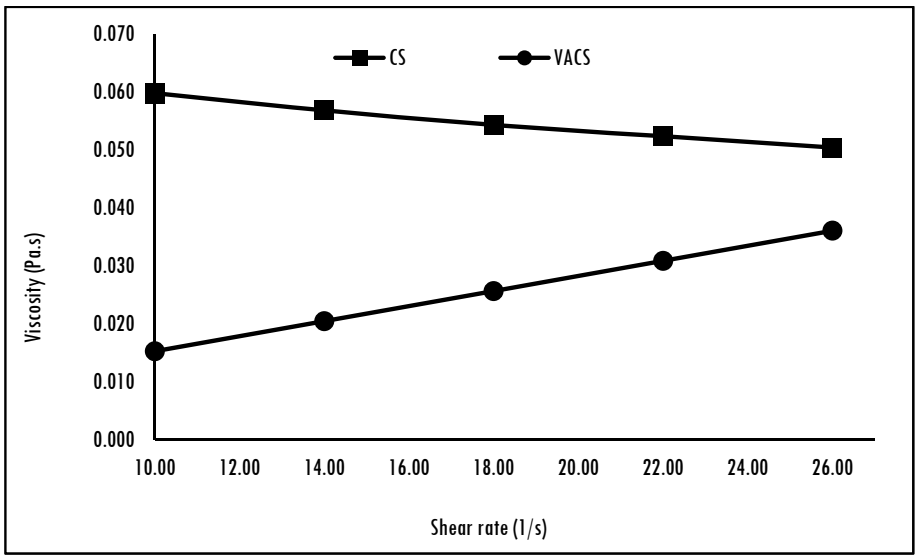

Fig.3.5 Changes in rheological properties of vanillic acid grafted chitosan as compared to chitosan

3.3.3 Optimization of oil in water emulsion

Chitosan in combination with anionic and non-ionic emulsifier stabilizes oil in water emulsion better than it does alone (Li *et al.,* 2013). Particularly Tween 20, a non- ionic emulsifier was found to be suitable in combination with chitosan for stabilisation of oil in water emulsion. It has been postulated that adsorption of the cationic chitosan to the oil droplet surfaces results from electrostatic interaction with negatively charged Tween 20-stabilized droplets (Mun *et al.,* 2005; Shunmugaperumal & Kaur, 2016). Accordingly, it was decided to use vanillic acid grafted chitosan in combination with Tween 20 for stabilization of fish oil in water emulsion. Effect of chitosan concentration on its emulsifying ability has been studied elaborately in published literature. It has been observed that emulsifying activity of chitosan is highest at a concentration of 0.75% and then decreases with increasing concentration (Li & Xia., 2011).Hence, in our study a concentration 0.8% of vanillic acid grafted chitosan was used for

preparation of oil in water emulsion. Various concentrations of Tween 20 (5, 2, 1, 0.5, 0.25 and 0.1% w/v) in combination with 0.8% vanillic acid grafted chitosan were evaluated to achieve optimum emulsion stability before spray-drying. The prepared emulsions were stored for one week in graduated stoppered test tubes and observed for phase separation and creaming (Fig 3.6 a). An emulsion, containing 0.25% and 0.1% Tween 20 was found to be stable, whereas all other emulsions showed clear phase separation (Fig 3.6 c). Fig 3.6 b and Fig 3.6 d presents the microstructure of the emulsion containing 0.25% Tween 20 at day one and after one week of storage. Though signs of droplet flocculation were observed after one week of storage, the micrograph was still typical of non-aggregated oil in water emulsion.

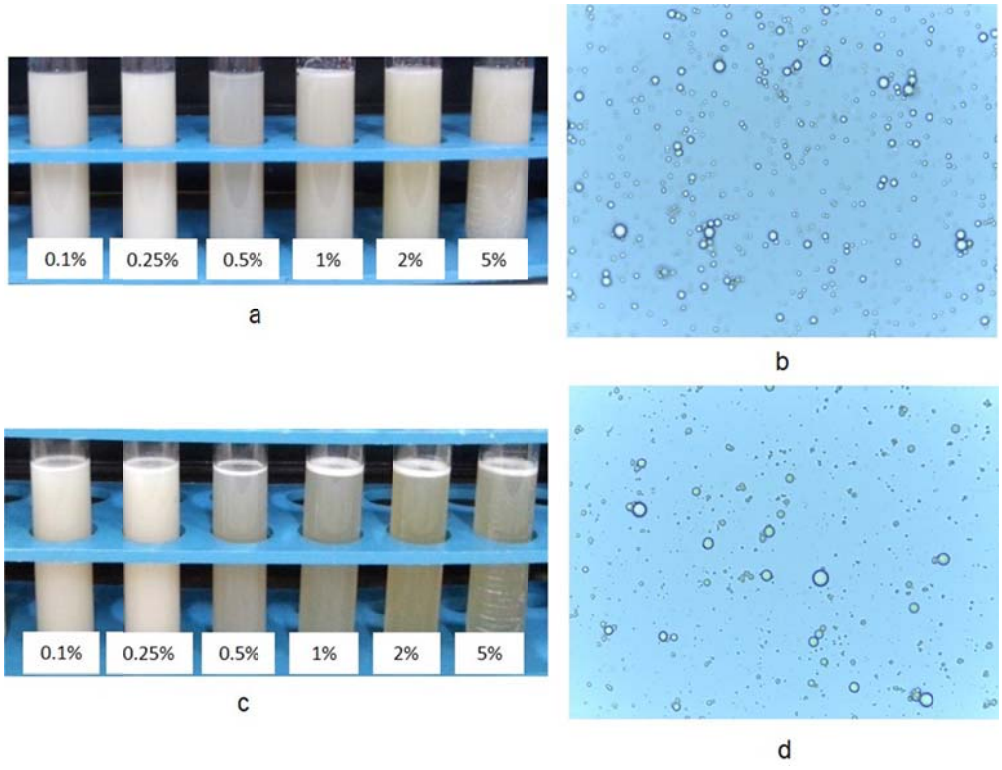

Fig.3.6 **a**. Stability of the emulsion intended for spray-drying. a. Emulsions prepared with different concentration of Tween 20 at day one; **3.6 b**. Micrograph of emulsion containing 0.1% Tween 20 at day one; **3.6 c**. Emulsions prepared with different concentration of Tween 20 at day seven; **3.6 d**. Micrograph of emulsion containing 0.1% Tween 20 at day seven

The droplet size distribution by number of the emulsion containing 0.25% Tween 20 after one week of storage is presented in Fig 3.7. The average diameter of the droplets was found to be 782 nm with a positive ζ potential of 14.3.

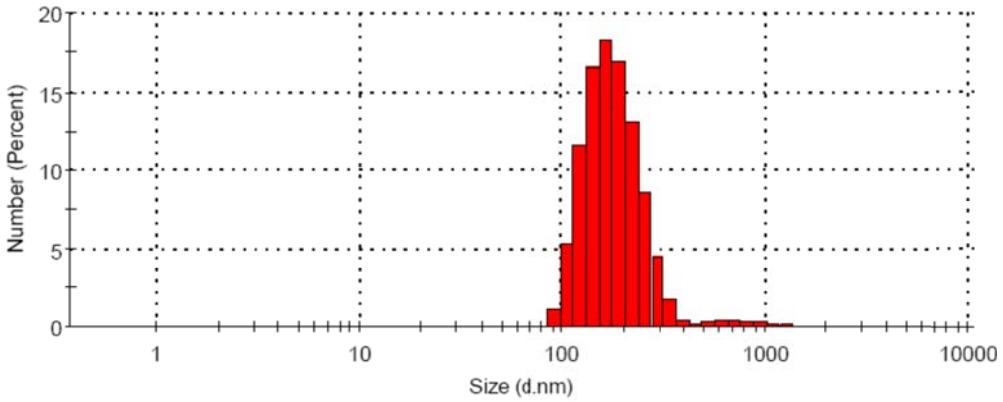

Fig. 3.7 a. Droplet "size distribution by number" of the oil in water emulsion

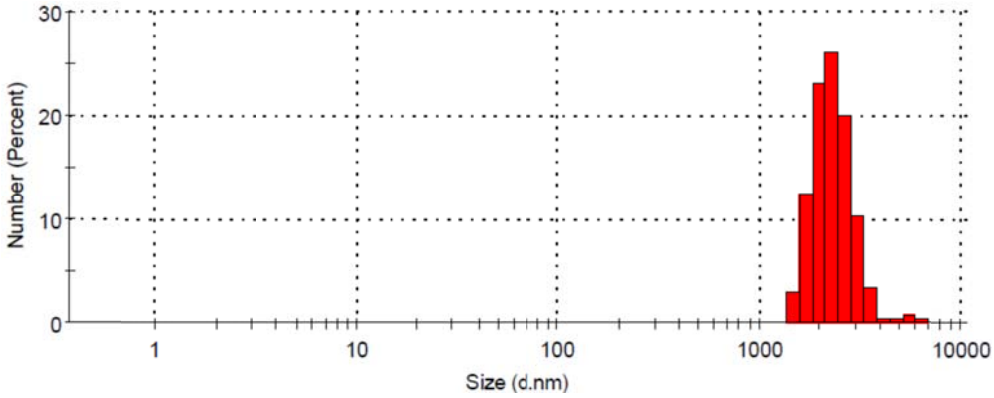

Fig. 3.7 b. Particle "size distribution by number" of re-dispersed microparticles

The PDI value was 0.582 which indicates that the emulsion droplet size dispersion was poly-disperse in nature. The positive ζ potential value is typical of phenolic acid grafted chitosan. In recent literature the ζ potential and PDI values of ferulic acid grafted chitosan and caffeic acid grafted chitosan nano-complexes were recorded in the range of ~25-27 and 0.268-0.368 respectively. The higher PDI values indicating polydisperse distribution of the emulsion droplets was attributed to heterogeneous distribution of phenolic acid that is grafted in chitosan molecular chain (Hu & Luo, 2016). Our observation agrees to this explanation and

also take into consideration the multiple scattering effect in case of concentrated emulsions. However, it is concluded that the prepared emulsion was not stable enough to be a potential emulsion based delivery system. This further justifies the need for spray drying of the emulsion to impart physical and oxidative stability. Long term stability of emulsion based delivery system has often been suboptimal and spray drying of the prepared emulsion has been suggested as an alternative measure to impart stability (Berendsen *et al.,* 2015; Esfanjani *et al.,* 2015).

3.3.4 Physicochemical characterization of microencapsulated fish oil powder

The physicochemical properties of microencapsulated fish oil powder have been presented in Table 3.1. The spray-drying yield, calculated as percentage weight of the spray dried powder with respect to the weight of total solids in the emulsion was around 75%. Moisture content and water activity of the spray dried powder were determined to be 2.5% and 0.259 respectively. Moisture content and water activity plays important role in determining the flowability, and storage life. In food industry, powdered food ingredients with water activity of less than 0.6 are considered to be microbiologically safe (Shafiur and Labuza, 2007). It is well known that low moisture content and water activity prevents release of encapsulated oil during storage, thereby prevents lipid oxidation (Velasco *et al.,* 2003). Drusch and Schwarz (2006) have also observed that the fish oil microcapsules with moisture content of less than 3% can ensure better product stability. Hygroscopicity of the powder was found to be 14.36% and considered acceptable. Hygroscopicity of fish oil encapsulate is important as the amount of water adsorbed has direct relation with the oxidative stability and flowability. Similar values of hygroscopicity in the range of 15.87 to 18.90% have been reported in recent literature for rosemary oil encapsulates using gum arabic as wall material (Fernandes *et al.,* 2013).

Higher bulk density of powdered food ingredient is a desirable property due to lower space required for storage and transport. A higher bulk density can also be related to the surface regularity of the microcapsules, smaller particle size and better stability against oxidative degradation. However a high bulk density

also means high compressibility and lower flowability. In our study, bulk density of the fish oil encapsulate was determined to be 0.41 g/ml which was slightly higher than the optimum of 0.31-0.35 g/ml (Bakry *et al.,* 2016).The higher bulk density obtained in our study could be due to lower inlet air temperature (140 °C) during spray drying. The bulk density of spray dried powders is reported to be affected negatively by inlet air temperature (Goula & Adamopoulos, 2010). A higher inlet air temperature may result in better bulk density; however the lower temperature was maintained to ensure better oxidative stability of the encapsulated oil. Colour is an important parameter for consumer acceptance of food and nutraceuticals products. The microencapsulated fish oil powder was pleasant vanilla coloured demonstrating the advantages of vanillic acid grafted chitosan as wall material. The lightness (L*) and yellowness (b*) values of 71.61 and 42.03 indicates the typical creamy whitish colour. The colour intensity (C*) and hue angle (H°) values were 42.62 and 80.40 respectively. Wan *et al.,* (2012) recorded similar values for microencapsulated menhaden oil and microencapsulated salmon oil using chitosan as wall material. However, the powder obtained was comparatively lighter indicating more whiteness. The C* and H° values recorded in our study were very similar to that of commercial baby food (Wan *et al.,* 2011) indicating suitability of fortification.

Table.3.1 Physical characterization of microencapsulated fish oil encapsulates

Parameter	Fish oil encapsulate
Water Activity	0.259
Colour	
L^*	71.61
a^*	7.11
b*	42.03
C*	42.62
H°	80.40
Bulk density (g/ml)	0.42
Tapped density (g/ml)	0.56
Flowability	25
Cohesiveness	1.33
Hygroscopicity (%)	14.36%
Induction point (at 110°C) (h)	7.67 ±0.05

3.3.5 Particle size distribution, morphology and crystallinity of microencapsulated sardine oil

Size distribution by number of the re-dispersed microencapsulated sardine oil has been presented in Fig 3.7 b. The average diameter of the particles was found to be 2.3 μ. Increase in particle size after spray drying as compared to oil in water emulsion micelle size was expected due to formation of larger droplets in the spray dryer nozzle. Similar ζ potential value of 16.5 was observed as in the oil in water emulsion. However a lower PDI value of 0.345 was recorded as compared to the oil in water emulsion. This indicates comparatively better homogeneity in particle size distribution. In a recent study, the authors (Hu *et al.,* 2015) reported similar PDI values for polymer nanoparticles composed with gallic acid grafted chitosan and bioactive peptides in the range of 0.288-0.530. Elsewhere, a PDI value of 0.386 has been recorded for ferulic acid grafted chitosan nanoparticles (Hu & Luo, 2016).

SEM images of sardine oil loaded micro particles showed spherical to irregular morphology (Fig 3.8) with a few small pores on the smooth surface. Broken particles at higher magnification clearly showed cavity inside the particle (Fig 3.8 c) indicating encapsulation of the oil inside the wall material. Similar irregular morphology of oil loaded chitosan microspheres has been noted in most of such literature (Zhang & Zhang, 2002; Duman and Kaya, 2016). It has been observed that chitosan when used in combination with other polysaccharide and protein result in better surface morphology of oil loaded micro particles (Dima *et al.,* 2016).

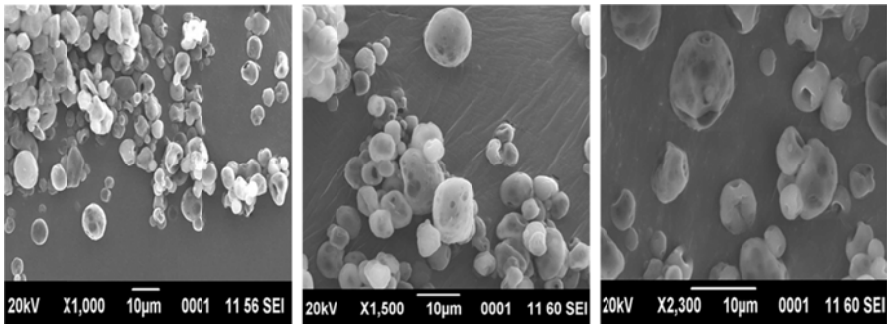

Fig. **3.8** **a** SEM images of the sardine oil encapsulates; a. spherical to irregular particles seen at X1000; **3.8 b**. spherical to irregular particles seen at X1500; **3.8 c**. broken particles showing cavity inside the particle seen at X2300

Further, crystallization behaviour of chitosan, vanillic acid grafted chitosan and sardine oil loaded micro particles was studied by XRD analysis (Fig 3.9). XRD spectra of chitosan showed strong reflection fall at 2θ 20° corresponding to crystal form II (Zhang *et al.,* 2006). In case of vanillic acid-grafted chitosan intensity of the reflection fall at 2θ 20° significantly decreased and the peak moved towards shorter angle. New low intensity peaks at 2θ 10°, 12°, 15°, 25° appeared due to introduction of vanillic acid in chitosan structure. These changes indicate loss in crystallinity due to formation of new co-valent bond and changes in structure of chitosan. This observation is consistent with earlier reports (Woranuch and Yoksan 2013) and might be attributed to the destruction of inter and intra molecular hydrogen bonds of chitosan by bulky vanillic acid. Similar changes in crystallinity and angle have also been observed elsewhere for cinnamic acid grafted chitosan derivatives (Yang *et al.,* 2016). Sardine oil loaded micro particles showed further reduction in intensity of the peak at 2θ 20° indicating loss in crystallinity. The low intensity peaks at 2θ 10°, 12°, 15°, 25° disappeared. This can be attributed to further folding of the polymer chains and successful encapsulation of the sardine oil inside the vanillic acid grafted chitosan microparticles. Similar observations have been made in XRD spectra of coriander essential oil loaded chitosan microparticles (Duman and Kaya, 2016).

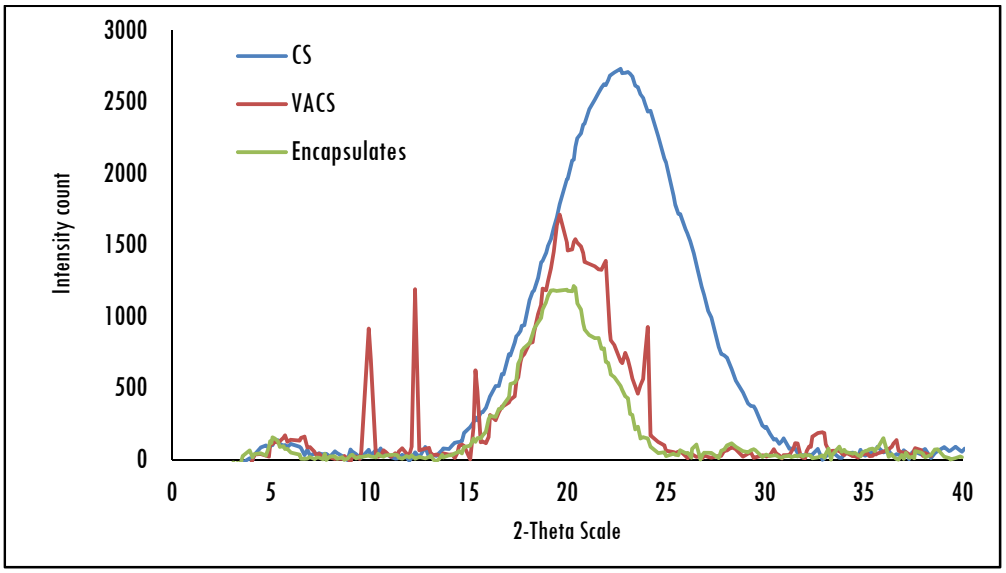

Fig.3.9 X-ray defractograms of chitosan, vanillic acid grafted chitosan and sardine oil encapsulates

3.3.6 Encapsulation efficiency of the process

The Encapsulation efficiency (EE) value of the sardine oil in vanillic acid grafted chitosan microparticles was 84±0.84 %. The LE value was determined as 67±0.51% (Table 3.1). Hence, around 33% of the initial oil was lost during spray drying process. Generally an EE value between 60-90% has been considered satisfactory in published literature depending on the type and composition of wall material, drying process used and the stability of the feed emulsion (Hardas *et al.,* 2000; Hogan *et al.,* 2001; Shen *et al.,* 2010).The EE value recorded in this study was better than literature reported values (~24-73%) where chitosan has been used as wall material for microencapsulation of oil following spray drying technique (Duman and Kaya, 2016; Binsi *et al.,* 2017). This could be due to better emulsifying property of vanillic acid grafted chitosan. A poor EE value indicates presence of high free surface oil and implies poor oxidative stability since the surface oil can get oxidized extremely rapidly. Hence, high surface oil has often been correlated with off-flavour of microencapsulated oil and poor stability during food processing. The satisfactory EE and LE (Loading efficiency) values obtained in our study prove successful encapsulation of sardine oil in vanillic acid grafted chitosan microparticles and suitability of further application in food.

3.3.7 Oxidative stability of microencapsulated sardine oil

Initial PV of the sardine oil was determined as 0.9±0.15 meq/kg oil which increased to 3.3±0.35 meq/kg oil in freshly prepared spray dried powder. This observation was expected since the emulsion droplets were temporarily exposed to air and high temperature during spray drying (Klinkesorn *et al.,* 2004; Anwar and Kunz, 2011). A few published reports suggest use of inert conditions (nitrogen as drying medium) to minimize lipid oxidation during spray-drying. However, this technique has not yet been used in the food industry and conventional spray drying remains most popular process for microencapsulation of oil (Encina *et al.,* 2016). PV of un-encapsulated sardine oil increased rapidly during storage and reached up to 27.6±1.7 meq/kg oil after 4 weeks of storage. In that same period PV of microencapsulated sardine oil increased slowly from

3.3±0.35 to 5.5±0.51 meq/kg oil (Fig 3.10). PV of microencapsulated fish oil, ranging from 5-100 meq/kg oil have been reported in published literature, depending on the wall material, drying condition and various other factors. Most often, a PV of less than ten has been considered optimum (Anwar & Kunz, 2011; Bakry *et al,* 2016). The excellent antioxidant activity of vanillic acid grafted chitosan as reported in the study (Chatterjee *et al.,* 2015), clearly explains the lower PV of microencapsulated sardine oil in the present study.This can also be correlated with the good encapsulation efficiency and loading efficiency achieved in the reported process. Lower the encapsulation efficiency, the higher is the amount of oil present in the particle surface. This surface oil, in direct contact with the oxygen, is much more susceptible to lipid oxidation than the encapsulated one (Tonon *et al.,* 2011). Changes in the TBARS value also followed similar trend; after four weeks of storage the determined value for the oil was 2.2 whereas for the microencapsulated oil the value was 0.89 mg malonaldehyde/Kg oil (Fig 3.10). The secondary oxidation products formed in the microencapsulated sardine oil was satisfactorily low and indicates that the powder is free from rancid odour (Shahidi and Wanasundara, 2002). The accelerated rancimat test also proved better oxidative stability of the microencapsulated sardine oil powder. Under these conditions the lipids are oxidised to short chain volatile acids like formic acid and acetic acid which are collected in distilled water increasing its conductivity. The Induction Point (IP) value indicates the time required to produce a sudden increase of conductivity, which can be defined as an indirect measure of oil stability. Freshly procured sardine oil presented an IP of 0.67±0.01h which is comparable to the value reported for fish oil (0.75h) (Gallardo *et al.,* 2013). Whereas freshly prepared microencapsulated oil showed IP value of 7.67±0.05 h (Table 3.1). IP values obtained for microcapsules clearly show a protective effect of the vanillic acid grafted chitosan wall material against oil peroxidation.

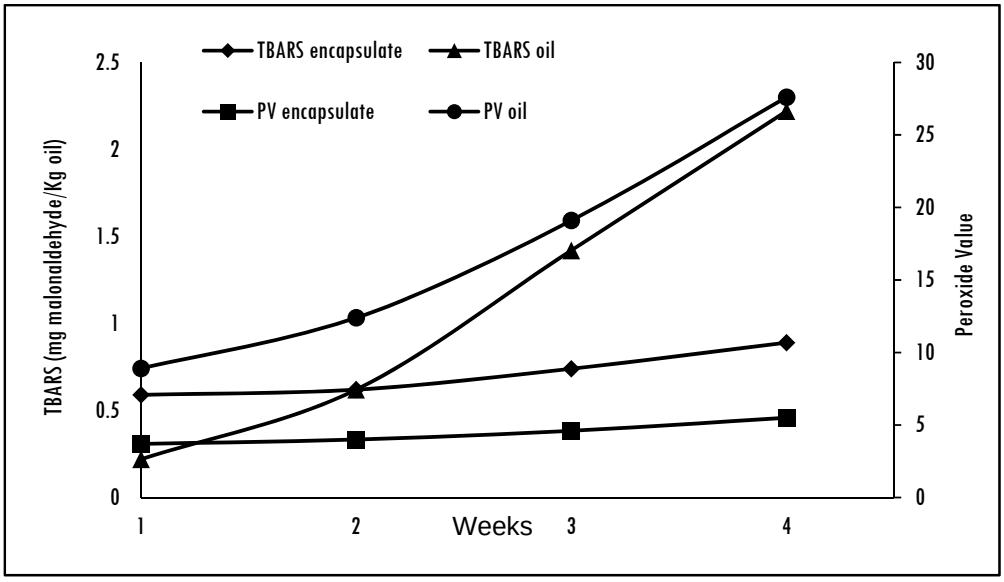

Fig. 3.10. Oxidative stability of sardine oil encapsulate and sardine oil during storage at room temperature

3.3.8 Changes in the fatty acid composition during storage

Fatty acid composition of sardine oil and microencapsulated sardine oil during storage has been presented in Table 3.2. The polyunsaturated fatty acid (PUFA) content of the sardine oil was 37.3±0.21% of which 27.3±0.19% was eicosapentanoic acid (EPA) and docosahexanoic acid (DHA). Since EPA and DHA are highly unsaturated and prone to peroxidation, their stability in bulk sardine oil and in the microcapsules was examined over a period of four weeks. The EPA and DHA content was comparatively less in freshly prepared microencapsulated sardine oil (25.4±0.07%) than in the freshly procured sardine oil. This is supposedly caused by the degradation of EPA and DHA due to the short exposure to air and high temperature during microencapsulation process. However, the EPA and DHA content in the bulk sardine oil steadily decreased during storage and after four weeks reduced to 12.1±0.19% (~56% decrease). Whereas, the EPA and DHA content in microencapsulated oil decreased slowly; from 25.4±0.07% to 22.4±0.17% (~12% decrease). In general, this observation is in agreement with published literature (Polavarapu *et al.,* 2011; Bakry *et al.*, 2016) and demonstrates the success of the microencapsulation process using vanillic acid grafted chitosan as an emulsifier and antioxidant wall material.

Table.3.2 Changes in fatty acid content of sardine oil and encapsulated sardine oil powder during storage. [a] Storage time in week (ST); [b] Saturated fatty acid, [c] Monounsaturated fatty acid; [d] Polyunsaturated fatty acid, [e] Ecosapentaenoic acid + docosahexaenoic acid.

Name	ST [a] (In weeks)	SFA [b]	MUFA [c]	PUFA [d]	EPA+DHA[e]
Sardine oil	0	40.5±0.09	21.5±0.16	37.3±0.21	27.3±0.19
	1	45.2±0.12	18.3±0.14	30.2±0.18	25.8±0.04
	2	51.9±0.21	16.2±0.09	22.4±0.10	21.9±0.22
	3	54.7±0.09	14.4±0.04	18.7±0.19	18.2±0.11
	4	60.8±0.18	10.0±0.19	10.8±0.25	12.1±0.19
Encapsulate	0	42.1±0.22	17.3±0.06	31.2±0.11	25.4±0.07
d Powder	1	42.9±0.29	17.0±0.09	30.7±0.10	25.0±0.11
	2	43.2±0.33	16.8±0.07	29.4±0.07	24.2±0.09
	3	45.6±0.15	16.0±0.13	28.9±0.22	23.5±0.14
	4	45.9±0.24	15.7±0.10	28.2±0.18	22.4±0.17

3.3.9 In vitro release study

Percentage release of sardine oil from SO-M on sequential expo-sure to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) is presented in Table 3.3. Percentage of oil released increased slowly as time of exposure increased from 2 to 6 h. Higher per-centage release of oil in case of sequential exposure to SGF and SIF than in SGF exposure alone was obvious and consistent with earlier observations (Goyal *et al.,* 2016). It was noted that after 6 h of exposure to SGF and SIF maximum 66% release of oil was recorded. This indicates sustained release of oil from the SO-M.

Table 3.3 Percentage release of sardine oil under simulated gastrointestinal condition

Formulation	Incubation Time (Hr)			
	2	4	6	Mean
SGF conditions	17.2±0.18[a]	22.4±0.22[b]	24±0.17[b]	21.24 [B]
Sequential exposure of SGF and SIF.	52.4±0.28[a]	60.8±0.32[b]	66±0.21[b]	60.18 [A]
Mean	35.13 [C]	41.55 [B]	35.13 [A]	

3.4 Conclusion

Vanillic acid grafted chitosan (Va-g-Ch) could be a new antioxidant wall material for microencapsulation of PUFA rich marine oils especially sardine oil. The antioxidant properties and improved rheological behavior of the wall material resulted in the good encapsulation efficiency during spray drying and maintained the oxidative stability of the encapsulated oil during storage. However, just passable flowability and irregular particle shape of the SO-M is a concern. This is a typical observation, where chitosan has been used for microencapsulation of oil using spray drying technique. Va-g-Ch, when used in combination with various other protein and carbohydrate wall material may solve this problem. So the present study signifies the efficacy of vanillic acid grafted chitosan as a potential encapsulant for fish oil encapsulation by offering better oxidative stability for further incorporation in food.

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Chapter 4

Cardioprotective effect of Microencapsulated Sardine oil against doxorubicin induced oxidative stress in cardiomyoblast cell lines (H9c2)

4.1 Introduction
4.2 Materials and Methods
4.3 Results & Discussions
4.4 Conclusion

4.1 Introduction

Cardiovascular disease (CVD) is one of the deadly diseases and its occurrence is ever increasing in world, with 17.3 million deaths per year (Heron, 2012). Numerous epidemiological studies have showed the relationship between CVDs and the consumption of fish oil, emphasizing reductions in the risk of many CVDs (Lavie *et al.,* 2009). Fish oil is associated with a lower incidence of CVDs mainly because of high content of polyunsaturated fatty acids i.e. Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA). Taking into consideration of the health benefits of fish oil, many health organizations have recommended the consumption of fish oil either directly or in the form of fortified food. The American Heart Association (AHA) recommends consumption of fish oil at 1 g per day; which may lower the CVDs mortality rate by 29% (Covington 2004; Shukla *et al*., 2010). Recognizing the potential, many food science laboratories and food processors have come forward with novel functional food products; fortified with PUFA rich oils. Microencapsulation has been considered as a promising technique for such fortification of highly unsaturated marine fish oils in food products, as it imparts controlled release behaviour and protects against oxidation during

processing, handling and storage (Ghorbanzade *et al.,* 2017; Caceres *et al.,* 2008; Hermida *et al.,* 2015). Chitosan and its derivatives have often been used for microencapsulation of various functional food ingredients including oils. Particularly, synthesis of antioxidant-chitosan conjugates by grafting of antioxidant molecules onto chitosan has received much attention. Recently application of gallic acid grafted chitosan in delivery of bioactive components has been reported (Hu *et al.,* 2015; Hu & Luo, 2016). Chatterjee *et al*., 2016 reported application of ferulic acid grafted chitosan for microencapsulation and controlled release of thiamine and pyridoxine.

Researchers have opined that the phenolic acid grafted chitosan derivatives could potentially be used as new food additives or even as functional foods (Xie *et al*., 2014). It has been reported that phenolic acid grafted chitosan derivatives demonstrate various bio-activities, such as antioxidant, antimicrobial, anti-diabetic. (Chatterjee *et al*., 2015; Liu *et al*., 2013; Lee *et al.,* 2014). Hence, application of phenolic acid grafted chitosan derivatives for microencapsulation and delivery of bioactive lipids may result in development of new food additives or novel functional foods. H9c2 cell line is widely regarded as the best *in vitro* model to study cardioprotection activity due to its high resemblance to primary cardiomyocytes (Pelloux *et al.,* 2006). Doxorubicin (DOX) is one of the anthracycline compounds with potent anticancer activity, however long term use of this compound can cause severe cardiac dysfunction leading to irreversible congestive heart failure (Zhang *et al.,* 2009). DOX induced cardiotoxicity will increase cardiac oxidative stress and lipid peroxidation, along with decreased levels of antioxidants. In addition, it downregulates the genes responsible for contractile proteins, and p53 mediated apoptosis (Chatterjee *et al.,* 2010). The possible protective effect of encapsulated fish oil with various biopolymers against DOX-induced cardiotoxicity and the underlying mechanisms are not known (Covington 2004; McLennan *et al.,* 2007; Lavie *et al.,* 2009). So the present study attempts to evaluate the cardioprotective potential of sardine oil loaded vanillic acid grafted chitosan (Va-g-Ch), as well as elucidate the underlying mechanisms of cardioprotection.

4.2 Materials and methods

4.2.1 Materials

H9c2 (cardiomyoblast cell line) was procured from National Centre for Cell Sciences (NCCS), Pune, India. Doxorubicin, 3-(4,5-dimethylthiazol-2yl)-2, 5 diphenyl tetrazolium bromide (MTT), 2, 5 dichlorofluorescindiacetate (DCF-DA), vanillic acid and Tween 20 were bought from Sigma Aldrich (St Louis, MO, USA). Dulbecco's modified Eagles medium (DMEM), penicillin–streptomycin, and fetal bovine serum (FBS) were purchased from Gibco (Gibco, Grand Island, NY, USA). High purity RNA isolation kit was from Invitrogen (Carlsbad CA). RT-PCR kit was bought from Thermo Scientific, USA. Chitosan from shrimp shells (MW = 100 kDa, 88 % degree of deacetylation) was prepared in the pilot plant facility of Central Institute of Fisheries Technology, Cochin, Kerala, India. Acetic acid, hydrogen peroxide (H_2O_2) and ascorbic acid were obtained from Merck Millipore (Germany). All the ingredients used for the preparation of microcapsules were of food grade.

4.2.2 Synthesis and characterization of Va-g-Ch

Vanillic acid grafted chitosan (Va-g-Ch) was synthesized, as previously described in the third chapter (Chatterjee *et al.,* 2015). Briefly, 10 g of chitosan was dissolved in 2 L of 2 % acetic acid solution (v/v) in a 5 L three necked round bottom flask. 20 ml of 1 M H_2O_2 containing 1.08 g of ascorbic acid was added dropwise to the chitosan solution, followed by addition of 10 g vanillic acid dissolved in 100 ml ethanol. The reaction was maintained under nitrogen environment for 24 h at 25°C with constant stirring. The reaction mixture was dialyzed against distilled water for 72 h to remove unreacted phenolic acids. The reaction mixture and free vanillic acid were developed in silica coated TLC plates, to confirm complete removal of free vanillic acid. Finally, the dialysate was freeze dried to yield Va-g-Ch derivative in solid form. Structural characterisation was carried out using various spectroscopic techniques.

4.2.3 Microencapsulation of sardine oil with Va-g-Ch

Va-g-Ch (0.8 % wt/v) was dissolved in acetate buffer solution (2 mM sodium acetate and 98 mM acetic acid in water, pH 3.0) with overnight stirring on a magnetic stirrer. Sardine oil in water emulsion was prepared by blending sardine oil (30 wt % of total solid), Tween 20 (0.25% w/v) and the Va-g-Ch solution using a high speed blender (Bio-Gen PRO-250 High speed homogenizer, Scientific Inc, USA) at 15,000 rpm for 30 min. The optimized emulsion was spray-dried using a pilot-plant spray dryer (S M Scientech, Kolkata) to yield sardine oil loaded microparticles (SO-M). The inlet and outlet temperature of the spray dryer were 140°C and 77°C respectively. The SO-M obtained was immediately transferred into a cold glass jar. The spray dried powder was then used for further studies.

4.2.4 Determination of in-vitro cardioprotective effect by MTT assay

4.2.4.1 H9c2 cell culture and seeding in 96 well plate

The cell lines (H9c2) were cultured in DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate, penicillin (100U/ml), streptomycin (100µg/ml), and amphotericin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). Confluent monolayer cells were then trypsinized and suspended in 10% growth medium and the cells were seeded with a seeding density of 5000 cells/well in a 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator. Sample stock was prepared by dissolving 1 mg of encapsulated fish oil powder in 1ml of DMEM.

4.2.4.2 Cardio protective activity of SO-M

Doxorubicin was used to induce toxicity as per methods described by Xiao *et al.,* 2012. After 24 h of cell seeding, the growth medium was removed; doxorubicin was added at a final concentration of 20mM to induce toxicity and incubated for an hour. After doxorubicin treatment, freshly prepared SO-M in 5% DMEM was added at different concentrations (25µg, 12.5µg, 6.25µg, 3.1µg in 100µl of 5% DMEM) and incubated at 37ºC in a humidified 5% CO_2 incubator. Experiment was conducted in triplicates for each concentration. After 24 h the

entire plate was observed in an inverted phase contrast microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Changes in the morphology of the cells, such as shrinking of cells, formation of cytoplasmic vacuoles and rounding of cells were considered as indicators of cell cytotoxicity.

4.2.4.3 Cytotoxicity Assay by MTT Method

The sample content in the wells was kept for 24h incubation period. After incubation period the samples were removed and 30µl of reconstituted MTT (15mg of MTT was reconstituted in 3 ml PBS solution) was added to all test and control wells, the plate was gently shaken and incubated at 37ºC in a humidified 5% CO_2 incubator for 4h. After the incubation period, the supernatant was removed and 100 µl of MTT solubilization solution was added and the wells were mixed gently for solubilizing the formazan crystals. The absorbance values were measured at 570 nm (Talarico *et al.,* 2004).

The percentage of growth inhibition was calculated using the Eq. 1

$$Percentage\ of\ viability = \left(\frac{Mean\ OD\ of\ sample}{Mean\ OD\ of\ control}\right) \times 100 \qquad (1)$$

4.2.4.4 Determination of apoptosis by acridine orange (AO) and ethidium bromide (EB) double staining

Acridine orange and ethidium bromide (Sigma, USA) are major DNA binding dyes mainly used in molecular biology for differentiating apoptotic and necrotic cells (Zhang *et al.*, 1998). Acridine orange stains both viable and non-viable cells. The intercalation of this dye into double stranded nucleic acid (DNA) will emit green fluorescence whereas ethidium bromide stains only non-viable cells and emits red fluorescence. The cells were cultured in DMEM and grown to 70-80% confluency and treated for 24 h, the cells were washed by cold PBS and then stained with a mixture of acridine orange (100 µg/ml) and ethidium bromide (100 µg/ml) at room temperature for 10min. The stained cells were washed with 1X PBS and observed by a fluorescence microscope (Olympus CKX41 with Optika Pro5 camera).

4.2.4.5 Relative expression of Caspase-3 levels in H9c2 cells by indirect ELISA

100µl of Cell lysate was added to the 96 well plates and kept in overnight incubation at 37°C. The next day the wells were drained and washed with PBS for 3 to 5 times. Then, 200 µl of freshly prepared blocking buffer (0.2% gelatin in 0.05% Tween-20 containing PBS) was added to the wells, and incubated for 1h at room temperature and washed two times with washing buffer (0.05% Tween-20 containing PBS) at room temperature. Followed by, 100 µl of primary antibody (Anti Caspase, Santhacruz, USA) was added and incubated for 2 h at room temperature. The 1º antibody was washed in washing buffer. Sequentially, secondary antibody (Anti HRP conjugated secondary antibody- 100 µl) was added and left for 1h at room temp. Finally, the wells were washed with PBS-Tween 20 for two times and incubated with 200 µl of chromogen for 30 min at room temperature in dark conditions. The colour was developed using O-dianizdine (composition – 1mg/100 ml methanol + 21 ml citrate buffer pH 5+ 60 ml H_2O_2) hydrochloride and the reaction was stopped by adding 5N HCl (50 µl). OD was read at 415 nm in an ELISA reader.

4.2.4.6 In-vitro ROS measurement using DCFDA

Total ROS activities in the cells were determined by a fluorogenic dye Dichloro dihydro fluorescien diacetate (DCFDA). Measurement of ROS was done using DCFDA- Cellular Reactive Oxygen Species Detection Assay Kit, Sigma Aldrich, according to the manufacturer's instructions. Briefly, cells were seeded on to 96 well culture plates at a cell density of 5000 cells/well and allowed to adhere overnight. Following treatment with samples, culture media was removed and cells were incubated with 50 µl of DCFDA for 30 min and excess dye was washed off with PBS. The fluorescence was measured at 470 nm excitation and emission at 635nm (Qubit 3.0, Life technologies, USA) by a microplate flourimeter (Qubit 3.0, Life technologies, USA).

4.2.4.7 Determination of mitochondrial membrane potential by flow cytometry

H9c2 cell were seed on to T25 flasks at a cell density of 5000cells/well and allowed to reach 70% confluency. The cells were then treated with 20mM

doxorubicin followed by 12.5µg/ml of sample. Doxorubicin treated cells were served as positive control. After 24h incubation the cells were harvested and cells were incubated with Muse™ MitoPotential Dye according to manufacturer's protocol. After staining cells were washed with cold PBS and subjected to flowcytometric analysis using a flow cytometer (Millipore, USA).

4.2.4.8 Relative expression of NF-kb in H9c2 cells and isolation of total RNA

H9c2 cells seeded on T25 flasks were grown to 70% confluency and one flask was treated with 20mM doxorubicin (Sigma Aldrich USA) followed by 12.5 µg/ml of samples. Doxorubicin (20mM) alone was kept as positive control and the samples were incubated for 24h. After incubation the RNA isolation was carried out using trizol reagent. Briefly, total RNA was isolated using the total RNA isolation kit according to the manufacturer's instruction (Invitrogen, USA). The RNA pellet was dried and dissolved in TE (Tris-EDTA) buffer. The purity of extracted RNA was determined using fluorometer Qubit 3.1 (Life Technologies, USA).

4.2.4.9 Reverse transcriptase PCR analysis

Verso One step RT PCR kit of Thermoscientific, USA was used for the cDNA synthesis and amplification. About 5 µL of RNA, 1µL of enzyme mix, 2.5µL of RT Enhancer, 2µL of forward primer and reverse primer were added to an RNAse free tube (Forward primer sequence: 5'-CCCACACTATGGATT TCCTACTTATGG-3'and reverse primer sequence: 5'-CCAGCAGCATCTT CACGTCTC-3'). To this mixture 25 µL of primer RT-PCR premix was added. Then the total reaction volume was made up to 50 µL with the addition of sterile distilled water. The solution was mixed by pipetting gently up and down. The thermal cycler (Eppendorf Master cycler) was programmed to undergo cDNA synthesis and amplification. The stained gel was visualized using a gel documentation system (E gel imager, Invitrogen) and the mean density was determined using ImageJ analysis software.

4.2.5 Statistical Analysis

Independent t-test was carried out to compare the means of caspase-3 level, ROS and NF-Kb expression. One way analysis of variance was carried out for

MTT assay. Means were compared by tukey's test at 5% level of significance. All the statistical analysis was carried out using SPSS version 16 software.

4.3 Results & Discussions

4.3.1. MTT assay

Cell viability, after treatment with different concentrations of SO-M (3.1, 6.25, 12.5, and 25) µg/ml is shown in Table 4.1. It is evident that there is no cytotoxicity up to a sample concentration of 25µg/ml and hence further experiments were carried out using 12.5 µg/ml concentrations. Simulated cardiotoxicity by doxorubicin for 1h caused a change in morphology of the H9c2 cells. The cells showed shrinkage, change in nuclear morphology and membrane blebbing which are characteristics of apoptotic cell death (Fig. 4.1). In our study we observed that 12.5 µg/ml of sardine oil loaded vanillic acid grafted chitosan microparticles decreased cytotoxic effects of doxorubicin in cardiomyoblast cell lines (H9c2). Around 84.9% cardiomyocytes cells were recovered from doxorubicin induced cardiotoxicity. It indicates the effectiveness of sardine oil loaded vanillic acid grafted chitosan microparticles against cardiotoxicity. Modern world lifestyle diseases such as cardiotoxicity due to various stress is becoming common, so it is highly recommended to include such functional food in diet to provide protection against cardio toxicity (Das *et al.,* 2016).

Table 4.1. Percentage of viability at different sample concentration.Values were calculated from the Mean OD of samples and control group. * A different alphabet superscript (a, b, c and d) indicates a significantly different percentage viability value than others by a Tukey test at $p \leq 0.05$

Sample Concentration (µg/ml)	Average OD at 540nm	Percentage Viability
Control	0.82	0.0^{a}
Doxorubicin	0.35	43.0^{b}
3.1 µg sample	0.45	54.9^{c}
6.25 µg sample	0.69	83.5^{d}
12.5 µg sample	0.70	84.9^{d}
25 µg sample	0.71	86.0^{d}

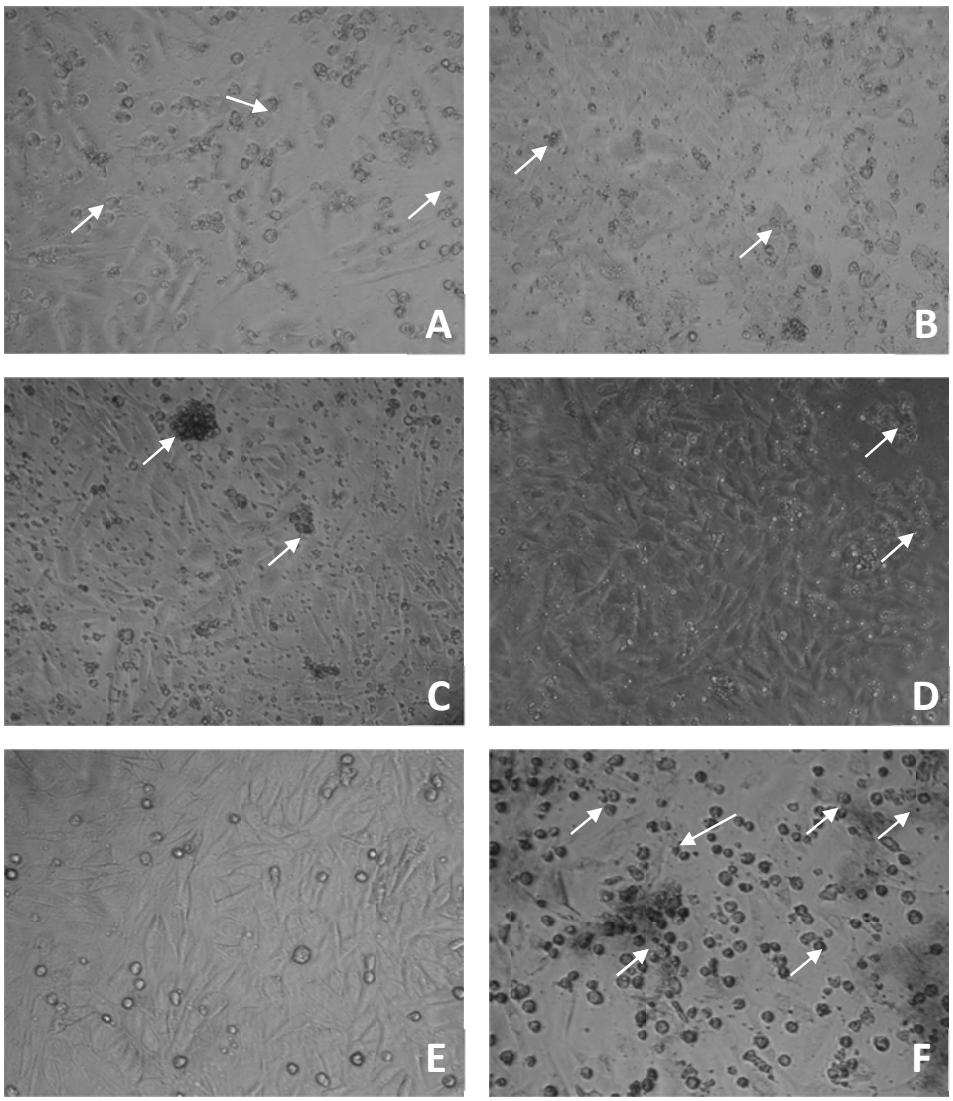


Fig.4.1. MTT images explaining the cellular morphological changes following treatment of doxorubicin and sample treatments Arrows highlight cellular rounding and detachment reflecting apoptotic cell death. A:Treated with 3.1 µg/ml concentration of sample, B: 6.25µg/ml concentration of sample, C: 12.5µg/ml concentration of sample and D: 25µg/ml concentration of sample, E: Control H9c2 cell lines, F: Doxorubicin treated cells.

4.3.2 Determinations of Apoptosis by Acridine orange (AO) and ethidium bromide (EB) double staining

If the sample is cytotoxic, the cells will undergo cell death following necrotic pathway. MTT assays cannot differentiate between the mechanisms of apoptosis and necrosis. It is possible to detect basic morphological changes in apoptotic cells by Acridine orange/Ethidium bromide fluorescent staining. AO/EB staining is one of the reliable methods to distinguish normal, early apoptotic, late apoptotic and necrotic cells (Biffl *et al.*, 1996). Acridine orange is a membrane permeable dye which can stain normal as well as apoptotic cells and has a characteristic green flourescence. On the other hand, only dead cells and late apoptotic cells with a damaged cell membrane are permeable to ethidium bromide which fluoresce orange-red (Ribble *et al.*, 2005). H9c2 cells were stained with AO/EB after 24h treatment with SO-M. No significant cell death was observed in the SO-M treated group on visualization under fluorescent microscope. Necrotic cells increased in volume and showed uneven orange-red fluorescence (Fig. 4.2). The cells appeared to be in the process of disintegrating.

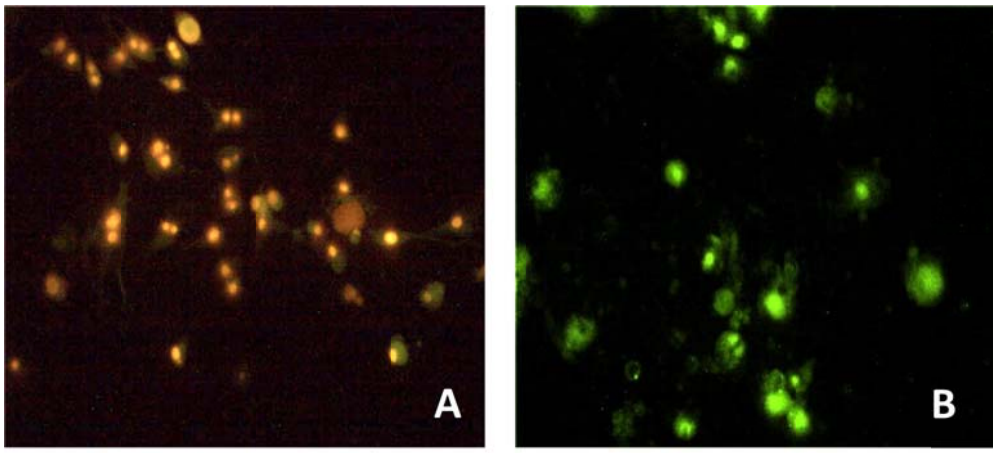


Fig. 4.2. AO/EB fluorescence staining for apoptotic assays- A: 1 % DOX treated cells showing uneven orange-red fluorescence and it is becoming dissolved or near disintegration which indicates non-viable cells by necrosis or late apoptosis. B: 12.5 µg/ml SO-M treated group, nucleus of the encapsulated oil treated cells showed yellow-green fluorescence by acridine orange. (After 24 h)

4.3.3 Relative expression of Caspase-3 levels in H9c2 cells

Caspases are the cysteine proteases widely considered as a significant marker of apoptosis during cell death. Elevated levels of caspases 3 clearly indicate the apoptosis mediated cell death (Hanahan & Weinberg, 2011). To investigate the effect of SO-M on the intrinsic pathway of apoptosis, caspase-3 activity in both treated and untreated H9c2 cells was measured (Fig. 4.3). It has been well documented that the activation of caspase-3 is essential for the occurrence of apoptosis in cardiomyocytes (Harrington *et al*., 2008). In the present study, the SO-M demonstrated to inhibit the subsequent activation of caspase-3. However, in doxorubicin induced control group, caspase 3 activation was markedly increased. The presence of SO-M significantly diminished the level of caspase-3 activity. Hence sardine oil loaded vanillic acid grafted chitosan microparticles could possibly inhibit the apoptosis and necrosis activation in cardiomyocytes.

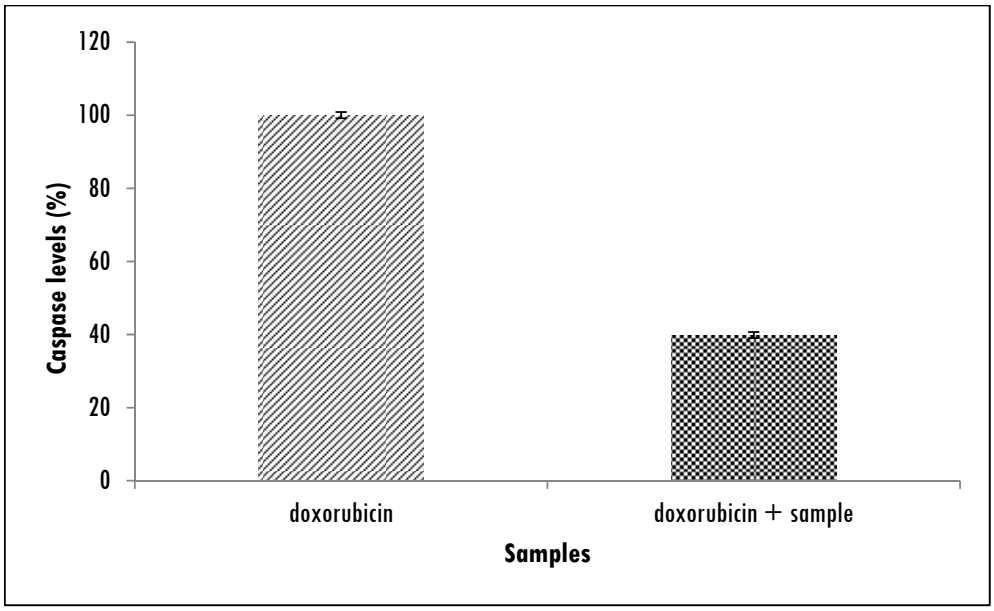


Fig. 4.3. Relative expression of caspase 3 levels in H9c2 cells determined by indirect ELISA by 1 % dox treated cells and 12.5 µg/ml SO-M treated group.

4.3.4 In vitro ROS measurement using DCFDA

ROS are the major agents which lead to pathological symptoms associated with cardiomyopathy (Penna *et al*., 2009). ROS originate in the body mainly by damaging the membrane permeability of mitochondria and in turn cause severity to cells such as cardiomycetes (Maack & Bohm, 2011). When ROS level is elevated, a series of signaling cascades are activated which in turn trigger the apoptotic pathway and ultimately leads to apoptosis. ROS mainly cause depolarization and bulging of mitochondria therefore accelerates apoptotic mechanism through mitochondrial involvement (Yamamoto *et al*., 1999; Suhara *et al*., 1998). Reactive oxygen species (ROS) includes H_2O_2 (hydrogen peroxide), NO (nitric oxide), O_2^- (oxide anion) and hydroxyl radical (OH^-). Mainly this oxidative species are produced in the conditions of cancers, neurologic, cardiovascular, infectious and inflammatory conditions (Lei *et al.,* 2015). To investigate whether the encapsulated fish oil powder triggers ROS production in H9c2 cells lines; the ROS status using fluorescent dye, DCFDA was analyzed. As expected, doxorubicin treatment increased ROS production in H9c2 cells lines whereas the addition of SO-M decreased ROS generation less than that of control (Fig. 4.4). Hence, SO-M treatment could possibly reduce the ROS production and in turn facilitated reduced cardiotoxicity during oxidative stress condition.

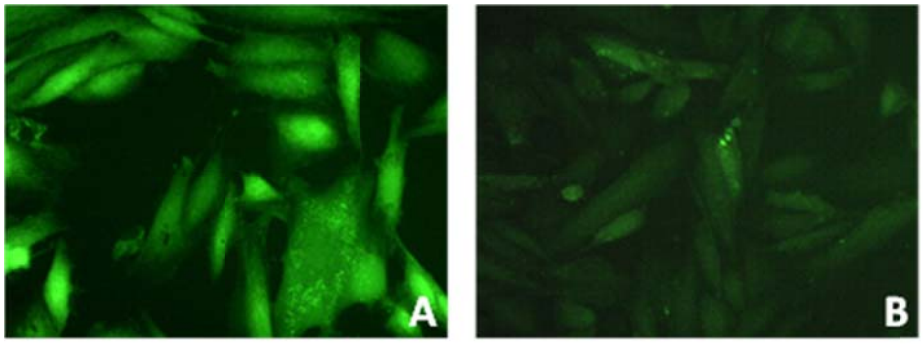
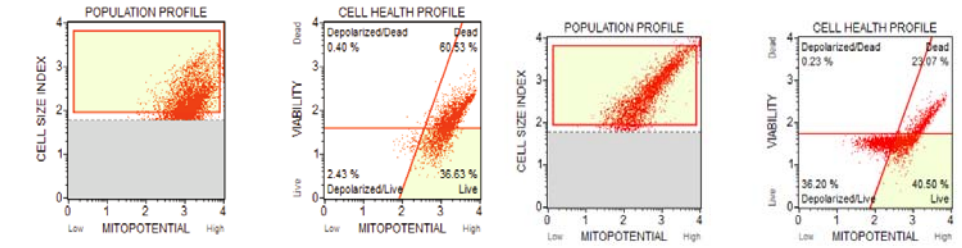


Fig. 4.4 Investigation of ROS potential in H9c2 cell lines. A: 20 mM doxorubicin-treated cardiomyoblast cell lines showing bright green fluorescence due to excess presence of ROS. B: 12.5 µg/ml SO-M-treated cells showing less fluorescence because of lower content of ROS

4.3.5 Mitochondrial membrane potential by flow cytometery

Inner mitochondrial membrane potential (MMP) is one of the indicators of mitochondrial dysfunction and it is very important in the study of apoptosis. Mitochondrial changes especially its MMP and viability are highly sensitive indicators of cell health (Landes & Martinou, 2011). MMP is an indicator of doxorubicin-induced apoptosis (Green & Leeuwenburgh, 2002). To determine whether doxorubicin induced apoptosis through disrupting MMP and how SO-M affected this process, changes in MMP were observed using flow cytometery with the help of Muse mitopotential assay kit. The mitopotential dye detects the changes in mitochondrial membrane potential and 7-AAD acts as a dead cell marker. Four populations of cells could be distinguished from the data; namely live cells with depolarized mitochondrial membrane, live cells with intact mitochondrial membrane, dead cells with depolarized mitochondrial membrane, and dead cells with intact mitochondrial membrane. From Fig. 4.5, it is evident that there is significant cell death due to disruption of MMP in doxorubicin treated group as indicated by increase in cell density in upper right quadrant. In SO-M treated samples the red fluorescence is very less in the right quadrant whereas sharp red fluorescence were observed in the lower left quadrant indicating the viability. The pre-treatment with SO-M increased the MMP to near normal levels, thereby confirming the protective effect of encapsulated fish oil powder on MMP integrity (Fig. 4.5).



	% Gated	Cell Conc. (Cells / mL)		% Gated	Cell Conc. (Cells / mL)
Live(LR) :	36.63 %	1.06E+06	Live(LR) :	40.50 %	3.63E+06
Depolarized/Live (LL) :	2.43 %	7.02E+04	Depolarized/Live (LL) :	36.20 %	3.24E+06
Depolarized/Dead (UL) :	0.40 %	1.15E+04	Depolarized/Dead (UL) :	0.23 %	2.09E+04
Dead (UR) :	60.53 %	1.75E+06	Dead (UR) :	23.07 %	2.06E+06
Total Depolarized :	2.83 %	8.17E+04	Total Depolarized :	36.43 %	3.26E+06
Total Cell Concentration :	2.88E+06 / mL		Total Cell Concentration :	8.95E+06 / mL	
Dilution Factor :	2.00		Dilution Factor :	2.00	

Fig. 4.5. Mitochondrial membrane potential by flow cytometery with 7-AAD, A: Doxorubicin treated cells and B: 12.5 µg/ml Sample (Encapsulated fish oil) treated cells.

4.3.6 Relative expression of NF-kb in H 9C2 cells

According to Wang *et al.,* 2002, DOX-induced apoptosis in cardiac muscle cells may be mediated through activation of NF-kB. NF-kB mediates cardiomyocyte damage by controlling the expression of apoptotic proteins such as Bcl-2 family proteins and caspases (Karin & Ben-Neriah, 2000). TNF-alpha is known to exert anti-apoptotic effects through the activation of NF-kB signalling and in turn prevent heart failure. Decreased expression of NF-kB in SO-M treated cardiomyocytes can be due to the TNF-alpha regulated mechanism which could have been inhibited activation of caspases expression and resulted in decreased production of reactive oxygen species (McGowan *et al.,* 2003). In the present study we examined the NF-kB expression by an RT-PCR method in nuclear extracts of H9c2 cells (Fig. 4.6 A). We observed that NF-kB activity in H9c2 cells was sharply increased by incubation in the presence of doxorubicin. However, the pretreatment with encapsulated fish oil samples markedly attenuated NF-kB activation induced by doxorubicin (Fig. 4.6 B).

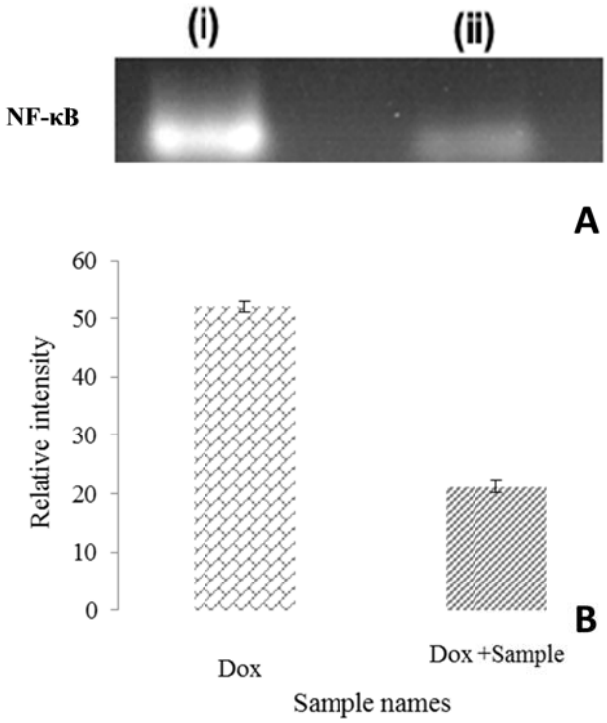


Fig. 4.6 **A**. (i) Significantly high expression of NF-κB in doxorubicin treated samples indicates cardiotoxicity (ii) decreased expression of NF-κB in SO-M treated cell lines indicates cardioprotection; **4.6 B.** Relative expression of NF-κB in H9c2 cells treated with SO-M.

4.4 Conclusion

Cardioprotective nutrients are vital nowadays due to increasing number of cardiotoxicity reports. Fish oil is conventionally used as a cardioprotection nutrient but undesirable if oxidized and it causes adverse effect rather than beneficial effects. Chemical preservatives added in order to prevent oxidation could be harmful and mask protective effect of fish oils. Encapsulation with chitosan and natural antioxidants could be efficiently preventing undesirable oxidation of fish oils to a great extent. Hence proven cardioprotection effect of fish oils are better retained or enhanced by encapsulation and derivatization with natural antioxidants agents such as vanillic acid. Effect exerted by this encapsulated oil microparticle protects cardiomyocytes through series of mechanisms namely, alleviated ROS actions, suppression of apoptosis and reduced expression of NF-kB.

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Chapter 5

Nutritional and metabolic influence of sardine oil loaded vanillic acid grafted chitosan microparticles in Wistar rats

5.1 Introduction
5.2 Materials and Methods
5.3 Results & Discussions
5.4 Conclusion

5.1 Introduction

Health benefits of long chain polyunsaturated fatty acids (PUFA) containing marine fish oils are well-known (Zulfakar *et al.,* 2007; Razack & Seidner, 2007; Stillwell & Wassall, 2003). Because of the presence of highly unsaturated fatty acids, it can undergo oxidation easily resulting in the formation of toxic off flavour compounds such as hydroperoxides and this can adversely affect its nutritional quality. Hence, fish oil needs to be protected from factors that promote oxidation such as oxygen, light, free radicals and pro oxidants to accomplish their original physiological functions (Kagami *et al.,* 2003). Microencapsulation is one of the potential methods to protect fish oil against oxidation and allow the manufacturer to handle and incorporate the oil in food products (Klinkesorn *et al*., 2004). Choice of emulsifier and wall material are important factors among others that determine the success of microencapsulation process (Encina *et al.*, 2016; Klinkesorn *et al.,* 2004). Chitosan is one of the amino polysaccharide with excellent emulsifying property and has often been used as a wall material, as well as an emulsifier for microencapsulation of highly unsaturated oils (Shen *et al.,* 2010; Klinkesorn *et al.,* 2005). Wide application of chitosan in the field of agriculture and pharmaceutics is already reported (Shahidi *et al*., 2002). Modified chitosan showed improved solubility and

emulsifying properties, (Jayakumar *et al.,* 2005). Modification of chitosan with phenolic acids has been reported previously (Chatterjee *et al.,* 2015). Phenolic acid-grafted chitosan derivatives and their applications in food is reported by Chatterjee, *et al.,* 2015 and their various bio-activities like antioxidant, antimicrobial and antidiabetic are also reported (Liu *et al.,* 2013; Lee *et al.,* 2014). The derivatives of chitosan will find potential applications in food industry as food additives or functional food ingredients (Xie *et al.,* 2014). The stability issues of PUFA rich oil could potentially be addressed through microencapsulation strategies (Chatterjee *et al.,* 2015). Grafting of bio-functional molecules on to chitosan aids in developing tailor made functional food ingredients or novel dietary supplements. Microencapsulation of fish oil with such chitosan derivatives will open up new avenues in food industry as potential novel functional ingredients. However, it is important to evaluate novel dietary supplements in experimental animal model to understand the mechanisms of action. In the present study, investigated the effect of dietary supplementation of sardine oil loaded vanillic acid grafted chitosan on growth performance, metabolic and immune responses in wistar strain albino rats.

5.2 Materials and Methods

5.2.1 Materials

Sardine oil (*Sardinella longiceps*) was obtained from Arbee agencies Kottayam, Kerala, India and was stored in dark amber coloured-glass bottles at -20° C, until use. Chitosan was prepared in Central Institute of Fisheries Technology, Cochin, Kerala, India. Vanillic acid, Tween 20 and all other chemicals used in the enzyme assay were obtained from Sigma-Aldrich (USA).

5.2.2 Synthesis and characterisation of sardine oil loaded vanillic acid grafted chitosan

Vanillic acid grafted chitosan was synthesized, as previously described in second chapter Briefly, 20 g of chitosan (Degree of deacetylation: 80-85%) was dissolved in 2.5 L of 2 % acetic acid solution (v/v) in a 5 L three necked round bottom flask. 20 ml of 1 M H_2O_2 containing 1.08 g of ascorbic acid was added drop wise to the chitosan solution, followed by addition of 20 g vanillic acid dissolved in

500 ml ethanol. The reaction was maintained under nitrogen environment for 24 h at 25°C with constant stirring. The reaction mixture was dialyzed against distilled water for 72 h to remove unreacted phenolic acids. Finally, the dialyzate was freeze dried to yield vanillic acid grafted chitosan derivative in solid form.

5.2.2.1 Emulsion Preparation

Vanillic acid grafted chitosan (0.8 % wt/v) was dissolved in acetate buffer solution (2 mM sodium acetate and 98 mM acetic acid in water, pH 3.0) with overnight stirring on a magnetic stirrer. Sardine oil in water emulsion were prepared by blending sardine oil (30 wt % of total solid), Tween 20 (0.1 % wt/v) and the vanillic acid grafted chitosan solution using a high speed blender (Bio-Gen PRO-250 High speed homogenizer, Scientific Inc, USA) at 15,000 rpm for 15 min.

5.2.2.2 Spray dried microparticle preparation

The homogenized emulsions were then converted into powder using a pilot-plant spray dryer (S M Scientech, Kolkata). The inlet and outer temperature of the spray dryer were ±140°C and outlet temperature was 77 ± 5^{o} C respectively. The fish oil powder obtained was immediately transferred into a cold glass jar. The spray dried powder was then used for further studies.

5.2.3 Experimental animals and design of the study

Wistar strain male albino rats (160-200 g) were used for this experiment. They were housed individually in polyurethane cages under hygienic conditions, maintained at room temperature (28±2^{o}C) and provided food and water ad libitum. Animal experiments were performed as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee (IAEC). The animals were randomly divided into 3 groups (n = 6 per group) Group I: Control, Group II: 1% sardine oil loaded vanillic acid grafted chitosan microparticles and Group III: 3% sardine oil loaded vanillic acid grafted chitosan microparticles. The feeds were prepared every week and packed in separate sealed plastic bags in quantities sufficient for one day's feed. The plastic bags were flushed with nitrogen, sealed and stored at -20^{o} C. The feed

which was not consumed by the animals was discarded daily. Food consumption was measured daily. At the end of the experiment (60 days), animals were anesthetized by chloroform and blood was collected. After blood collection, liver, kidney, muscle and heart were removed. All the tissues were flash-frozen and stored at -20^{o} C, tissue part was homogenized in appropriate buffers for analysis of biochemical parameters.

5.2.4 Growth Study

The animals were weighed at the start and every 7 days' intervals thereafter till the termination of the experiment on the 60th day. The growth performance was evaluated in terms of weight gain (%) and specific growth rate (SGR).

Weight gain (%) = (final weight – initial weight)/initial weight × 100

SGR = 100 (logeaverage final weight − loge average initial weight)/number of days

5.2.5 Lipid profile

Total cholesterol, HDL (High density lipo-protein), LDL (Low density lipo-protein) and TG (Triglycerides) were determined by using automated lipid analyzer (Abacus-250).

5.2.6 Blood profile

Complete blood profile was determined by completely automated clinical analyzer (Minbray-3200 BC).

5.2.7 Blood Glucose

Blood glucose was estimated by the method of Nelson-Somogii (Somogyi, 1945).

5.2.8 Enzyme assay

Liver specific enzymes like aspartate amino transferase (AST) and alanine amino transferase (ALT) activities were measured by the estimation of oxaloacetate and pyruvate released, respectively, after incubation of the reaction mixture at 37 °C for 60 min (Wooten, 1964). Superoxide dismutase (SOD) activity was estimated as

detailed by Misra & Fridovich (1972). Catalase activity was assayed following the method described by Claiborne (Claiborne, 1985). Glutathione-S-transferase (GST) activity was measured spectrophotometrically by the method of Habig *et al.*, 1974. Methods were explained in second chapter. Alkaline phosphatase (ALP) and acid phosphatase (ACP) activities were determined as described by Garen & Levinthal, 1960. Tissue protein content was determined according to Lowry et al. (Lowry *et al*., 1951).

5.2.9 Statistical Analysis

Two way analysis of variance was carried out to compare the direct and interaction effect of different treatments. Tukey's test was performed to compare the marginal means and t-test used to compare the interaction means at 5% level of significance. All the statistical analysis was done using SAS 9.3 software.

5.3 Result and Discussion

5.3.1 Characterization of microparticles

Sardine oil microparticles were developed by spray drying (Already explained in chapter 3). Size distribution by number of the re-dispersed SO-M has been presented in Fig. 5.1 The approximate diameter of the developed particles was found to be 2.3 µ. Increase in particle size after spray drying as compared to oil in water emulsion micelle size was expected due to formation of larger droplets in the spray dryer nozzle. ζ potential value of 16.5 was observed, which was similar to that of oil in water emulsion. However a lower PDI value of 0.345 was recorded as compared to the oil in water emulsion. This indicates comparatively better homogeneity in particle size distribution.

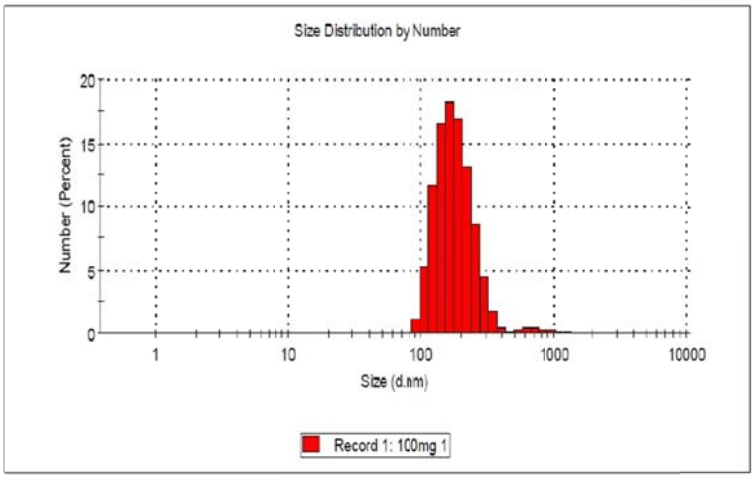

Fig. 5.1. Particle size distribution of sardine oil encapsulate

SEM images of SO-M showed spherical to irregular morphology (Fig. 5.2) with a few small pores on the smooth surface. Broken particles at higher magnification clearly showed cavity inside the particle indicating encapsulation of the oil inside the wall material. The Loading efficiency (LE) value was determined as 67±0.51%. Hence, around 33% of the initial oil was lost during spray drying process

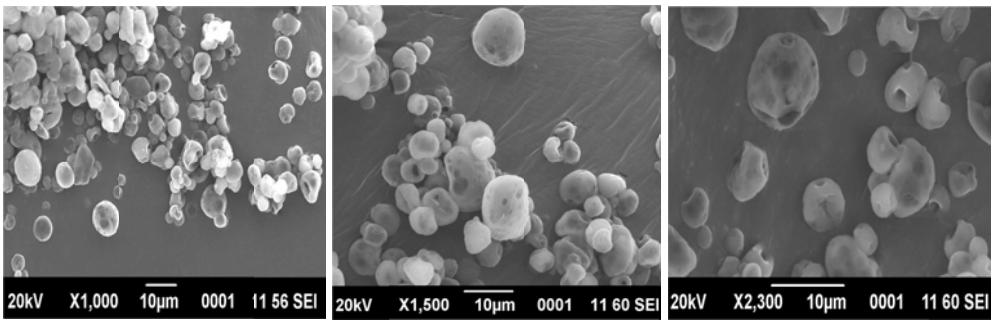

Fig.5.2 SEM images of the sardine oil encapsulates

5.3.2 Growth performance

The weight gain % and specific growth rate (SGR) of the experimental rats are given in Table 5.1. Animals from the treatment group fed with sardine oil-loaded vanillic acid-grafted chitosan (SO-VGC) showed improved weight gain percentage and SGR compared to control animals. The major contents in the fish oil microparticles were sardine oil, vanillic acid and chitosan. The health

promoting potential of fish oil and carbohydrate-phenolic acid conjugate may be the reason for the improved weight gain. This will find a very good application in the development of fish oil supplements with added benefit of vanillic acid and chitosan. The previous studies showed that incorporation of polysaccharide phenolic compounds in the diet will result in the improved growth performance (Tejpal *et al.,* 2017). Hence it can be concluded that the developed sardine oil powder is showing better growth rate and weight gain percentage and that can be used as a health promoting component.

Table 5.1 Impact of dietary supplementation of sardine oil loaded vanillic acid grafted chitosan micro-particles on weight gain% and SGR in Wistar albino rats. Value represents the mean ± SD.

Parameter	Control	1% SO-M	3% SO-M
Weight gain%	300.8±5.28	324.6±6.49	320±7.21
SGR	2.98±0.02	3.19±0.07	3.12±0.02

5.3.3 Blood Glucose

The glucose content was estimated at the end of experimental period. Fig.5.3. shows the blood glucose levels of control and experimental group of wistar rats. Supplementation of sardine oil loaded vanillic acid grafted chitosan microparticles significantly decreased blood glucose level. The blood glucose level of control group was 117 mg/ml. Dietary supplementation with sardine oil loaded vanillic acid grafted chitosan at 1 and 3% in rats showed slight decrease (98 and 82 mg/ml) in blood glucose level. Risk of diabetes is a big concern and the health products which are able to lower the glucose level has gaining attention recently. Our findings revealed the ability of our compounds to lower the glucose levels. Reports suggest the anti-diabetic activity of omega-3 rich fish oil and the activity might be due to the action of omega-3 fatty acids such as DHA and EPA (Soltan, 2012). Anti-diabetic activity of vanillic acid, an important secondary metabolite is well known (Chang *et al.,* 2015). So the reason behind glucose lowering activity of our compounds could be the synergic action of omega-3 rich fish oil and vanillic acid.

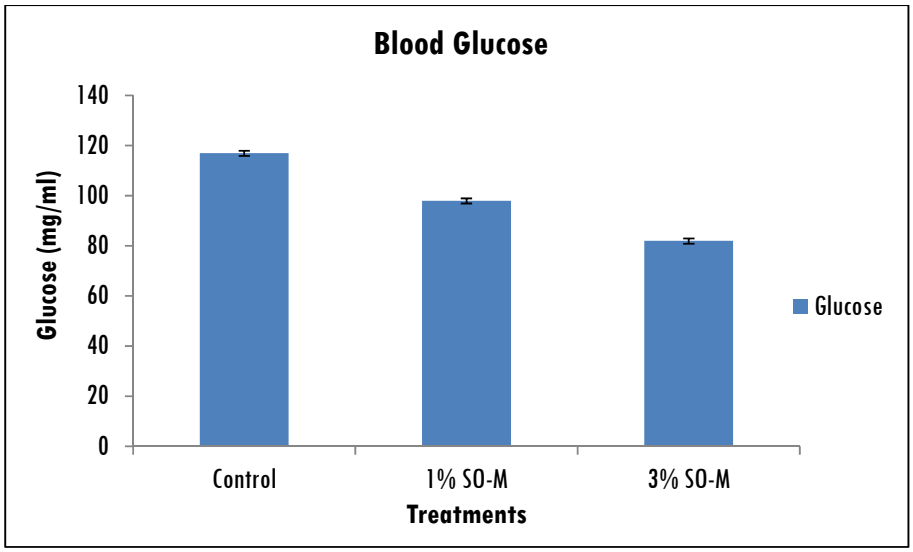

Fig.5.3. Effect of dietary supplementation of sardine oil loaded vanillic acid grafted chitosan micro-particles on blood glucose levels in albino rats

5.3.4 Effect of Microparticles on lipid profile

The present results elucidated high significant decrease in total cholesterol, triglyceride and LDL-cholesterol concentrations in the sardine oil microparticles treated groups compared to control rats and highly significant increase in HDL-cholesterol levels (Fig 5.4). The results showed that serum cholesterol concentration in control group is 62 mg/dl and in sardine oil microparticles (SO-M) treated group the serum cholesterol level is reduced to 55 mg/dl in 1 % SO-M treated group and 60 mg/dl in 3% SO-M treated group. Decreased triglyceride (TG) level is observed in 1% (68 mg/dl) and 3% (71 mg/dl) treated groups compared to control group (74 mg/dl). In case of HDL cholesterol increased amount is observed in treated group (24 mg/dl for 1% and 22 mg/dl for 3 %) compared to control (18mg/dl) whereas LDL is decreased in the sardine oil powder treated groups (19 mg/dl for 1% and 21mg/dl for 3%) compared to control (25mg/dl).

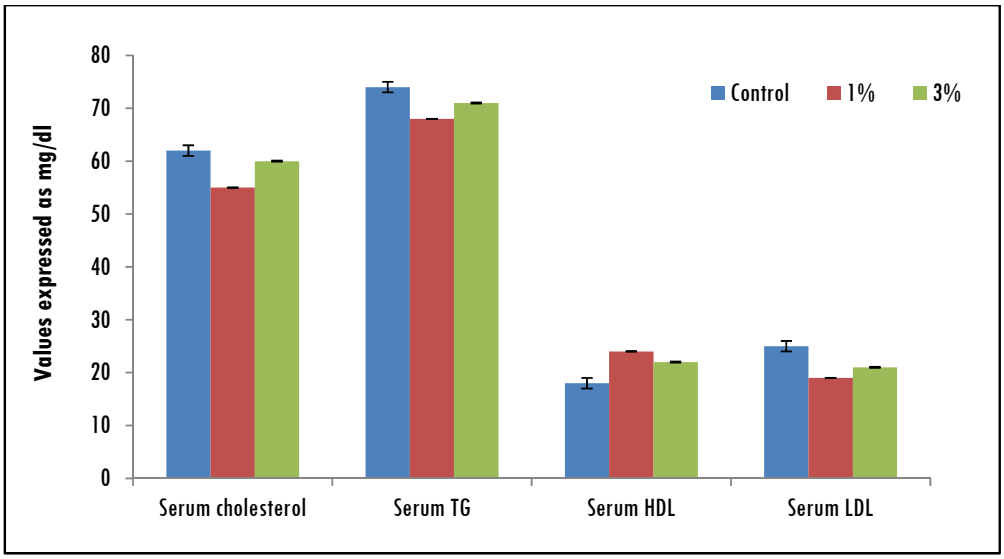

Fig. 5.4 Effect of dietary supplementation of sardine oil loaded vanillic acid grafted chitosan microparticles on lipid profile in wistar rats

Coronary diseases are mainly associated with the imbalance in lipid metabolism in the body. High concentration of cholesterol, triglyceride, LDL-cholesterol and VLDL-cholesterol were found to increase the cardiac problem many folds (Gordon *et al.,* 1989). Wide ranges of health products are available in market demanding the ability to reduce the bad cholesterol and TAG level in the body. At the same time health products derived from natural compounds always have the advantage of less or no side effects and enhanced cardio-protection activity (Oyinloye *et al.,* 2016). Lipids which are rich in omega-3 fatty acids are well known for their cardio-protection activity whereas, highly oxidized omega-3 fatty acid reverse this positive effects and cause several health issues in the body (Albert *et al.,* 2015). Our study mainly focused on the production of omega-3 rich fish oil powder with negligible oxidation. In our feeding study using animal model we observed decreasing trend in the serum cholesterol, triglyceride, LDL-cholesterol and VLDL-cholesterol concentrations and this activity may be due to the action of intact omega-3 rich fish oil. We have observed the cardio- protective activity of vanillic acid grafted fish oil powder in H9c2 cardiomyocetes cell line studies. Hence this product can be included as a vital health product with commendable cardio-protection activity.

5.3.5 Effect of Microparticles on Hematological pattern

Nutritional benefits of the consumed products can be better monitored by analyzing hematological parameters since any biochemical change ultimately reflects in clinical parameters (Boots *et al.,* 2008). Hemoglobin, platelets, RBC and packed cell volume are the major parameters analysed to observe effect of sardine oil microparticle on clinical parameters. Increased content of hemoglobin has good accordance with the better health since Hb is the vital molecule to transport oxygen (Page *et al.,* 1998). Details of the Hb content were illustrated in figure 5.5. Hemoglobin content was increased to 15.3 g/dl in 1 % treated group compared to control group (12.6g/dl). RBC is the major blood cell which participates in respiration and normal RBC level is very much important for a healthy person. RBC concentration was depicted in figure 5.6. Noticable increase in RBC concentration was noticed in 1% treated group compared to control group, whereas no such increase was observed in 3% treated groups. Increase in RBC diecltly influence the increased Hb production and this observation was noticed in our work too. So both increased production of Hb and RBC cells could be beneficial to enhance the health of a person. So our product can be a good nutritional supplement to many people who suffer from decreased Hb and RBC. Platelets play vital role in blood coagulation, clotting, host defence etc. and decreased platelets severely affects various physiological functioning of the body. In our study we observed the change in the platelet content after treating with different concentration of fish oil powder. Platelet count was depicted in figure 5.7. There was an increased platelet count noticed in 1% treated group compared to control group, whereas no such increase was observed in 3% treated groups. PCV is the percentage of red blood cells in circulating blood. A decreased PCV indicates red blood cell reduction due to variety of reasons like cell damage, blood loss, and failure of bone marrow synthesis. Increased PCV indicates the better hematological status of the body which is very essential for healthy body (Ots *et al.,* 1998) Effect of dietary supplementation of sardine oil loaded vanillic acid grafted chitosan microparticles on PCV in wistar rats is depicted in figure 5.8. An increase in PCV was observed in 1% sardine oil loaded

vanillic acid grafted chitosan microparticles treated group and no significant change is observed in control. So our results substantiate the role of fish oil powder to increase PCV leading to better health.

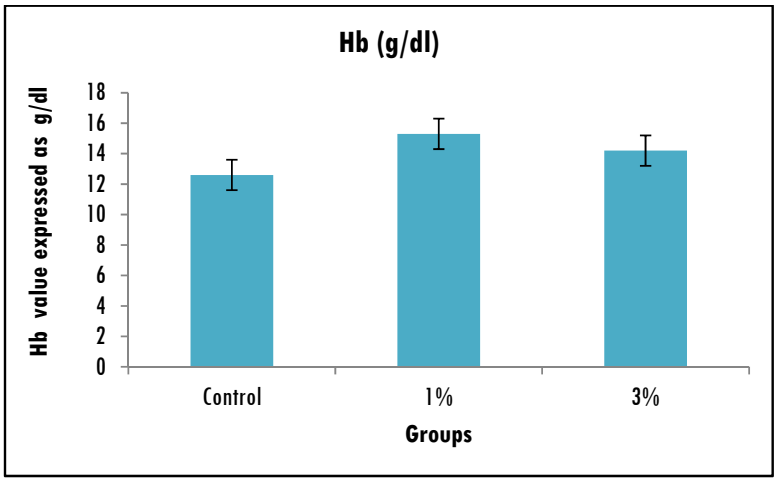

Fig.5.5 Effect of dietary supplementation of sardine oil loaded vanillic acid grafted chitosan microparticles on hemoglobin profile in wistar rats

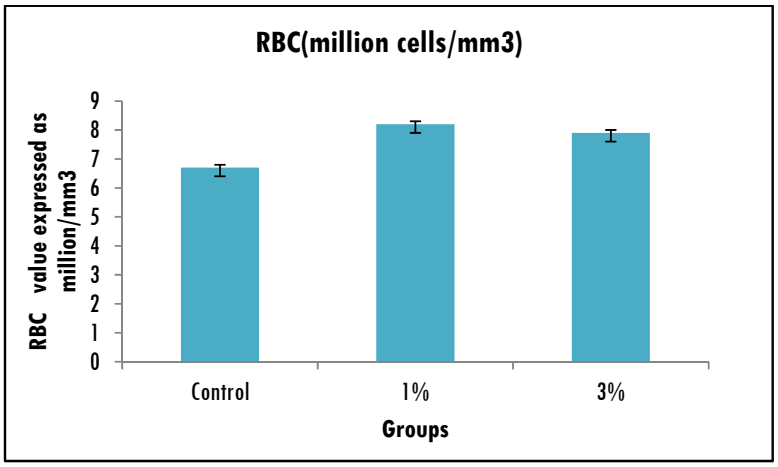

Fig.5.6 Effect of dietary supplementation of sardine oil loaded vanillic acid grafted chitosan microparticles on RBC changes in wistar rats

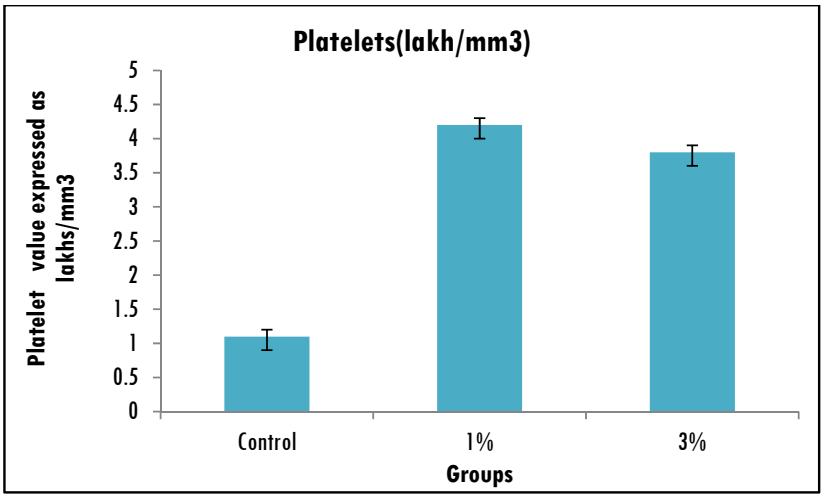

Fig.5.7 Effect of dietary supplementation of sardine oil loaded vanillic acid grafted chitosan microparticles on platelet profile in wistar rats.

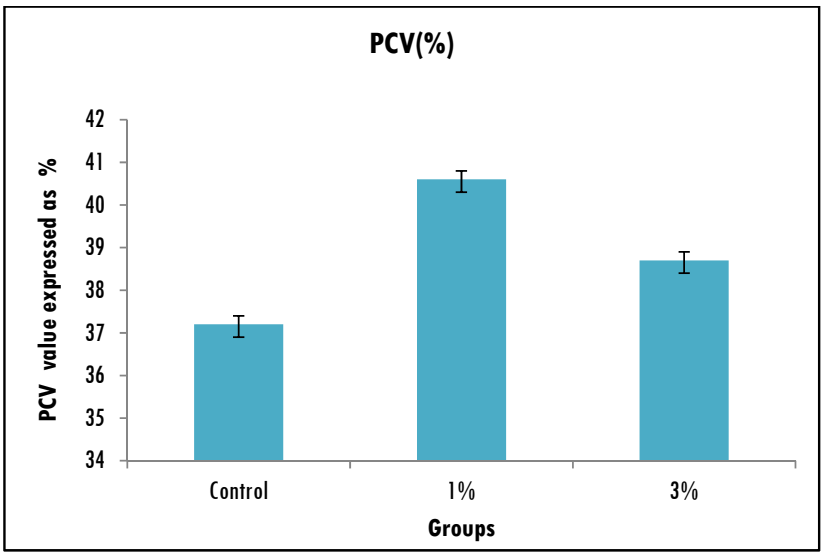

Fig.5.8 Effect of dietary supplementation of sardine oil loaded vanillic acid grafted chitosan microparticles on PCV in wistar rats.

5.3.6 Enzyme Assay

5.3.6.1 Aspartate amino transferase (AST) and Alanine amino transferase (ALT)

In the present study the activity of AST and ALT was studied in muscle, liver, kidney and heart **(Table.5.2)**. Results show that both AST and ALT activities were decreasing in the group which treated with increased sardine oil microparticles

in the feeding diet. In the organs, highest AST and ALT activity were observed in muscle. Previous studies reported that increased activities of AST and ALT accelerate the metabolism of amino acids to form TCA (Tricarboxylic acid) intermediates and finally leads to decrease in the availability of amino acids for muscle growth. Instead these amino acids intermediate enter into gluconeogenesis and gradually decrease the bioavailability for growth and development which may lead to lack of growth performance (Taha *et al.,* 2014; Tejpal *et al.,* 2017). So it is highly essential to have health products or compounds that can reduce the activities of AST and ALT to normal levels and in turn help in growth performance. In our study we observed a decrease in the activity of these enzymes in studied organs and concluded that efficiency of SO-VGC is very good to enhance growth performance. Weight and SGR levels were increased remarkably in the group which was treated with SO-VGC and it can be correlated with the above findings in our study. Similar kind of work like growth performance study of nutraceuticals product in animal model were published previously (Tejpal *et al.,* 2017).

Table. 5.2 **Activi**ty of dietary supplementation of sardine oil loaded vanillic acid grafted chitosan microparticles on AST and ALT activity in muscle, liver, kidney and heart tissues of albino rats. Different superscript (a, b, c, d) in the row indicate significant difference ($p<0.05$) between the control and treatment groups. Marginal means given in bold (A, B, C, D). Values are expressed as mean ± SE (n=6).**Units:** nano moles oxalo acetate released/ mg protein/ minute at 37^{o}C (AST); nano moles of sodium pyruvate formed/mg protein/minute at 37^{o}C (ALT).

Response	Tissue	Treatments			
		Control	1% SO-VGC	3% SO-VGC	Mean
AST	Liver	18.04±00.21^{a}	11.26±0.31^{b}	10.03±0.28^{b}	**13.86** ^{B}
	Kidney	10.26±0.20^{a}	7.43±0.18^{b}	5.52±0.17^{c}	**8.61** ^{D}
	Muscle	61.26±3.47^{a}	48±2.89^{b}	40.25±3.12^{c}	**50.07** ^{A}
	Heart	14.25±0.34^{a}	10.38±0.37^{b}	7.95±0.41^{c}	**11.36** ^{C}
	Mean	**26.48** ^{A}	**19.94** ^{B}	**16.50** ^{C}	
ALT	Liver	14.21±0.28^{a}	11.35±0.34^{b}	9.64±0.22^{c}	**12.23** ^{B}
	Kidney	6.34±0.31^{a}	5.21±0.27^{b}	3.56±0.22^{c}	**5.09** ^{D}
	Muscle	54.26±3.10^{a}	50.12±3.24^{b}	46.4±2.01^{c}	**51.81** ^{A}
	Heart	9.12±0.41^{a}	7.68±0.35^{b}	5.32±0.31^{c}	**8.43** ^{C}
	Mean	**21.88** ^{A}	**19.42** ^{B}	**16.87** ^{C}	

5.3.6.2 Alkaline Phosphatase (ALP) and Acid Phosphatase (ACP)

Both ALP and ACP have a very significant role in immunity. ALP and ACP activities were measured in the liver, muscle, kidney and heart tissue of experimental rats. The results are presented in Table 5.3. Results showed that dietary supplementation of sardine oil loaded microparticles decreased the activity of ALP and ACP in liver, muscle, kidney and heart tissue. Higher activity of ACP was observed in muscle whereas higher activity of ALP was observed in kidney. Both these enzymes are associated with different diseased condition. Increased level of ACP activity is mainly associated with diseased conditions like carcinoma, and some skin disorders (Hillmann, 1971). Mohapatra *et al.*, 2012 observed decreased amount of ALP and ACP in the fish fed with probiotics and that is correlated with improved immune system.

Table.5.3 Activity of dietary supplementation of sardine oil loaded vanillic acid grafted chitosan microparticles on ACP and ALP activity in muscle, liver, kidney and heart tissues of albino rats**.** Different superscript (a, b, c, d) in the row indicate significant difference ($p<0.05$) between the control and treatment groups. Marginal means given in bold (A, B, C, D). Values are expressed as mean ± SE (n=6). **Units:** Mole of PNP released min-1 mg-1 protein (ACP) and Mole of PNP released min^{-1} mg^{-1} protein (ALP)

Response	Tissue	Treatments			
		Control	1% SO-VGC	3% SO-VGC	Mean
ACP	Liver	9.02±0.52^{a}	7.83±0.64^{b}	8.14±0.32^{b}	**8.50 ^{D}**
	Kidney	12.43±0.31^{a}	10.25±0.28^{b}	10.94±0.32^{b}	**11.28 ^{C}**
	Muscle	48.5±0.28^{a}	36.54±0.29^{b}	30.21±0.31^{c}	**38.89 ^{A}**
	Heart	18.61±0.51^{a}	11.26±0.58^{b}	9.32±0.49^{c}	**13.09 ^{B}**
	Mean	**22.03 ^{A}**	**16.91 ^{B}**	**14.88 ^{C}**	
ALP	Liver	8.42±0.18^{a}	6.12±0.22^{b}	5.92±0.24^{b}	**6.67 ^{C}**
	Kidney	29.3±0.29^{a}	26.40±0.31^{b}	22.5±0.33^{c}	**25.57 ^{A}**
	Muscle	4.27±0.21^{a}	3.46±0.22^{b}	3.05±0.21^{b}	**3.97 ^{D}**
	Heart	9.25±0.19^{a}	8.34±0.18^{b}	8.01±0.11^{b}	**8.65 ^{B}**
	Mean	**12.58 ^{A}**	**11.05 ^{B}**	**10.01 ^{C}**	

5.3.6.3 Superoxide Dismutase (SOD) and Catalase

SOD and catalase are the main antioxidant enzymes present in body. Its activity is increased in stressed condition. SOD and catalase activities in liver, kidney and muscle of male rats are shown in Table 5.4. SOD activity in liver tissue showed significant ($p<0.05$) difference between control and experimental groups, but a significant difference in SOD activity was not observed in muscle, kidney and heart tissue. Among the tissues, relatively higher SOD activity was recorded in the liver followed by kidney, muscle and heart. Detoxification of many chemical compounds occurs in liver and that may be the reason for high SOD activity in liver. The catalase activity in liver, kidney, muscle and heart tissue showed significant ($p<0.05$) decrease between control and treated groups. Higher catalase activity was observed in kidney. In the biological system, both SOD and catalase protect the cell from free radical damage generated during oxidation metabolism. The antioxidant enzyme level is highly associated with stress. SOD and catalase activity is high when the body is under stress. Previous studies proved that feeding of some probiotics or nutraceutical antioxidant compounds will lower the level of SOD and catalase (Shahbaz *et al.,* 2010; Mohapatra *et al.,* 2012; Tejpal *et al.,* 2017). From the above findings it can be concluded that supplementation of sardine oil loaded microparticles will help to maintain the integrity of self-defense mechanism and act as an antioxidant compound against various oxidants

5.3.6.4 Gluthathione-S-transferase (GST)

Glutathione-S-transferase (GST) is the xenobiotic metabolizing isozyme and involved in many detoxification processes. Activity of GST in liver, kidney, muscle and heart of experimental rats was analyzed and the results are presented in Table 5.4. The action of Glutathione-S-transferase is mainly by preventing the interaction of endo and exo toxic substances with crucial cellular proteins and nucleic acids. Also it catalyses the conjugation of GSH with a sulfhydryl groups in order to make the compounds more water soluble. This activity detoxifies the unknown compounds. The expression of this enzyme varies with tissue. In the

present study, the activity of GST decreased in kidney, liver, muscle and heart tissue of rats in the sardine oil microparticles treated groups. The previous studies already showed the antioxidant properties of chitosan and vanillic acid (Rice-Evans *et al.,* 1996; Xie *et al.,* 2001). Our results is very good accordance with the findings of Stone *et al.*, 1980. He reported that the dietary supplementation of selenium lowered GST activity. Hence, the present study reveals that supplementation of sardine oil-loaded vanillic acid-grafted chitosan in the diet improves the GST activity in rats.

Table 5.4 Impact of dietary supplementation of sardine oil loaded vanillic acid grafted chitosan microparticles on SOD, Catalase and GST activity in muscle, liver, kidney and heart tissues of albino rats. Different superscript (a, b, c) in the row indicate significant difference ($p<0.05$) between the control and treatment groups. Values are expressed as mean ± SE (n=6). Units: milllimols H_2O_2 decomposed min^{-1} mg^{-1} protein at 37 ºC (Catalase); 50 % inhibition of epinephrine auto oxidation mg^{-1} protein min^{-1} (SOD) and µ moles of CDNB-GSH conjugate formed min^{-1} mg^{-1} protein (GST).

Response	Tissue	Treatments		
		Control	1% SO-VGC	3% SO-VGC
SOD	Liver	138.47±0.44[a]	129.04±0.58[b]	120.58±0.49[c]
	Kidney	86.41±0.38[a]	80.18±0.52[b]	78.57±0.54[b]
	Muscle	49.21±0.77[a]	44.27±0.61[b]	41.51±0.68[c]
	Heart	32.85±0.08[a]	30.05±1.30[b]	28.06±0.94[b]
Catalase	Liver	30.01±0.82[a]	26.82±0.59[b]	23.51±0.71[c]
	Kidney	60.24±3.21[a]	58.54±4.2[b]	56.30±3.3[b]
	Muscle	19.01±0.21[a]	14.26±0.27[b]	10.04±0.22[c]
	Heart	22.89±1.21[a]	20.04±1.30[b]	18.95±1.14[c]
GST	Liver	1.89±0.02[a]	1.34±0.07[b]	1.21±0.02[b]
	Kidney	0.28±0.01[a]	0.21±0.03[a]	0.17±0.02[a]
	Muscle	0.31±0.07[a]	0.28±0.05[a]	0.21±0.02[a]
	Heart	0.24±0.07[a]	0.22±0.08[a]	0.16±0.04[b]

5.4 Conclusion

Vanillic acid grafted chitosan can be used as a better encapsulant for fish oil and it will offer better oxidative stability for further incorporation in food. However, it is important to evaluate novel dietary supplements in experimental animal model to understand the mechanisms of action. The present study was to evaluate the nutritional benefits of developed sardine oil loaded vanillic acid-grafted chitosan microparticles using Wistar albino strain rats. Study revealed that the dietary supplementation of sardine oil loaded vanillic acid-grafted chitosan microparticles effectively boosted the growth performance, metabolic and immune responses in rats. The beneficial effect was found to be dose dependent. So the developed microparticles will find its application as a potential health promoting compound or as a food ingredient.

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Chapter 6

Co-encapsulation of PUFA through water in oil in water (w/o/w) multiple emulsification with chitosan-whey protein as wall material

6.1 Introduction

6.2 Materials and Methods

6.3 Results and Discussion

6.4 Conclusion

6.1 Introduction

There is a growing demand for polyunsaturated fatty acids, particularly long chain ω-3 fatty acids. The addition of ω-3 polyunsaturated fatty acids to functional food ingredients and their consumption in dietary supplements have experienced significant increases (Sanguansri & Augustin, 2006). These omega-3 polyunsaturated fatty acids have been associated with a variety of health associated benefits, such as reducing the risk of cardiovascular diseases, hypertension, arthritis, and several immune response disorders (Rubio-Rodriguez *et al.,* 2010). However, omega-3 PUFA are susceptible to oxidation due to their high rate of unsaturation a process which involves the formation of off-flavor compounds (Kolanowski & Wei Brodt, 2007) as well as toxic products like peroxides and hydroperoxides (Guillen & Ruiz, 2005). Microencapsulation technology is mainly used to protect sensitive compounds from adverse effects caused by light, moisture, and oxygen and enable its controlled release (Desai & Park, 2005). The process converts the PUFA into a free flowing powder which can be easily handled and can be used for food application. Microencapsulation can be defined as a process in which tiny droplets of sensitive compounds are surrounded

by a coating of a micro encapsulating agent. This wall material can be made of a wide variety of food grade materials and protects the sensitive compounds by providing a strong barrier against adverse environmental conditions. Spray-drying is one of the most common methods used for micro encapsulation of oils due to its low cost and efficiency (Gharsallaoui *et al.,* 2007). The wall material can be made of variety of food grade materials like gum arabic, whey protein isolate, chitosan and pectin (Bertolini *et al.,* 2001; Fang *et al.,* 2005; Krishnan *et al.,* 2005). Proteins and polysaccharides are mainly used as wall material for oil emulsions for increasing the oxidative stability of oil-in-water emulsions (Hu *et al.,* 2003). Protein-polysaccharide complexes have better physical and oxidative stability than those coated with proteins alone because of the difference in interfacial charge, structure, and thickness of layers (Guzey & McClements, 2007). Whey proteins prevent the droplets from coming close enough to aggregate (Sun *et al.,* 2007). They stabilize the formed droplets from aggregation by increasing the repulsive interactions between the droplets (Surh *et al.,* 2006). Whey proteins also inhibit lipid oxidation in emulsions when they are at the surface of the emulsion droplets (Hu *et al.,* 2003). Chitosan, which is the deacetylated form of chitin is being reported to have many biopharmaceutical characteristics such as pH sensitivity, biocompatibility and low toxicity (Hirano *et al.,* 1989). Moreover, it is a biodegradable material as it can be metabolized by certain human enzymes like lysozyme (Muzzarelli, 1997). It is also being widely used as a wall material for encapsulation because of its emulsification property. Taking into consideration of these properties of chitosan, it has been used for encapsulation technique and in drug delivery in the recent past. In this chapter it is aimed to investigate the co-delivery of betalain and PUFA using water in oil in water (w/o/w) multiple emulsification process with chitosan-whey protein complex for high efficiency and an increased protection from oxidation.

6.2 Materials and Methods

6.2.1 Materials

Polyunsaturated fatty acid (PUFA) was obtained from Arjuna Chemicals, Ernakulam, Kerala, India and was stored in dark amber coloured-glass bottles at -20° C, until use. Chitosan (MW = 100 kDa, 88 % degree of deacetylation) was prepared in the pilot plant facility of Central Institute of Fisheries Technology and the source is shrimp shell, Cochin, Kerala, India. Whey protein and all the other chemicals used in the study were of analytical grade and were obtained from Sigma-Aldrich (USA).

6.2.2 Preparation of emulsions

A water in- oil- in- water emulsion was prepared using whey protein isolate (WPI) and chitosan as the wall material and polyunsaturated fatty acids (PUFA) as the core material. The W1 (water phase) was prepared by mixing 8.16 ml betalain extract (contains 10mg/ml betalain) mixed with 31.84 ml of solution containing 0.1% Acetic acid, 2% NaCl, 3% Gelatin, 0.2% Ascorbic acid. The primary (W/O) emulsion was prepared by mixing 2.5 ml of W1 and 7.5ml of PUFA. 5% PGPR (Polyglycero polyricinoleic acid) added to this solution as an emulsifier. After preparing primary emulsion, it was subjected to homogenization at a speed of 6, 000 rpm for 10 minutes using a high speed homogenizer. Final emulsion (W/O/W) was prepared by mixing 10 ml of primary emulsion and 40ml of outer W2 phase contains 10% WPI (in acetate buffer p^H 5.4) and 1% chitosan (in acetate buffer p^H 4). The emulsion prepared was then transferred to 25 ml graduated test tubes, sealed and kept in the dark for further studies.

6.2.3 Microstructure of emulsion

The microstructure of emulsion was determined using optical microscope (Leica ICC50 HD) and observed at an objective magnification of 40X. An image of the emulsion was acquired using digital image processing software (image-pro plus^{Tm}, version6).

6.2.4 Emulsion rheology

The rheology of the emulsions prepared was studied using a DV Brookfield viscometer (cone plate model).all the measurements were done at a temperature of 25^0C. The spindle used was of CP-41 model. For measuring the rheology, about 2 ml of the emulsion sample was taken and loaded onto the rheometer. The viscosity of emulsion was measured using a steady state flow program and the flow curves were obtained by plotting the shear stress against shear rate. The data thus obtained were fitted to power law and Bingham model to study the emulsion rheology.

6.2.5 Particle size and Zeta potential of the emulsions

The particle size and the zeta potential of the emulsions were measured by taking the Dynamic light scattering (DLS) and zeta potential measurements using the zeta sizer (DLS-ZP/Particle SizerNicompTM 380 ZLS). The reading was taken in triplicates.

6.2.6 Determination of emulsion stability

Emulsion stability of eight different oil emulsions was determined according to the method of Klinkesorn *et al.,* 2004. Immediately after the emulsion preparation, 25ml of emulsion was placed in a test tube, sealed and stored at room temperature for one day. The evaluation was determined by the depths (cm unit) of a distinctive clear serum lower phase. The stability was compared by the % of separation and expressed as Creaming Index (%) of total emulsion height in the tubes (Keogh & O'Kennedy, 1998).

$$\text{Creaming Index (CI)} = 100\times \frac{\text{The height of formed cream layer}}{\text{Total height of emulsion}}.$$

6.2.7 Spray drying

The homogenized emulsions were then converted into powder form using a spray dryer (S M Scientech, Kolkata). The emulsion was fed by a peristaltic pump at a fixed flow rate (20 ml/min). The dryer was operated at an air inlet temperature of 180^o C ($\pm 2^o$ C) and outlet temperature of 90^0C. The powder was

then kept in amber colored bottle and stored at cool temperature for further characterization.

6.2.8 Microcapsule characterization

6.2.8.1 Moisture content

The moisture content of micro encapsulated oil was determined gravimetrically (AOAC, 2000)

$$\% \text{ of moisture} = \frac{W1-WD}{W1} \times 100$$

6.2.8.2 Hygroscopicity

Hygroscopicity is the measurement of ability of a material to absorb or release water as a function of humidity i.e. water activity. Hygroscopicity was determined according to the method proposed by Cai & Corke (2000). The encapsulated oil sample were (1g) placed in a container with saturated NaCl solution at 25^0C. After one week the samples were weighed and hygroscopicity was expressed as g of adsorbed moisture per 100g dry solids.

6.2.8.3 Bulk density and tap density

1g of encapsulated powder was added into the 50ml tarred graduated cylinder and measure the height of powder. This volume was then used to calculate the bulk density according to the equation:

$$\text{Density} = \frac{M}{V}.$$

For tapped density 1g powder was added into the 50 ml tarred graduated cylinder and the samples were repeatedly tapped manually by lifting and dropping the cylinder under its own weight at a vertical distance of 5cm until negligible difference in volume between succeeding measurements was observed. The tapped density was computed as:

$$\text{Tapped density} = \frac{M}{TV},$$

TV is the tapped volume.

6.2.8.4 Solubility

The powder was weighed (1g) and stirred in 25ml of distilled water for 5 minute using a blender. The solution was then centrifuged at 4000 rpm for 15 minute. An aliquot of 2ml was taken in petridish and oven dried at 105^{o}C overnight. Solubility % was calculatated as the percentage of dried supernatant in reaction to the amount of powder originally added.

6.2.8.5 Surface oil content of the microcapsule

The surface oil also known as free oil fraction or non-encapsulated oil fraction was determined according to Sankarikutty *et al.,* (1988). About 200 ml of n-hexane was added to 2 g of the spray dried powder, followed by stirring at room temperature for 15 minutes. This was then filtered through Whatman no: 1 filter paper and the solvent collected was evaporated in a rotary evaporator. The oil thus extracted was dried to a constant weight by flushing with a stream of nitrogen.

6.2.8.6 Total oil content of the microcapsule

The total oil content of the microcapsule was determined according to Li *et al.,* 2013. About 1g of the dried sample was placed in the cellulose thimble and was subjected to soxhlet extraction using 150 ml of petroleum ether for 5h. The oil thus extracted was concentrated using vacuum rotary evaporator and dried at 105^{0}C until constant weight.

6.2.8.7 Encapsulation efficiency

The encapsulation efficiency of encapsulated PUFA powder was determined by the method of Shen *et al.,* 2010) as follows:

Encapsulation efficiency = (TO-SO/TO)*100

Where,

TO– Total oil content

SO- Surface oil content

6.2.8.8 Wettability

Wettability was determined using the method of Fuchs & Schweizer, 2002 with some modification. The powder sample (100 mg) was sprinkled over the surface of 100ml of distilled water at 20^0C without agitation. The time it took until the last powder particles to submerge was recorded.

6.2.8.9 Water activity of encapsulated powder

The water activity of microcapsules was measured using an electric hygrometer (Novasina IC – 500 AW – LAB, Novasina). A sample dish was carefully filled halfway with microcapsules, Measurement was performed at room temperature and the results were recorded after the equilibrium was reached.

6.2.8.10 Color analysis

Color of the powder was determined using Hunter colorimeter (Hunter lab, Reston, VA, USA) (Hunter & Harold, 1987). The readings were made with illuminant D65, 10° standard observer and 1 mm viewing size. Before measurement the machine was calibrated against white and black tile. The tri stimulus L* a* b* measurement mode was used as it relates to the human eye response to colour. The L* variable represents lightness (L*=0 for black, L*=100 for white), a* scale represents the red/green (+a* intensity in red and –a* intensity in green) and the b* scale represents the yellow/blue (+b* intensity in yellow and –b* intensity in blue). The value was measured in triplicates and the mean value was taken.

6.2.8.11 Morphology of microcapsules by scanning electron microscopy (SEM)

The surface morphology of the particle was studied with the help of scanning electron microscopy (SEM) (JEOL Model JSM - 6390LV).

6.2.8.12 Peroxide value and Accelerated rancimat test

The primary oxidation product (peroxides) was determined using the method of Kolanowski *et al.,* (2007). About 30ml of 2:1 methanol/chloroform (v/v) solution was added into 2g of sample taken in a 250ml Erlenmeyer flask, followed by the

addition of 5ml saturated NaCl. After vigorous shaking to destroy the encapsulants, it was kept for 2 hours separation. The oil dissolved in the chloroform was collected separately and the solvent was evaporated in a vacuum evaporator. Fifteen milliliters of acetic acid-chloroform was added into the extracted oil and was stirred well. 5ml of saturated potassium iodide (KI) solution was added and the solution was kept in dark for 30 minutes. To this, about 30 ml of distilled water was added and titrated with 0.01N sodium thiosulphate ($Na_2S_2O_3$) until the yellow color of the solution disappeared. The titration was continued after the addition of starch indicator to the solution until the blue colour disappears. A blank was run simultaneously. PV was calculated as:

$$PV = (S-B) \times N \times 1000/W$$

Where, S is the volume of $Na_2S_2O_3$ added to the sample, B is the volume of $Na_2S_2O_3$ of the blank; N is the normality of $Na_2S_2O_3$ solution, and W is the sample weight (g).

The accelerated rancimat test was conducted to determine the stability of PUFA to oxidation after the process of microencapsulation. The oxidative stability of encapsulated PUFA was measured as per method described by Gallardo *et al.*, (2013) using rancimat apparatus (Metrohm, Herisau, Switzerland). Briefly, 5 g of microencapsulated powder was taken in each reaction vessel and pure PUFA (5g) was used as control. Induction period values were then automatically deducted and registered. Analyses were performed in triplicates to avoid the occurrence of error.

6.2.8.13 Statistical analysis

All the experiments were performed in triplicates and the data were expressed as mean ± SD.

6.3 Results and Discussion

6.3.1 Emulsion stability of PUFA

Microencapsulation of PUFA by multiple emulsification-spray drying technique was carried out for the stabilization of PUFA. In this present study, we

aimed to investigate the co-delivery of betalain and PUFA in a water in oil in water (w/o/w) multiple emulsion and spray dried microencapsulate thereof. The ability of chitosan-whey protein as a wall material to encapsulate PUFA was also assessed in this study. The emulsion stability (Percentage) was observed in the treatment prepared was 82%. The amount and nature of wall materials are very important for the preparation of a stable emulsion. Wall materials play an important role in preserving the integrity and stability of emulsions (Mcclements *et al.,* 2007). If the core material to wall material ratio is less, there will be sufficient wall material to cover the core compound completely. This might be the reason for higher emulsion stability in this treatment. Similar findings were also reported in case of microencapsulation of fish oil, flax seed oil, linoleic acid etc. (Tan *et al.,* 2005; Tonon *et al.,* 2011) Hence, we concluded that the multiple emulsion prepared with chitosan-whey protein was more stable.

6.3.2 The microstructure of the emulsion

The microstructure of emulsion was determined using optical microscope (Leica ICC50 HD). An image of the emulsion was acquired using digital image processing software (image-pro plus Tm, version 6). The microstructure of the PUFA emulsion is shown in Fig.6.1 It is clear from the microscopic image that, one particle was surrounded by another one and also there was less evidence of aggregation and the oil droplets were seemed to be separated from each other. It exhibited higher stability by generating more electrostatic repulsion and steric hindrance among the oil droplets and formed lesser flocculation and aggregation. Apart from this, chitosan-whey protein could have possibly adsorbed to the oil-water interface to form a protective coating around the droplets, reduced the interfacial tension and thereby prevented the oil droplets from aggregation (Whitehurst, 2008). Here, chitosan might have acted both as an emulsifier and stabilizer. The Figure 6.1 presents the microstructure of emulsion (100x).

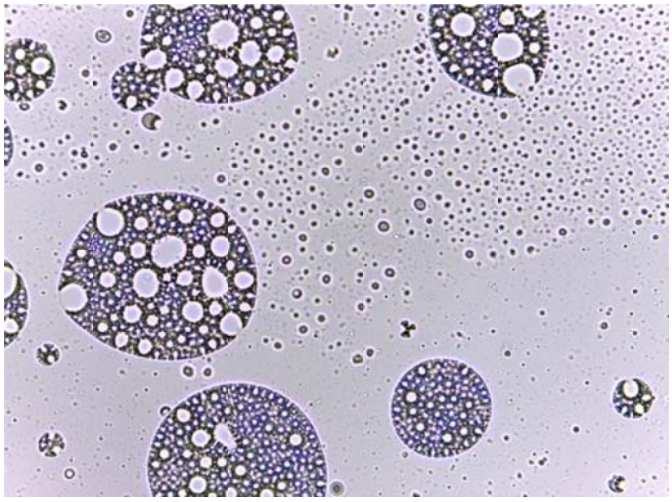

Fig. 6.1 Microstructure of PUFA multiple emulsion

6.3.3 The rheological properties of the emulsions

The viscosity of the emulsion prepared with chitosan-whey protein wall material was determined. The viscosity of the prepared emulsion was 37.21cP. There is a correlation between core material and wall materials used in the emulsion preparation. If the core to wall material ratio was decreased, there was a considerable increase in the viscosity (Kumar *et al.,* 2017). The higher viscosity will reduce the rate at which particles sediment or cream or coalescence and thereby we can increase the emulsion stability (Mcclements *et al.,* 2007). The flow properties of the emulsion were determined with the help of power law model. From the results, it is observed that as the shear rate increased, there was a considerable increase in the emulsion viscosity (Fig. 6.2). This shows that the emulsions had a shear thickening effect. Hence, it was concluded that the PUFA multiple emulsion prepared with chitosan and whey protein had a shear thickening behavior.

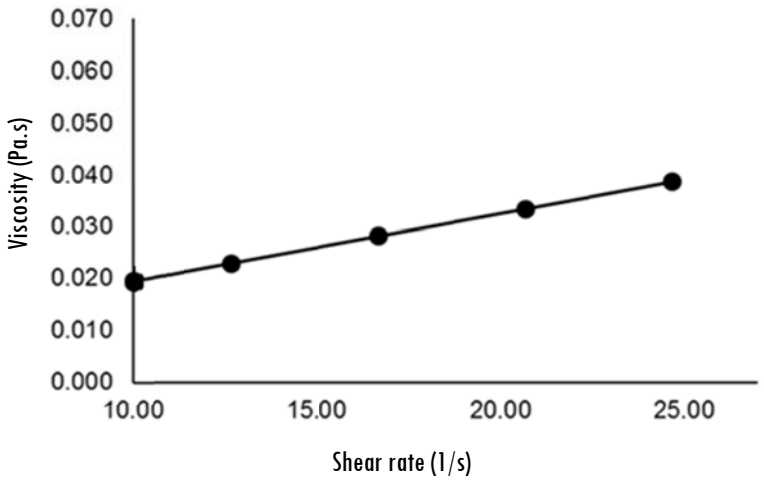

Fig.6.2 Power law model of PUFA multiple emulsion

6.3.4 Particle size and zeta potential

In our study the zeta potential of the particle obtained -24.1mv.The emulsion remained in a stable form. Zeta potential had a lower negative charge and thus remained stable .The zeta deviation recorded was 5.17mv (Fig 6.3). The droplet size distribution by number of the emulsion containing 1% chitosan and 10% whey protein was found to be 982 nm.

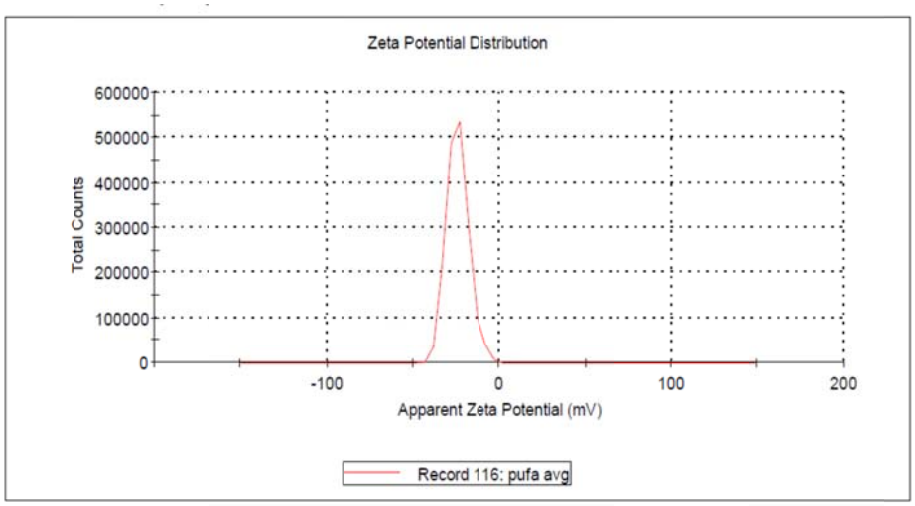

Fig 6.3 Zeta potential of PUFA multiple emulsion

The result is in very good accordance with the study of Luo *et al*., 2011 who have reported that when chitosan was used to coat the fish oil, the zeta potential was

in the range of ±20 to ±40 mV. The zeta-potential of the stable emulsion is always lies between ±20 to ±40 mV. (Jayme *et al.*, 1999).

6.3.5 Microparticle Characterization

6.3.5.1 Moisture

Moisture is an important parameter mainly used to evaluate the shelf life of encapsulated powders. The powder weight loss percentage after oven drying at 105^0C until a constant weight was obtained and moisture content (%) was calculated. High moisture content will make the powder sticky, it will result in the aggregation of the microcapsules and the subsequent agglutination and oxidation of the encapsulated powders, the moisture of the powders varied between 0.17% and 3.87. Literatures showed that low moisture content and water activity will prevent the release of encapsulated oil during storage, thereby prevents lipid oxidation (Velasco *et al.*, 2003). According to Drusch & Schwarz (2006) fish oil microparticles with moisture content of less than 3% can provide better stability.

6.3.5.2 Hygroscopicity

Hygroscopicity is the capacity of powders to absorb moisture in a relatively moist environment. Absorption of water is a critical factor as its presence may influence the lipid oxidation process and the loss of flavoring compounds. Value obtained was 13.07%. Similar values of hygroscopicity in the range of 15.87 to 18.90% have been reported in recent literature for rosemary oil encapsulates using gum arabic as wall material (Fernandes *et al.*, 2013).

6.3.5.3 Bulk density and tap density

Tapped & bulk density is an important factor related to packaging and commercializing powders. A dry product with high density can be stored in smaller containers than a similar product with low density (Quispe- Condori *et al.*, 2011). Tapped & bulk density correlated with wall material concentration, which provided a negative effect and consequently a value where this density is maximum. However, the model did not present a good fit for the data variation. The result obtained varied between 0.66 and 1g ml^{-1}. Kagami *et al.*, 2003 found

value of 0.35 – 0.41 gml^{-1} and Jinapong *et al.,* 2008 obtained measurement from 0.30 to 0.35 g ml^{-1} for the tapped densities of fish oil and soymilk, respectively. There was a tendency for tapped density to increase at higher wall material concentrations or with a decrease of the aqueous phase in the feed emulsion.

6.3.5.4 Encapsulation efficiency and solubility

Microencapsulation efficiency of encapsulated powder was defined by measuring the total oil and surface oil contents of the powder. The encapsulation efficiency of powder was found to be 87.73 ± 0.21 %. The loading efficiency of encapsulated powder was found to be 67%. The higher encapsulation efficiency indicated the presence of lower proportion of surface oil content in the encapsulated powder. The higher encapsulation efficiency can be due to the fact that 1% chitosan and 10% whey protein is sufficient to act as a wall material for PUFA encapsulation. Previous studies showed that the MEE (Microencapsulation efficiency) could be affected by coatings and the method of microencapsulation (McClements, 2007*).* It was found that encapsulation efficiency is very high and it indicates that the entire encapsulation process has succeeded. Powders used as ingredients in the industries should provide good solubility. Solubility is the last step of particle dissolution and is considered to be an important factor in the quality of the powder. (Jayasundera *et al.,* 2011).

6.3.5.5 Wettability

Wettability is one of the handling properties associated with flowability. Wettability is defined as the ability of bulk powder to absorb liquid under the influence of capillary forces. Wettability depends on particle size, density, porosity, surface charge, surface area, and surface activity of powder (Vega & Roos, 2006*)*. In the present study, the times obtained for the powders to become completely wet varied from 10 sec. The concentration of wall and encapsulated material influenced the values of this property. The highest instantiation time of the particles occurred with high concentrations of oil and wall material. The present study produced a range of values for the concentration of the encapsulated materials in which the wettability is minimum with an increase in oil

concentration and because of the hydrophobic nature of the microcapsules; particle instantiation time was higher which is a function of lower affinity in relation to water. The concentration of wall material likely increased at higher concentrations, masking the powder more compressed and thus hindering water penetration into the particle. The composition of the surface plays an important part in the powder reconstitution process. In general, the presence of hydrophobic components on the surface results in poor wettability.

6.3.5.6 Water activity

The final effect of drying a product is to lower moisture content along with water activity (Barbosa & Vega, 1996). The moisture content (1%) and water activity (0.211) of spray dried emulsion powders decreased with increasing air inlet temperature from 165 to 180^0c. These results are constant with the observation of Barbosa & Vega, 1996, who found that the moisture content of spray dried products was highest when operated at lowest temperature. The maximum moisture specification for most dried powders in the food industry is between 3% and 4%. In the present study a lower moisture content was attained; may be due to the operation of high inlet temperature (180^o C)

6.3.5.7 Color and surface morphology

Color of powders is another important criterion that shows the aesthetic appeal of the product and its acceptability. Lightness of the powder showed that it is having almost a whitish yellow color with an L* value of 80.12 ± 0.22 and b* value of 12.49 ± 0.19. The whitish yellow color of encapsulated microcapsule can be due to chitosan. This result shows the developed powder is having an acceptable color for the consumer acceptance. SEM (Scanning electron microscopic) images of PUFA loaded micro particles showed smooth spherical morphology (Fig 6.4) with a few small pores on the smooth surface. Similar smooth morphology of oil loaded chitosan microspheres has been noted in most of the literature (Zhang & Zhang, 2002; Duman & Kaya, 2016). From the literature it is clear that chitosan when used in combination with other polysaccharide and protein result in better surface morphology of oil loaded micro particles (Dima *et al.*, 2016).

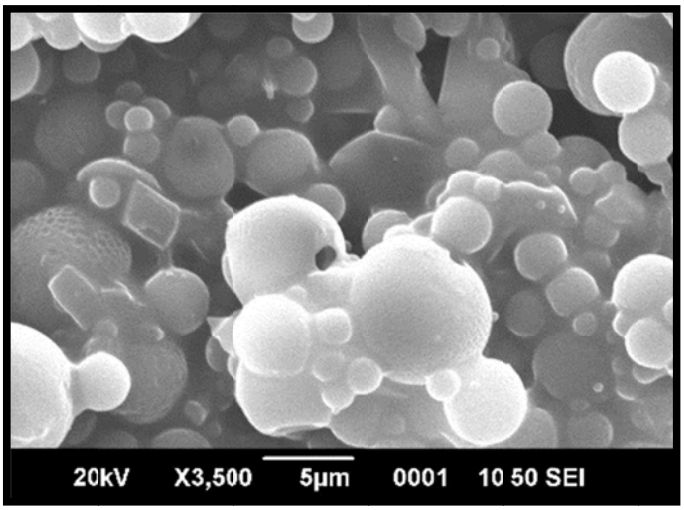

Fig 6.4 SEM image of PUFA microparticles

6.3.5.8 Peroxide value and Accelerated rancimat

Initial peroxide value of PUFA was determined as 2.00±0.08 meq/kg which increased to 2.8 ±0.15 meq/kg in freshly prepared spray dried powder. This slight increase of peroxide value is mainly because of air exposure and high temperature during spray drying (Klinkeseron *et al.,* 2004). PV of un-encapsulated PUFA increased rapidly during storage and reached up to 56.6±1.3 meq/kg oil after 6 weeks of storage. In that same period PV of microencapsulated PUFA increased slowly from 2.8 ±0.15 meq/kg to 9.2±0.31 meq/kg oil (Fig. 6.5). PV of less than ten has been considered optimum (Anwar & Kunz, 2011). The excellent wall material nature of chitosan whey protein complex clearly explains the lower PV of microencapsulated PUFA in the present study. This can also be correlated with the good encapsulation efficiency and loading efficiency achieved in the reported process. The results of accelerated rancimat showed encapsulated PUFA was stable compared to PUFA. The Induction Point (IP) value indicates the time required to produce a sudden increase of conductivity, which can be defined as an indirect measure of oil stability. Freshly procured PUFA presented an IP of 0.57±0.01h which is comparable to the value reported for fish oil (0.75h) (Gallardo *et al.,* 2013). Whereas freshly prepared microencapsulated PUFA showed IP value of 5.37±0.03 h (Table 6.1). IP values obtained for microcapsules

clearly show a protective effect of the chitosan whey protein wall material against PUFA peroxidation.

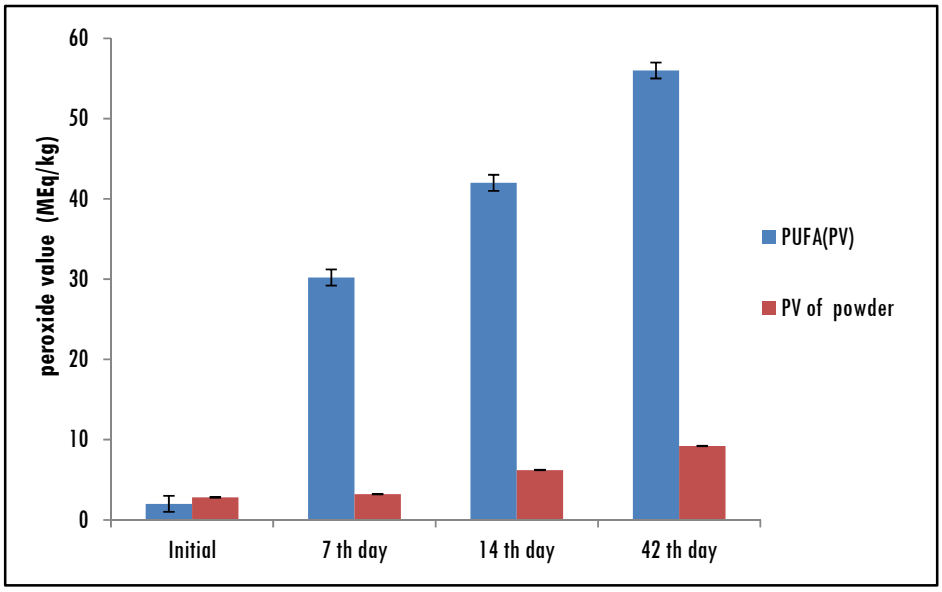

Fig. 6.5 Peroxide value of PUFA and encapsulated PUFA

Table 6.1 Accelerated rancimat results of PUFA and PUFA encapsulate

Formulation	Induction Point (IP) in hr
PUFA	0.57±0.06
Encapsulated PUFA	5.37±0.03

6.4 Conclusion

Based on the present study that focused on the co-delivery of betalain and PUFA by multiple emulsification and spraydrying, the microcapsules obtained were subjected to physico-chemical characterizations like microencapsulation efficiency, solubility, peroxide value, powder water activity and bulk & tap density and were found to be highly stable and effective as a food supplement. The betalain formed the inner aqueous phase, PUFA formed the oil phase and chitosan-whey protein emulsifier combination formed the outer aqueous phase in a two-step emulsification procedure. The encapsulation efficiency of PUFA was observed to be 87%. The oxidative stability of PUFA was observed higher in this study than the normal recorded values because the PUFA was encapsulated and

therein enriched by betalain. The antioxidant property of betalain also added to the higher stability of encapsulated PUFA. Thus the resulting highly stable spray dried powder is rich in omega-3 and betalain and on further food fortification of this encapsulated powder yield functional foods enriched in omega-3 and betalain.

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Chapter 7

Summary & Conclusion

Fish oil undoubtedly remains as one of the excellent dietary sources of polyunsaturated fatty acids (LC-PUFA) such as EPA and DHA. This itself made fish oil as a suitable candidate in food fortification. The health benefits of LC-PUFA have been well documented by researchers all over the world. Some of them include its ameliorative effects on psoriasis, bowel diseases, inflammation, cardiovascular diseases, mental illnesses, asthma, developmental coordination disorders, movement disorders, obesity etc. The subsequent increase in awareness of the health benefits associated with the consumption of fish oil fostered its demand among the general public. However, it posed certain serious issues such as poor insolubility in water and most importantly its susceptibility to oxidation. Prevention of fish oil from oxidation has thus become highly indispensable for allowing its direct incorporation into foods has become a major concern to the food industry. Apart from this, if included directly in a food product, it will undergo oxidation and may affect the sensory acceptance of the final product. Hence, certain intervention strategies have to be adopted to preserve the integrity of fish oil and to increase its overall sensory acceptance when added into a food system.

Microencapsulation is one of the promising technologies to protect fish oil from oxidation and to make it more oxidatively stable. In this method, fish oil is packed into a wall matrix and thereby preventing its further interaction with pro-oxidants. The technology even helps in masking the off-flavor of fish oil and converts it into a free flowing powder for better handling and storage. There are several technologies available for the microencapsulation of sensitive compounds such as freeze drying, spray drying, coacervation (simple and complex), in situ polymerization, fluidized bed drying etc. Among this, the most commonly used microencapsulation technology is spray drying which is economical, flexible, and efficient and can produce microcapsule with low water activities. Apart from the

technology used, another important aspect that might affect the success of encapsulation is the wall material used. The major role of wall material is to protect the core material from oxidative deterioration and release it under the desired conditions. Hence the wall material has to be appropriately selected keeping in mind the properties of material to be protected. The most commonly used wall materials include gum arabic, maltodextrin, whey protein, guar gum, sodium caseinate, gelatin, chitosan, sodium alginate etc.

Chitosan, a naturally occurring and biocompatible polysaccharide, is finding wide applications in food as well as the pharmaceutical industries owing to its inherent properties such as antioxidant, antimicrobial, biodegradable, non-toxic and emulsifying nature. However, its application in the biomedical industries is often restricted due to insolubility in water, high viscosity and molecular weight. This has prompted researchers to work on the chemical modification of chitosan to improve its physico-chemical properties for widening its application in food industries. Vanillic acid which is the oxidized form of vanillin is found to have diverse pharmacological activities. It is also considered safe and licensed as a food additive. Till date, grafting of vanillic acid with the amino functionality of chitosan has not been reported and it is apparent that these chitosan derivatives will open up new applications in innovative functional food and nutrient delivery. In the present study, an attempt has been carried out to optimize the microencapsulation of omega 3 rich sardine oil using emulsification process with vanillic acid grafted chitosan. The fish oil powder thus developed was characterized in terms of encapsulation efficiency, oxidative stability, in-vitro digestibility, particle size, Z-potential etc. The sardine oil powder was found to be very much stable compared to the pure sardine oil. Accelerated rancimat test and peroxide value depicted the oxidative stability of the product. The shelf life stability study proved that it can be stored upto a period of 1year. Cardio protective benefits of the microparticles were also evaluated in the doxorubicin-induced cardiotoxicity in H9c2 cell lines. Results of MTT, Caspase-3 level, ROS potential, mitochondrial membrane potential and relative expression of NF-κB by PCR also confirmed the protective effect of encapsulated sardine oil against doxorubicin induced cardiotoxicity. The effect of

dietary supplementation of sardine oil loaded vanillic acid grafted chitosan on growth performance, metabolic and immune responses in wistar strain albino rats has also been carried out. Dietary supplementation of sardine oil powder seemed to significantly improve the anti-oxidant markers such as catalase, SOD and Glutathione system. There are no adverse effects on clinical parameters such as lipid profile, blood profile, ALT and AST after feeding with sardine oil powder. It was concluded that the supplementation of sardine oil loaded vanillic acid grafted chitosan favors the growth performance of rats. It has been observed that sardine oil loaded Va-g-Ch microparticles can offer a wide range of solutions such as the oxidative stability of PUFA present in the sardine oil and increased bioavailability which was also confirmed by in-vivo and in-vitro. The above results suggest the health importance of microencapsulated sardine oil powder and the beneficial effects can be attributed to the synergic effect of bioactive compounds present such as sardine oil, chitosan and vanillic acid. Microencapsulation of PUFA was attempted with various biopolymers, among which chitosan and whey protein was found to be the best wall.

Fish oil largely known for its medicinal use is being widely used as a nutrient in many food supplementations. This study point out the damage caused to body by oxidised fish oil because of the presence of high degree of unsaturation. High demand for fish oil resulted in the mushrooming of fish oil supplements in the markets with less quality due to oxidation or excessively added chemical anti-oxidants. Development of fish oil powder has emerged as a nascent technique to avoid oxidative damage with the aid of biofunctional natural wall material either conjugated or non-conjugated form. The fish oil powder developed with vanillic acid grafted chitosan was observed to be superior to the commonly available fish oil supplements due to the negligible oxidation and enhanced stability of PUFA. The cell line study has confirmed the cardio-protective action of PUFA. Fish oil powders possess an advantage as it can be consumed for variety of population because many of them don't like to consume fish oil as such. Added natural phenolics like vanillic acid will enhance the sensory acceptability of developed fish oil powder and can be used as an ideal nutrient supplementation

especially for infants and cardiac patients. We observed that fish oil powder product developed with vanillic acid grafted chitosan could be a promising, stable and safe dietary supplement for the growth and development of nutritionally deprived population

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