BIOPROSPECTING MARINE BIVALVE MOLLUSKS AND CEPHALOPODS FROM SOUTH WEST COAST OF INDIA FOR POTENTIAL BIOACTIVE MOLECULES

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By

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Certificate

This is to certify that this thesis entitled ***"BIOPROSPECTING MARINE BIVALVE MOLLUSKS AND CEPHALOPODS FROM SOUTH WEST COAST OF INDIA FOR POTENTIAL BIOACTIVE MOLECULES"*** *submitted by Ms. SELSA J CHAKKALAKAL, Junior Research Fellow of Marine Biotechnology Division of Central Marine Fisheries Research Institute, for the award of the degree of Doctor of Philosophy in Chemistry is the result of bonafide research work carried out by her in the Marine Biotechnology Division of Central Marine Fisheries Research Institute in Chemistry, Cochin-682018, under my guidance and direct supervision. I further certify that this thesis or part thereof has not previously formed the basis for the award of any degree, diploma, associateship of any other University or Institution.*

Kajal Chakraborty

Place: Cochin
Date: 17/11/2018

Declaration

*I do hereby declare that the thesis entitled "**Bioprospecting marine bivalve mollusks and cephalopods from southwest coast of India for potential bioactive molecules**" is an authentic record of research work carried out by me under the guidance and supervision of Dr. Kajal Chakraborty, Senior Scientist (Organic Chemistry), Marine Biotechnology Division, Central Marine Fisheries Research Institute, Cochin-682018 and the same has not previously formed the basis for the award of any degree or diploma.*

Whenever the work described is based on the findings of other researchers, due acknowledgement is made in keeping with the general practice of reporting scientific observations. However, errors and unintentional oversights, if any are regretted.

Selsa J Chakkalakal

Place: Cochin
Date: 17/11/2018

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Abstract

The phylum molluska represents one of the largest and most diverse groups of marine animals and are considered to be an important source to derive bioactive compounds. Mollusks contain rich nutrients that are beneficial to people of all ages. Large populations, particularly those living in coastal areas relied on these animals for a substantial portion of their diet and few reports available in the public domain deal with the traditional use of mussels against diseases. The consumption of mollusks as popular seafood has increased steadily over the past decades and extensive research efforts initiated to derive bioactive molecules that promote health in that field. Bivalve mollusks and cephalopods are widely used in different parts of the world for various studies, but only recently they have been recognized as potential sources for bioactive compounds.

Free radicals and other reactive species are constantly generated in the human body. Uncontrolled generation of free radicals is associated with lipid and protein peroxidation, resulting in cell structural damage, tissue injury, or gene mutation and ultimately led to the development of various health disorders. The roles of antioxidants are to neutralize the excess of free radicals, to protect the cells against their toxic effects, and to contribute to disease prevention. Antioxidant defense systems scavenge, and minimize the formation of, reactive oxygen species, but they are not fully effective. ROS mediated inflammation is treated mainly by non-steroidal anti-inflammatory drugs (NSAIDs) that exert anti-inflammatory actions by inhibiting cyclooxygenase and lipoxygenase enzymes. Therefore, in recent years, there are interests in finding naturally occurring antioxidant and anti-inflammatory compounds in food or medicine to replace synthetic products. Nutrient antioxidants belong to the exogenous antioxidants compounds, which cannot be produced in the body, and must be provided through foods or supplements. As mollusks live in the permanent pro-oxidant conditions, greater levels of antioxidant defenses are generally measured in these species.

Bivalves comprising one of the major marine fishery resources, and consumption of bivalve mollusks, particularly the south Malabar area has increased in the recent years in response to the higher availability under wild and cultured conditions. The cephalopods represent important economic seafood for human consumption due to their nutritional and market value, cephalopod aquaculture has also shown an increase during the past few years. Among the bivalves, *Perna viridis* and *Crassostrea madrasensis* constitute an important part of marine fishery and aquaculture at the south west coast of India and are the preferred food items among the people living in this area. *Octopus dolffusi* is predominant among the cephalopod species and therefore, these candidate species have been taken into account to isolate and characterize the bioactive molecules with reference to their antioxidant and anti-inflammatory potential. This work relates to the isolation and characterization of bioactive antioxidant and anti-inflammatory components of marine

mollusks, and further contemplated to develop enriched antioxidant and anti-inflammatory formulation(s) with individual or combination of natural ingredients with potential antioxidant properties to form a stabilized anti-inflammatory formulation.

The present study provides insights in different biochemical and fatty acid variation of three different mollusks collected from different geographical locations on the south-western coast of India. This work also explored the influence of the growth conditions on the essential nutritional compositions of the bivalves harvested from different sites in south western coast of India. It is apparent that spacial and growth conditions play a vital role in physiological mechanisms of bivalves, guiding fatty acid metabolism. High levels of PUFA including *n*-3 PUFAs and low levels of *n*-6 PUFA and relatively high *n*-3/*n*-6 PUFA ratio values characterized *P. viridis* and *C. madrasensis.* The nutrient profiling of octopus indicated that *O. dolffusi* collected south-west coasts during the post-monsoon season have superior quality with respect to their nutritional and fatty acid profile, especially as a protein source with low in fat content.

The natural environment has provided a wealth of chemically diverse, bioactive compounds that are responsible for over half of the medications currently available for a multitude of diseases. Extracts both aqueous and solvent fractions of different polarities were generated from these species and screened for the promising antioxidant and anti-inflammatory activities. The ethylacetate extracts of the mollusks were found to possess higher antioxidant and anti-inflammatory potential than other solvent extracts when the *in-vitro* and *in-vivo* antioxidant and anti-inflammatory properties were considered. These crude extracts were subjected to undergo repeated purifications with the aid of various chromatographic techniques and a total of ten compounds with potential antioxidant and anti-inflammatory activities were isolated from these mollusks. The chemical structures of the pure compounds, as well as their relative stereochemistries, were established by means of detailed spectroscopic experiments. Chromatographic separation of *P. viridis* extract led to the isolation of six new derivatives. The molecular structures of the purified compounds was proposed on the basis of comprehensive analysis of the ^{1}H NMR, ^{13}C NMR, including 2D-NMR experiments (^{1}H-^{1}H-COSY, HMQC, HMBC, and NOESY), and mass spectra. The compounds belonging to phenanthrenone derivatives, such as 3-hydroxy-13-vinyl-dodecahydro-11-phenanthrenone (1) and 4,4,9-trimethyl-13-vinyl-dodecahydro-2-phenanthrenone (2), chromene derivatives such as 11,20-dihydroxy-6,6-dimethyl-decahydro-5H-benzo[h]naphtho[1,2-c]chromene-16-carbaldehyde (3) and 16-acetyl-20-hydroxy-6,6-dimethyl-dodecahydro-5H-benzo[h]naphtho[1,2-c]chromen-12-one (4) and compounds such as cholest-5-en-3β-3-yl-(30-hydroxy-3-methyl-36-methyleneundeca-30E,34E-dienoate (5) and cholest-5-en-3β-3-yl-((E)-33-oxooct-31-enoate (6) were isolated from the chloroform partioned methanolic extract of *P. viridis*. These compounds were demonstrated to possess potential antioxidative anti-inflammatory properties. In the present study, it was observed that these derivatives can inhibit anti-inflammatory enzymes, and suggested that the compounds substantiate the wet lab

experiments. The ethylacetate-methanol extract of *Crassostrea madrasensis* was fractionated chromatographically to yield methyl-3-(26-phenylacetyloxy)-icosahydro-19-hydroxy-4,4,20-trimethylpicene-23-carboxylate (7) and methyl-3-(26-phenylacetyloxy)-icosahydro-1,19-dihydroxy-4,4,20-trimethylpicene-23-carboxylate (8). The ethylacetate-methanol extract of *Octopus dolffusi* yielded two pure compounds, namely, cholesta-5-en-3β-yl-(32-methyl-(30-((E)-34-amino-36-ethyl-39-oxohept-36-enyl)-pentanedioate (9) and 7-ethyl-9-vinyl-octahydroazuleno[1,8-bc]pyran-3,12-dione (10). The effective antioxidant and anti-inflammatory properties of these compounds indicated that they have potential as natural lead molecules in the food industry. The anti-inflammatory potential of these compounds are proved to be comparable to the synthetic anti-inflammatory agents, aspirin and indomethacin.

Marine-derived bioactive components have excellent potential as functional food ingredients as they possess advantageous physiological effects, with medicinal characteristics and added health benefits such as anticancer or anti-inflammatory activities. Considering the importance and easy availability of *P. viridis*, compared with other species, an anti-inflammatory concentrate from *P. viridis*, for use as nutraceuticals or functional food, in combating oxidative stress-induced and inflammatory diseases has been formulated. The harmful reactive oxygen species (ROS), which attack the biological macromolecules were arrested by natural antioxidants in different compositions of oleoresins (*Rosmarinus officinalis (ROO)* and *Curcuma longa(CLO)* and other natural antioxidant additives in different proportions were blended with mussel extract (FDPE) in different combinations and were subjected to accelerated shelf life study for a period of 90 days (d_{90}) to find their individual and synergistic effects, which were able to reduce the free radicals causing oxidation reactions thereby deteriorating the nutritional compositions of the mussel extract.

The present work involved the extraction and isolation of anti-inflammatory principles from *P. viridis.* Specifically the polysaccharides and anti-inflammatory lecithin along with glycolipoprotein and *n*-3 polyunsaturated fatty acids were isolated from this bivalve mollusk in an attempt to enrich the anti-inflammatory activity of the formulations. The oligosaccharide (**11**) characterized as di-(N-acetyl-β-D-mannosamine) -(1→4)-2-N-acetyl-β-D-galactosamine-(1→) - (4-N-acetyl p-phenoxyl) motifs. IR spectrum of (→4)-2, 4 Di-(N-acetyl-b-D-mannosamine) -(1→4)-2-N-acetyl-b-D-galactosamine-(1→) - (4-N-acetyl p-phenoxyl) unit was isolated from the aqueous extract of *P. viridis.* The down field-shifts in carbon signals of the sugar units compared to the native glucan might suggest the possible linkage information of the compound. The linkage information of the sugar units were further confirmed by NOESY and HMBC experiments. In the HMBC spectrum, the intra- and inter-residual connectivities of both anomeric protons and carbons of each of the glycosyl residues. The compound **12** contain C-28 carbon lysolecithin isolated from the chloroform-methanol extract of green mussel has been assigned to the choline and glycerol systems. Its mass spectrum exhibited a molecular ion peak at m/z 550

($C_{28}H_{57}NO_7P$). ^{1}H NMR in conjugation with ^{13}C NMR recorded the signature peaks of choline, glycerol and fatty acid portion of the lysolecithin. ^{13}C NMR spectra displayed three close peaks at δ 54.31(C-1), δ 54.21(C-2) and δ 53.56(C-3) that were attached to the nitrogen represented singlet at δ 3.19 (s) with an integral value of 9. The potential anti-inflammatory combination was enriched with bioactive components, isolated from *P. viridis* which was found to possess higher anti-inflammatory activity as compared to other mollusks extracts. A time-dependent accelerated shelf life studies were conducted in order to find the effects of different compositions of additives in the enriched anti-inflammatory composition. Significant *in vivo* activities also indicates better therapeutic profile and fewer side effects of the anti-inflammatory combination as compared to the synthetic non-steroidal anti-inflammatory drugs.

The anti-inflammatory combination formulated in this study, were found to be an effective alternative to the synthetic non-steroidal anti-inflammatory drugs used against inflammatory diseases in the mammalian model. The optimized procedure to prepare the stabilized anti-inflammatory combinations developed in the present study would contribute to the commercial application to produce the stabilized anti-inflammatory as nutraceuticals and dietary supplements.

Abbreviations

^{1}H NMR	-	Proton Nuclear Magnetic Resonance
$^1O_2$	-	Singlet Oxygen
1D-NMR	-	One Dimensional-NMR
2D-NMR	-	Two Dimensional-NMR
AA	-	Arachidonic Acid
ABTS	-	2, 2-Azino-Bis-3ethylbenzothiozoline-6-Sulfonic Acid
AcOH	-	Acetic Acid
AI	-	Atherogenicity Index
ALA	-	α-Linolenic acid
ANOVA	-	Analysis of Variance
ASTM	-	American Society for Testing and Materials
BHA	-	Butylated HydroxyAnisole
BSA	-	Bovine Serum Albumin
CAT	-	Carnitine AcylTransferase
CHD	-	Coronary Heart Disease
CNS	-	Central nervous system
COSY	-	Correlation Spectroscopy
COX	-	Cyclooxygenase
DCM	-	DichloroMethane
DEPT	-	Distortionless Enhancement By Polarization Transfer
DHA	-	Docosahexaenoic acid
DMSO	-	Dimethyl Sulfoxide
DPPH	-	2, 2'-Diphenyl-1-picryl hydrazil
TEAA	-	Total essential amino acid
EDTA	-	Ethylenediaminetetra Acetic Acid
EI-MS	-	Electron Ionisation Mass Spectra

EPA	-	Eicosapentaenoic acid
EtOAc	-	Ethyl Acetate
FAME	-	Fatty Acid Methyl Esters
FFA	-	Free Fatty Acid
FTIR	-	Fourier Transform Infra Red
GC-MS	-	Gas Chromatography-Mass Spectrometry
GAE	-	Gallic Acid Equivalence
GLC	-	Gas Liquid chromatography
H_2O_2	-	Hydrogen Peroxide
HETE	-	HydroxyEicosaTetraEnoic acid
HH	-	Hypocholesterolaemic/ hypercholesterolaemic
HMBC	-	Heteronuclear Multiple Bond Correlation
HPLC	-	High-Performance Liquid Chromatography
HRMS	-	High Resolution Mass Spectroscopy
HSQC	-	Heteronuclear Single Quantum Coherence
IC_{50}	-	Inhibition Concentration at 50 %
LC-PUFA	-	Long Chain Polyunsaturated Fatty Acids
LOX	-	Lipoxygenase
LU	-	Unit of Lipase
m/e or m/z	-	Mass-to-Charge Ratio
MAG	-	MonoAcyl Glyceride
MDA	-	Malondialdehyde/Malonaldehyde
MDAEQ	-	Malondialdehyde Equivalent Compounds
MDC	-	Dichloromethane
Me_2O	-	Acetone
MeOH	-	Methanol
MUFA	-	MonoUnsaturated Fatty Acid

NADP	-	Nicotinamide Adenine Dinucleotide Phosphate
NMR	-	Nuclear Magnetic Resonance
NOESY	-	Nuclear Overhauser Effect Spectroscopy
PCA	-	Principal Component Analysis
PDA	-	Photodiode Array
PGE_2	-	Prostaglandin E_2
PGF_2	-	Prostaglandin F_2
PGG_2	-	Prostaglandin G_2
ppm	-	Parts Per Million
PTC	-	Phenylthiocarbamyl
PUFA	-	PolyUnsaturated Fatty Acid
Rf	-	Retardation Factor
$RO^{\cdot}$	-	Alkoxyl Radical
$ROO^{\cdot}$	-	Peroxyl Radical
ROS	-	Reactive Oxygen Species
RP	-	HPLC - Reverse Phase High-Performance Liquid Chromatography
Rt	-	Retention Time
RT	-	Room Temperature
$R^{\cdot}$	-	Alkyl Radicals
SD	-	Standard Deviation
SFA	-	Saturated Fatty Acid
SFE	-	Supercritical Fluid Extraction
SPSS	-	Statistical Program for Social Sciences
TAA	-	Total amino acids
TAG	-	Triacylglycerol
TArAA	-	Total aromatic amino acids

TBA	-	Thiobarbituric Acid
TBARS	-	Thiobarbituric Acid Reactive Species
tBHQ	-	Tertbutylhydroquinone
TCA	-	Trichloroacetic Acid
TEAA	-	Total essential amino acids
TI	-	Thrombogenicity Index
TLC	-	Thin Layer Chromatography
TNEAA	-	Total non-essential amino acids
TNF-α	-	Tumor Necrosis Factor-α
TOTOX	-	Total Oxidation
TPC	-	Total Phenolic Content
TAG	-	TriAcylGlyceride
TSAA	-	Total Sulphur containing Amino Acid
UV-VIS	-	Ultra Violet-Visible

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Chapter 1

INTRODUCTION

Contents

1.1 Importance of mollusks

Marine organisms are considered as vast untapped resources of bioactive molecules having enormous pharmaceutical potential, which led to the growing interest in investigation of useful therapeutics. Among different marine organisms, mollusks are widely distributed, and have many representatives in the marine and estuarine ecosystems. The phylum Molluska comprises highly diverse group of invertebrates, with 50,000 to 1,20,000 reported species, and form the largest marine phylum, comprising about 23% of the named marine organisms (Chapman, 2009).The total annual production of mollusks has been estimated as 1.4 lakh tonnes, which is 10% of total marine fish landing (CMFRI, 2014). Cephalopods, bivalves and gastropods are the principal groups of mollusks exploited from coastal, estuarine and backwater environments. The annual landing of octopus, bivalves and gastropods were 9761, 6015 and 2005 tonnes, respectively during 2012 and constitute a valuable fishery resource in various sectors of coastal India (CMFRI, 2014). Mollusks have been used as highly palatable and nutritious food source for humans. Mollusks were also reported to be one of the important

sources to derive bioactive compounds that exhibit antitumor, antimicrobial, anti-inflammatory and antioxidant activities (Chellaram *et al*., 2009). Marine bivalves belong to the class Bivalvia of phylum Molluska, and are widely distributed in the coastal sea beds.

The commercially important bivalves along the Indian sea coasts are clams, mussels, edible oysters, and pearl oysters. The bivalves were exploited for shell, meat, industrial purposes and for the pearl. The edible bivalves became more popular and the average quantity of bivalve products exported per annum was 580 tonnes during the year 1995-1999. Clams and cockles form 73.8%, followed by oysters (12.5%), mussels (7.5%) and windowpane oysters (6.2%) (Appukuttan, 2008). They are good source of proteins and novel bioactive compounds. They are cultured to large numbers in coastal waters as a source of food, and for the identification of novel molecules.

Cephalopod mollusks constitute squid, cuttlefish, octopus, and are the most neurologically advanced of all invertebrates. Cephalopods make up only a small proportion (nearly 3 %) of the world capture fisheries landings, but there have been substantial increase during the last three decades. Cephalopods are landed in all the maritime states in India, and the state of Kerala ranked first among others, accounting for 37.70 % of the total cephalopod landings followed by Maharashtra (28.98 %) and Tamil Nadu (13.8 %) during 1992 (Appukuttan,2008). Cephalopods are generally carnivorous, highly versatile, and active predators at all stages of their life consuming mostly crustaceans, mollusks, and to a lesser extent other invertebrates or fish (Rodhouse and Nigmatullin, 1996). Due to their ecological importance, greater nutritional and market value, excellent palatability and increasing demand throughout different regions of the world, cephalopods have received considerable attention in recent years.

1.2 Marine environment as a potential source of bioactive compounds

Marine environment encompasses a massively complex ecosystem with diverse assemblage of life forms and extreme variations in pressure, salinity, thermal stress and exposure to ultraviolet radiation. The extreme environment can be stressful to the resident organisms, and are able to withstand up to certain limits. The increased stress can be highly toxic to aquatic organisms including mollusks, often resulting in oxidation of lipids in membranes, protein oxidation and DNA damage. The adaptation to these stress factors could involve efficient defense strategies that allow them to limit the harmful consequences of oxidative stress. Absence of oxidative damage in the stress-induced biochemical parameters suggested that their cells are equipped with powerful anti-stress secondary metabolites. These inhabitants are known to possess bioactive compounds, formed as a part of highly toxic defense mechanisms, which is a reflection of the highly competitive and solute environment in which the organisms reside. Moreover, considering its great taxonomic diversity, investigations related to the search of new bioactive compounds from the marine environment can be seen as an almost unlimited field.

1.3 Antioxidant and anti-inflammatory potentials to compact plurality of disorders

1.3.1 Free radicals as major causative agent of life threatening diseases

Free radicals are highly reactive molecules with one or more unpaired electrons, produced as part of normal cellular function, can cause damage to cell structures and consequently may adversely affect immune functions. Reactive oxygen species (ROS) is a collective term that includes all reactive forms of oxygen, both radical and non-radical species that are oxidizing agents or can be easily converted into radicals. The sources of ROS includes

endogenous free radicals, that are generated from immune cell activation, inflammation, mental stress, excessive exercise, ischemia, infection, cancer and aging. Endogenous antioxidant systems dysfunction is due to the low amount of non-enzymatic antioxidants such as gluthatione, vitamins A, C and E, reduced enzymatic activity. Exogenous ROS result from air and water pollution, cigarette smoke, alcohol, heavy or transition metals (Cd, Hg, Pb, Fe, As), certain drugs (cyclosporine, tacrolimus, gentamycin, bleomycin), industrial solvents, cooking (smoked meat, used oil, fat) and radiation. After penetration into the body by different routes, these exogenous compounds are decomposed or metabolized into free radicals. ROS, comprising the superoxide anion radical, the peroxide anion and singlet oxygen, are highly reactive entities, produced from molecular oxygen by the gain of electrons, or the realignment of the electron spins. The hydroxyl radical, the highly reactive species of ROS, is formed by dismutation of peroxide catalysed by Fe^{2+}. The mechanisms of oxidative cellular damage are summarized in **Fig 1.1.**

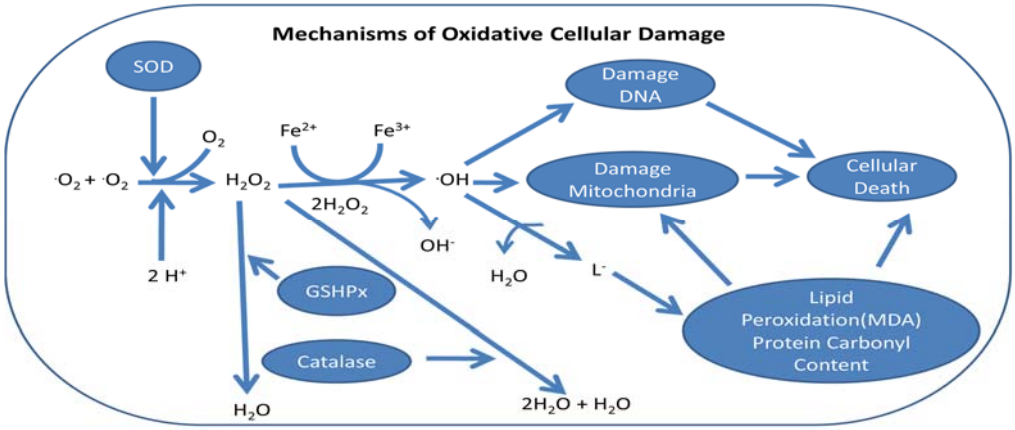


Fig 1.1 Mechanisms of oxidative cellular damage. Free radicals are reduced into water with the cooperation of the three main antioxidant enzymes: SOD, catalase, and GSHPx. The generation of hydroxyl radicals from hydroperoxide produces the development of oxidative cell injury: DNA damage; carboxylation of proteins; and lipid peroxidation, including lipids of mitochondrial membranes. By these pathways, oxidative damage leads to cellular death.

Uncontrolled generation of free radicals is associated with lipid and protein peroxidation, resulting in cell structural damage, tissue injury, or gene mutation and ultimately led to the development of various health disorders. The ROS are the mediators of inflammation, and through this their interaction with platelets, neutrophils, macrophages and other cells can involve the synthesis of eicosanoids and the activation and release of various cytokines, propagating the inflammatory process from one organ system (liver) to another (kidney, lungs, etc.). (The **Fig 1.2** illustrates the free radical induced diseases in human).

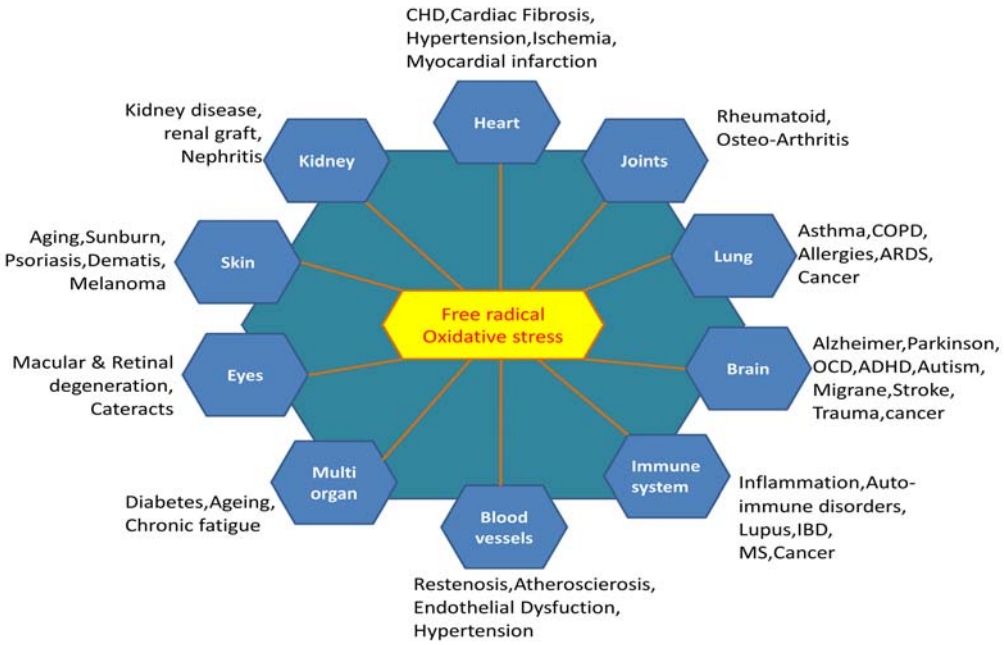


Fig 1.2 Free radical induced diseases in humans

These often results in tissue oxidative stress and multiple-system organ failure (Parke and Parke, 1995). The ROS-mediated inflammation is involved in immune and autoimmune diseases, for example, rheumatoid arthritis and inflammatory bowel disease (Parke *et al*., 1991), cancer (Witz, 1991; Ames *et al*., 1993), atherosclerosis (Halliwell, 1994), hepatitis (Elliot and Strunin, 1993), AIDS (Baruchel and Wainberg, 1992), Alzheimer's dementia (Evans,

1993), multiple-system organ failure (Fry, 1992; Parke and Parke, 1995), and respiratory distress syndrome (ARDS). Molecular mechanisms of ROS toxicity and ROS-mediated disease include: (i) oxidation of vital thiol compounds to disulphides, (ii) loss of tissue GSH, (iii) impairment of energy generation (ATP, NADH, NADPH), (iv) inhibition of Ca^{2+} transport and electrolyte homeostasis, (v) oxidation of cytochromes (vi) DNA strand cleavage, and (vii) the initiation and promotion of mutations and carcinogenesis (Parke and Parke, 1995).

1.3.2 Biological defense against ROS injury

The body has several mechanisms to counteract oxidative stress by producing antioxidants, either naturally generated *in situ* (endogenous antioxidants), or externally supplied through foods (exogenous antioxidants). The enzymatic and non-enzymatic antioxidants defenses overwhelmed the ROS production resulting in the adaptation and prevention of diseases are illustrated in **Fig 1.3.**

The roles of antioxidants are to neutralize the excess of free radicals, to protect the cells against their toxic effects, and to contribute to disease prevention. The human body constantly quenches excessive oxidants through various scavenging mechanisms, such as, use of antioxidant enzymes and molecules. The major antioxidant enzymes directly involved in the neutralization of ROS are: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRx). SOD, the first line of defense against free radicals, catalyzes the dismutation of superoxide anion radical ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2) by reduction. The oxidant formed (H_2O_2) is transformed into water and oxygen (O_2) by catalase (CAT) or glutathione peroxidase (GPx). The endogenous antioxidant are produced in the body, such as lipoid acid, glutathione, L-ariginine, coenzyme Q_{10},

melatonin, uric acid, bilirubin, metal-chelating proteins, transferring, and a variety of nutritional factors, primarily the antioxidant vitamins. These antioxidants could attenuate the oxidative damage of a tissue indirectly by increasing cells natural defenses or directly by scavenging the free radical species. The resulting increased oxidative damage to biomolecules may play an important role in the pathology of several human diseases, and is amenable to therapeutic intervention with appropriate antioxidants.

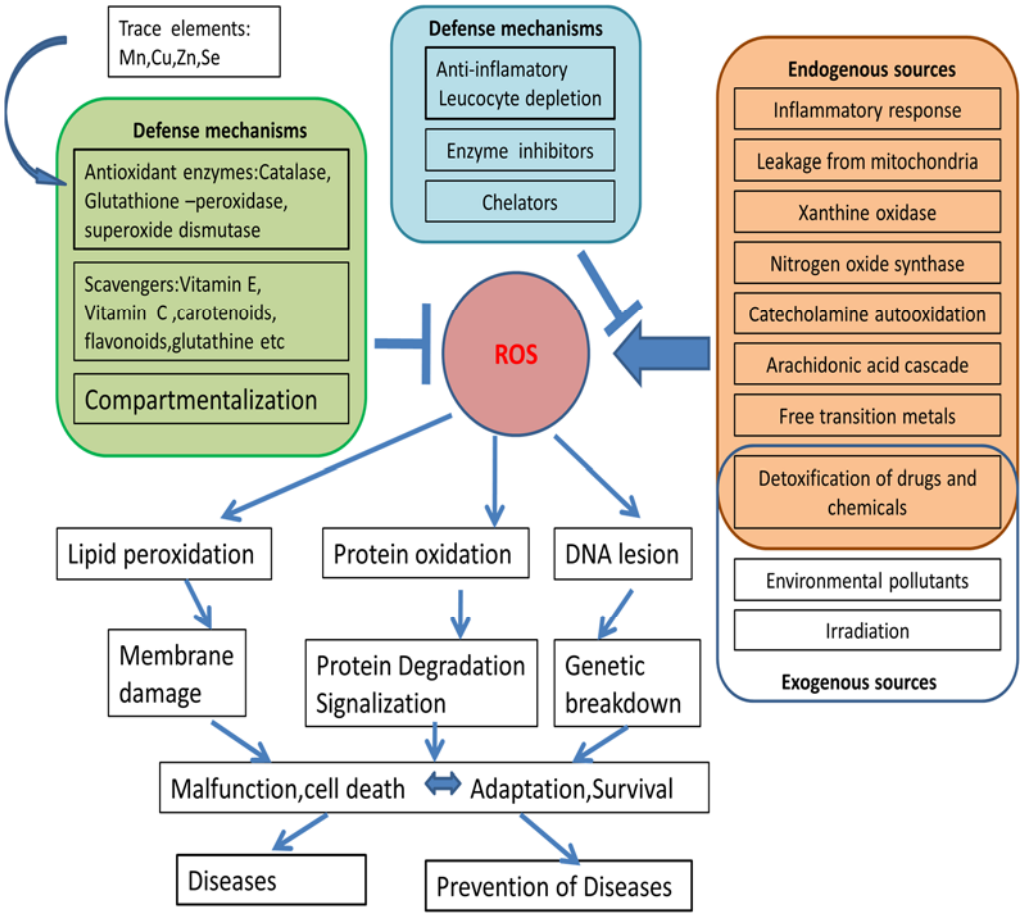


Fig 1.3 Reactive oxygen species (ROS) are derived from multiple sources (endogenous and exogenous) and are counterbalanced by enzymatic and non-enzymatic antioxidants. The antioxidant defenses are overwhelmed ROS production resulting in prevention of diseases.

1.3.3 Natural alternatives

Free radicals and other reactive species are constantly generated in the human body. Antioxidant defense systems scavenge, and minimize the formation of, reactive oxygen species, but they are not fully effective. Free radicals are known to take part in lipid peroxidation in foods, which is responsible for rancid odors and flavors, which decrease the nutritional quality. Antioxidant defenses minimize the damage levels of food ingredients containing unsaturated fatty acids that are quite susceptible to quality deterioration, especially under oxidative stress. Therefore, synthetic antioxidants, such as, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ) are widely used in the food industry as potential inhibitors of lipid peroxidation. They are extensively used to control rancidity in lipid-containing foods, and formation of peroxidation products, cosmetic, and pharma industries. Synthetic antioxidants are associated with side effects. BHT was reported to react with other ingested substances to cause the formation of carcinogens. BHA and BHT accumulate in the body and result in liver damage and carcinogenesis. ROS mediated inflammation is treated mainly by non-steroidal anti-inflammatory drugs (NSAIDs) that exert anti-inflammatory actions by inhibiting COX-2, which induces the pro-inflammatory agents such as TNF-α, LPS and tumor-promoting factors (Jaggi *et al*., 2004). The main undesirable side-effects of NSAIDs are due to the inhibition of COX-1 (constitutive isoform), along with COX-2. The side effects of these drugs are often deleterious, which include gastrointestinal ulcers, cardiovascular diseases, drug dependency etc (Quan *et al*., 2008). Therefore, in recent years, there are interests in finding naturally occurring antioxidant and anti-inflammatory compounds in food or medicine to replace synthetic products. This trend has increased considerably as the synthetic

antioxidants are being restricted due to the consumer preference for natural products, and concern about the potential toxic effects of synthetic medicines (Zheng *et al*., 2001). These natural antioxidants prevent oxidation of proteins, lipid peroxidations and prevent generation of ROS, thus act as upstream therapeutic barrier to oxidation stress.

1.3.4 Nutritional antioxidants

The nutritional antioxidants take care of the nutritional part along with the defense mechanisms to suppress various metabolic disorders. Nutrient antioxidants belong to the exogenous antioxidants compounds, which cannot be produced in the body, and must be provided through foods or supplements. Dietary antioxidants namely, phenolic compounds and low molecular mass antioxidants (Se, Zn, vitamins E, C, & A) were found to arrest the oxidation process by ROS (Fang *et al*., 2002; Gorinstein *et al*., 2002). The various mechanisms for nutritional defense and disease prevention are: scavenging of ROS; reduction of peroxides and repair of per oxidized biological membranes; sequestration of iron to decrease the formation of ROS; utilization of dietary lipids for rapid energy production; scavenging of ROS by short-chain fatty acids, scavenging of ROS by cholesteryl esters and alternative biological pathways as occur in stomach cancer, multiple system organ failure and diabetes. The potential of dietary antioxidants to reduce the risk of coronary vascular disease (CVD) and inflammation, is gaining a great deal of interest in the medical and scientific communities. In this contest the concept of nutraceuticals having antioxidant and anti-inflammatory potential play a vital role in eliminating the disorders associated with the free radicals. Marine-derived nutrients and other marine bioactive components have great potential as functional food ingredients as they possess advantageous physiological

effects, with medicinal characteristics and added health benefits, such as, anticancer or anti-inflammatory activities.

1.4 Mollusks as potential sources of bioactive compounds

The marine invertebrate species are one of the most interesting phyla with diverse chemical classes to biosynthesize pharmacologically active marine compounds. Among different marine invertebrates, mollusks are the potential sources of bioactive substances . Bivalve mollusks and cephalopods possess protective antioxidative defense mechanisms to avoid the cellular damage in the structural components of cells. The protective mechanisms against oxidative stress include specific antioxidant systems (e.g., vitamins, antioxidant enzymes, glutathione, etc.) and nonspecific molecules which provide competitive advantages against various oxidative stress factors leading to the development of harmful reactive oxygen species. As mollusks live in the permanent pro-oxidant conditions, greater levels of antioxidant defenses are generally measured in these species. The absence of cellular damage in the structural components of bivalve mollusks and cephalopods implies that their cells possess protective antioxidative defense mechanisms. This makes them specific for prevention of free radical accumulation, supporting the immune system in eliminating the proliferation of radicals. These sessile species are the richest natural sources of bioactive compounds, many of which belong to novel chemical classes not found in terrestrial the sources Therefore it is imperative to explore these organisms for potential antioxidant molecules.

The nutritional antioxidants are of particular interest in the case of marine invertebrates particularly bivalve mollusks. Bivalves are preferred food items in the Malabar region of India. Among various bivalves, green mussel is a delicious food item for the people living in this region from the time

immemorial. These species constitute a major source of nutritional elements, such as, fatty acid, amino acid, proteins, vitamins and minerals along with the vitamin E, K and Se etc. The seasonality related variations were reported for antioxidant activities (Power & Sheehan, 1996). Bivalves were reported to repair the oxidative damage caused by ROS by increasing the expression of antioxidant molecules. This makes them specific for prevention of free radical accumulation, supporting the immune system in eliminating the proliferation of radicals. Various studies on bioactive compounds from mollusks exhibiting antitumor, anti-leukemic, antibacterial and antiviral activities have been reported worldwide (McPhee *et al*., 2007). Although the coastline of South Indian subcontinent is bestowed with large assemblage of mollusks and cephalopods, have not been explored in detail regarding their potential to be used against inflammatory and oxidative stress induced disorders. Marine mollusks have evolved biochemical and physiological mechanisms for the production of bioactive compounds against oxidative stress. Hence mollusks can be considered as an important source to derive bioactive compounds with anti-inflammatory and antioxidant activities.

1.5 Antioxidant and anti-inflammatory potentials of mollusks and cephalopods

Bivalve mollusks and cephalopods are widely used in different parts of the world for various studies, but only recently they have been recognized as potential sources for bioactive compounds. Bivalve mollusks and cephalopods are abundant in the coastal brackishwater and sea. The culturing of these mollusks is initiated on a commercial scale, and experimental farming methods of a number of species have been carried out and suitable farming techniques evolved (Kuriakose and Appukuttan, 1980; Narasimham, 1980). Marine bivalves are virtually untapped resource for the discovery of novel

bioactivities and were reported to contain abundance of bioactive principles (Chellaram *et al*., 2004). Ziconotide isolated from the cone snail *Conus magus* venom was approved to treat chronic pain. Ziconotide shows its effect by blocking N-type voltage gated Ca^{2+}channels (Schroeder *et al*., 2004). In recent years, the *n*-3 PUFAs from bivalve mollusks provide an almost unlimited variety of long chain PUFAs, with beneficial roles in human health (Nestel, 2000; James *et al*., 2000; Emelyanov *et al*., 2002). The antioxidant potential of mollusks measures the biological resistance towards ROS, thus providing useful indications to predict oxyradical-mediated adverse effects on the physiological condition of the organisms. Bivalve mollusks and cephalopods are rich sources of structurally diverse bioactive compounds with valuable pharmaceutical and biomedical potential. These compounds frequently produce structural and functional changes in lipids, proteins and other biomolecules, and are derived from the partial reduction of O_2. However, no detailed chemical studies to identify the active principle(s) have been reported from the bivalve mollusks and cephalopods available from the south western coast of India. Further investigations intending to purify these active compound(s) should be considered to invent their chemical nature responsible for the target bioactivities. Therefore, a systematic search for the development of new sources of chemical compounds from bivalve mollusks and cephalopods will be helpful for the isolation of anti-inflammatory and antioxidant molecules.

In the present study we have considered *Perna viridis* and *Crassostrea madrasensis* among the bivalves and *Octopus dolffusi* as the candidate cephalopod. These species are abundantly available at the south west coast of India and are the preferred food items among the people living in the Malabar coast. Based on this background, the present work is focused in the isolation of

potential anti-inflammatory and antioxidant principles from these candidate bivalve mollusks and cephalopods, and their characterization them by different spectroscopic techniques. The products containing active anti-inflammatory and antioxidant principles with suitable stabilizers are developed from the candidate bivalve mollusks and cephalopods in an attempt to deliver potent medication and nutraceutical supplements.

Based on this background the objectives of the thesis are as follows:

1) To develop an optimized procedure for the extraction of antioxidant and anti-inflammatory substances from bivalve mollusks, such as, *Perna viridis* and *Crassostrea madrasensis* and cephalopods, such as, *Octopus dolffusi* available in the south-west coast of India.
2) To document the *in vitro* antioxidant and anti-inflammatory potential of the crude extracts from these mollusk species.
3) To purify the active principles by bioassay-guided chromatography, and to elucidate the structure of purified molecules by spectroscopic experiments.
4) To develop a product containing the active principles from selected bivalve mollusks and cephalopods with antioxidant and anti-inflammatory activities as nutraceutical supplements and medication.

1.6 Thesis outline

Based on the above objectives the present thesis is summarized into a total of seven chapters. The importance of the study on the nutritional and bioactive properties of mollusks and cephalopods, with objectives, were discussed and explained in the introduction under Chapter 1. Chapter 2 deals with the detailed review of the works carried out regarding the significance of

mollusks and cephalopods as potential sources particularly the antioxidative and anti-inflammatory bioactivities have been considered to explain the pharmaceutical and medicinal values of these species. Subsequently Chapter 3 described the nutritional parameters of the candidate species to understand the seasonal and spacial variation in the nutritional constrains. Chapter 4 described the evaluation of the anti-oxidant and anti-inflammatory potentials of *Perna viridis, Crassostrea madrasensis* and *Octopus dolffusi* to assess the in-build antioxidant and anti-inflammatory capacity to inhibit free radical formation and inflammation in the body. The isolation and characterization of secondary metabolites responsible for antioxidant and anti-inflammatory activities for the development of new sources of bioactive pharmacophores from these mollusks has been described in Chapter 5. A stabilized anti-inflammatory formulation for use as nutraceutical supplement has been developed from *P. viridis* was described in Chapter 6. The summary of the work carried out along with the major research findings from the present study were summarized in Chapter 7.

Chapter 2

REVIEW OF LITERATURE

Contents

Background of the study

Mollusks are heterogenous group of animals both in shape and diversity, and are mainly represented by amphineura, gastropods, bivalves, cephalopods and scaphopods. Most of the mollusks inhabit the marine environment and very few dwells in the terrestrial and freshwater habitats. The Indian coast is blessed with number of ecosystems, such as, coral reefs, mangroves, lagoons, estuaries, sandy beaches and rocky shores. Among these, the coral reef and rocky shores have rich assemblage of molluskan fauna. They are the largest and most diverse phylum in the tropical seas (Venkatraman and Venkataraman, 2012). The majority of mollusks inhabit marine biotopes, and they occur from the backwater zone, mangroves, intertidal, shelf and down to deeper waters. The mollusks are abundant in rocky shores, as it provides a better substratum, and act as a feeding and breeding ground, ensuring a favorable environment for their survival. A total of 80,000 to 100,000 species of mollusks recorded from various parts of the world. An aggregate number of 3271 mollusks are known to occur in coastal Indian waters, belonging to 220 families and 591 genera, of which 1900 were reported as gastropods

(Venkatraman and Venkataraman, 2012). Although many species of the phylum molluska are indigenous to the continent, the most important classes of commercial interest are the bivalves (oysters, clams, scallops and mussels), gastropods (conchs, whelks, abalones and periwinkles) and cephalopods (cuttle fish, squid and octopus) (Periyasamy, 2012).

Fig 2.1 Abundance of bivalves along the coastal waters of the Indian subcontinent

Fig 2.2 Cephalopods in Fortkochi harbor, collected from coastal waters of the Indian subcontinent

2.1 Marine environment as a prolific source of bioactive compounds

Marine environment is a rich source of both biological and chemical diversity. Marine ecosystem covers a wide thermal, pressure and nutrient ranges, and it has extensive photic and non-photic zones. These marine organisms live in complex habitats, and are exposed to the extreme conditions and intensive ecology pressure. The mollusks are soft - bodied, heterogeneous group of animals with great antiquity and diversity (Venkatraman and Venkataraman , 2012). Despite the presence of a shell in some molluskan groups, all mollusks are essentially soft-bodied, making them vulnerable to predators and pathogens. Mollusks often live in microbial rich habitats, such as, soil and leaf litter on land and amongst marine benthic sediments and hard reef communities (Benkendorff *et al*., 2009). As they thrive in a different kind of climate and habitat, these organisms develop certain adaptation mechanisms, which may be useful for their defense, and this result in the production of bioactive metabolites, which cannot be found in other organisms (Ira Bhatnagar *et al*., 2010). The marine environment, which contains a vast array of organisms with unique biological properties, is one of the most underutilized biological resources. Moreover, considering its great taxonomic diversity, investigation related to the search of new bioactive compounds from the marine environment has seen in almost unlimited field (Rasmussen *et al.*, 2007, Plaza *et al*., 2008).

Marine invertebrates synthesize primary and secondary metabolites. The primary metabolites include amino acids, simple sugars, nucleic acids, and lipids, whilst secondary metabolites are alkaloids, terpenoids, and other compounds, have known bioactivities and biological functions. Mollusks are considered as one of the important sources to harbor valuable bioactive compounds that exhibit antitumor, antimicrobial, anti-inflammatory, and

antioxidant activities (Anbuselvi *et al*., 2009; Chellaram and Edward, 2009b; Benkendorff *et al*., 2011). These animals usually produce structurally diverse substances to form a chemical defensive system, representing a different source of bioactive compounds from marine organisms. A series of metabolites with complex structures and intriguing biological activities have been isolated from mollusks (Zhen and Yue, 2012). Compounds isolated from mollusks were also used in the treatment of rheumatoid arthritis and osteoarthritis (Chellaram and Edward, 2009a). Mollusk extracts also exhibited antiviral and antibacterial activity against fish pathogenic bacteria, and therefore may be applied in aquaculture other than medicinal purpose (Defer *et al*., 2009). Compounds isolated from marine organisms even show promising activities within the nanomolar range, and can be developed as drug candidates and lead pharmacophores.

2.2 Bivalves and Cephalopods

Bivalves are highly abundant group of organisms in majority of coastal and marine environments. Till date about 7500 bivalves were identified and found from the intertidal zone to the abyssal. They inhabit different marine ecosystems including temperate, tropical and polar seas, brackish estuary, hydrothermal vent etc. Kerala, a coastal state in the southwestern peninsula, dominates in the production of bivalves, which include oysters, mussels and clams. The fishing of bivalves is pursued as a small-scale activity, mostly at subsistence level in various estuaries and inshore seas. The total bivalve production in the Malabar region of India was estimated at 9695 t during 2007 (**Fig. 2.3**). The annual average clam production is about 57,000 t, oysters about 18,800 tonnes, and mussels about 14,900 tonnes (CMFRI, 2007-08). Mollusks are important for marine ecology and human diet, and considered to be an important source of nutrients.

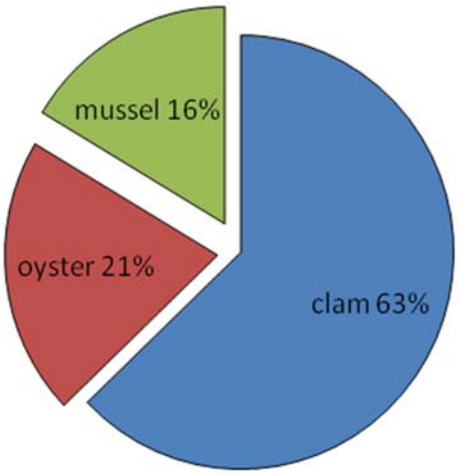


Fig 2.3 Total bivalve production (%) in Malabar region during 2007-08

Class Cephalopoda represents the most advanced class of the phylum Molluska, and include some intriguing individuals like squids, octopods, cuttle fishes and so forth. These species are exclusively marine, and are distributed throughout the seas of the world. The vast majority of them are free swimming shallow water predators described by rapid growth. More than 700 species are depicted today, and their number has a tendency to expand consistently as the researcher discovers new species, especially in the tropical and polar oceans (Sweeney *et al*., 1998). These species occupy a leading place among the exploited marine fishery resource on the planet, and are receiving greater importance due to their increasing demand in export. The expanding interest of cephalopods in the global business sector is primarily because of the increased awareness of the nutritional qualities (Zlatanos *et al*., 2006). The cephalopods were accounted to be a rich source of long chain *n*-3 polyunsaturated fatty acids, essential amino acids, which are deficient in cereals and pulses, along with rich antioxidants and minerals, for instance,

selenium, which could not be acquired from other sources. Notwithstanding this, their great sensory properties make them a favoured human delicacy.

The progression of cephalopods as an imperative business fishery commodity in the global business occurred only during the recent decades (Boyle, 1990). Cephalopods are by far the most important group with an average annual production of about 1,05,000 tonnes. They are landed as by-catch and as a targeted fishery mostly in mechanized trawlers operating up to 200 m depth, and beyond in some areas. Cephalopod catch in Kerala during 2007 was estimated at 23,392 tonnes and showed a decline of 25% compared to previous year (CMFRI, 2007-08).

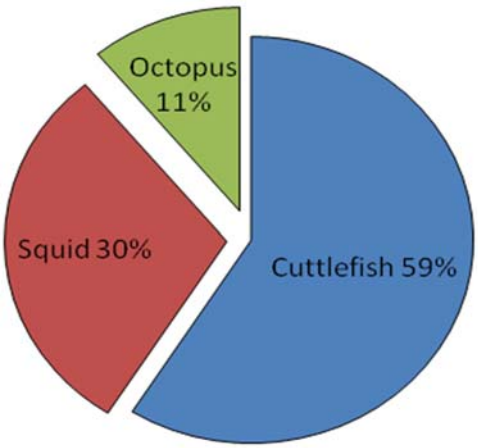


Fig 2.4 Cephalopod landing (%) in Kerala. Cuttlefishes contributed 59% followed by squids (30%) and octopus (11%) *Sepia pharaonis, S. aculeate* and *Sepiella inermis* (cuttlefish), *Urothethis duvauceli, Doryteuthis sibogae* (squids), *Amphioctopus marginatus, O. dolfusi, Cystopus indicus* were observed in the fishery.

2.3 Nutritional importance of mollusks

The knowledge on biochemical composition of any edible organisms is extremely important since the nutritive value is reflected in its biochemical

content. Phytoplanktons are considered as a main food source for bivalves and many studies looked at the influence of bivalves on phytoplankton community (Arapov *et al*., 2010).Bivalve mollusks and cephalopods are highly delicious seafood items because of their nutritive value next to finfishes and crustaceans. They are also very good source for biomedically important products (Shenoy, 1988). Mollusks, as well as the invertebrates, in general, constitute a source of lipidic bioactive compounds offering a variety of nutraceutical and pharmaceutical applications (Benkendorff *et al*., 2010). Among them, the *n*-3 polyunsaturated fatty acids (PUFA), such as, eicosapentaenoic acid, 20:5*n*-3, and docosahexaenoic acid, 22:6*n*-3, are known for their beneficial effects on human health (Simopoulos *et al*., 2008). These long chain unsaturated *n*-3 fatty acids are widely known for their capacities for cardioprotection. They were reported to reduce triacylglycerol and cholesterol levels, and have anti-inflammatory and anticancer effects. Numerous experiments on animals confirmed the cancer preventive properties of *n*-3 fatty acids from marine sources (Wendel *et al*., 2009; Candela *et al*., 2011).

2.3.1 Nutritional importance of bivalves

Bivalve mollusks are aquatic mollusks (Phylum: *Molluska*; Class: *Pelecypoda* or *Bivalvia*), and comprise major marine fishery resources, mussels being a prominent member (Murphy *et al*., 2002). Bivalves in coastal area are good sources of *n*-3 PUFAs, including the long-chained eicosapentaenoic acid (20:5*n*-3; EPA) and docosahexaenoic acid (22:6*n*-3; DHA) with valuable pharmaceutical and biomedical potential (Taylor and Savage, 2006). The consumption of bivalve mollusks in India, particularly south Malabar area has increased in the recent years in response to the higher

availability under wild and cultured conditions. Bivalves are considered vital next to the fish and prawns from the nutritive point of view. Bivalve mollusks were reported to contain bioactive lipids, which include fatty acids; sphingolipids, phytosterols, diacylglycerols, etc., and many of these can influence human health and diseases linked to alleviate the symptoms of inflammatory conditions (Li and Sinclair, 2007). The green mussel *Perna viridis* (family: Mytilidae) is a bivalve mollusk native of the Indian coast and available throughout the Indo-Pacific and Asia-Pacific (Benson *et al*., 2001). It forms a significant fishery and contributes nearly 50% to the total bivalve production of the area (Laxmilatha *et al*., 2011).

Mussels are consumed as seafood in several European countries including Belgium, the Netherlands, and France. In India, two species of mussels (green mussel *P. viridis* and brown mussel *P. indica*) support a traditional sustenance fishery in Malabar and Konkan areas of Penninsular India.

The Malabar Coast of Kerala is popularly called as the 'Mussel fishery zone of India', which accounts for the bulk of the mussel production in the country. Mussels are commercially valuable species, easy to cultivate or collect in coastal areas. They are very important for marine ecology and for human diet, since they are an important source of nutrients (Astorga-Espana *et al*., 2007).

Fig 2.5 (A) Photographs of *P. viridis* (green mussel) with meat (B) *P. viridis* shells are kept near the seahore (C) *P. viridis* along the southwestern coast of Kerala

The edible oyster *Crassostrea madrasensis* is a commercially important bivalve mollusk distributed all along the east and west coasts of India. The Indian Backwater Oyster (*Crassostrea madrasensis*) locally known as 'Kadal muringa' is found in the backwaters and estuarine regions of Kerala. *Crassostrea madrasensis* is endemic to brackishwaters, such as, estuaries, creeks, bays and backwaters (Rao and Nayar, 1956; Joseph and Joseph, 1983; Velayudhan *et al*., 1998). *Crassostrea madrasensis* is a good source of *n*-3 PUFAs, including the long-chained eicosapentaenoic acid (20:5*n*-3; EPA) and docosahexaenoic acid (22:6*n*-3; DHA). Consumption of these bivalve mollusks provides an inexpensive source of protein with a high biological value, essential minerals and vitamins (Astorga-Espana, *et al*., 2007; Karakoltsidis *et al*., 1995).

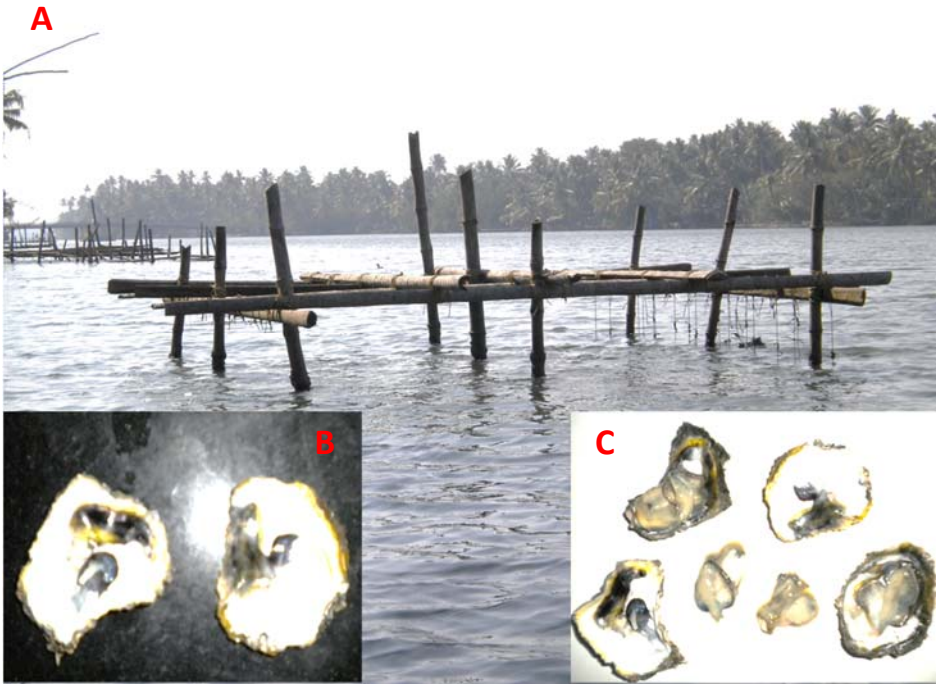


Fig 2.6 (A) Photographs of culturing sites of *C. madrasensis* (oyster), (B) *C. madrasensis* with meat inside the shell (C) *C. madrasensis* with shell and meat separately.

2.3.2 Nutritional importance of cephalopods

The cephalopods represent important economic seafood for human consumption and contribute to 14% of the world fisheries (FAO, 2004). This is due to its high nutritional and market value, excellent palatability, and increasing demand throughout different regions of the world. Cephalopods including cuttlefish, squid and octopus are now among the most interesting inhabitants of the seas for both scientific and economic reasons. They are considered as an important marine resource since they are rich in taste and have few inedible parts. These species are also cheap sources of well-balanced protein and lipid (Kreuzer, 1984; Sinanoglou & Miniadis-Meimaroglou, 1998; Sinanoglou & Miniadis-Meimaroglou, 2000; Zlatanos *et al*., 2006). According to Boucaud-Camou, cuttlefish is composed mainly of water (81%) and protein

(16.1%); it has no carbohydrates and lesser than 1% lipids (Boucaud-Camou, 1990). It is also a source of mineral salts and vitamins, and is highly digestible. Its nutritional profile, as a high protein and EPA/DHA lipid source, makes it one of the most suitable and healthy forms of human food. An overall increase in world cephalopod consumption is predicted, since an open market with high growing potential is foreseen in the future.

Fig 2.7 Photographs of cephalopods collected from southwestern coast of Kerala

Cephalopods are fairly good sources of protein with low in fat content and rich in *n*-3 polyunsaturated fatty acids (Sinanoglou and Miniadis-Meimaroglou, 1998). The characteristic PUFAs were found to be docosahexaenoic acid (DHA; 22:6*n*−3) and eicosapentaenoic acid (EPA; 20:5*n*−3), which ranged between 20% and 36%, and 8.3%, and 17.6% of total fatty acids, respectively (Culkin and Morris, 1970; Gibson, 1983; Sinanoglou and Miniadis-Meimaroglou, 1998). Passi *et al*. studied the fatty acid composition of several species of teleosts, cephalopods and crustaceans from the Mediterranean Sea and found that all of them had an *n*-3/*n*-6 ratio of more

than 1, confirming the great importance of these fish and shellfish as a dietary source of n-3 PUFA for humans (Passi *et al*., 2002). The essentiality of *n*-3 highly unsaturated fatty acids (HUFA) in fish diets is directly related to their role as components of biomembrane phospholipids.

Octopus and cephalopods in general are characterized by low lipid contents, with relatively large phospholipid and sterol fractions, and triacyglycerides as minor components (Nash *et al*., 1978; Hayashi and Yamamoto, 1987; Navarro and Villanueva, 2000). Thus, the changes in the fatty acid composition of total lipids observed in the octopus reflected those that occurred mainly in the composition of phospholipids. The nutritional composition of cuttlefish has been reported by some researchers (Thanonkaew *et al*., 2006; Villanueva *et al*., 2004; Ozyurt *et al*., 2006; Phillips *et al*., 2002). However, this composition could vary among species due to geographical differences of fishing grounds.

2.4 Bioactive potentials of mollusks

Mollusks are considered as one of the important sources to derive bioactive compounds that exhibit antitumor, antioxidant activities (Benkendorff *et al*., 2011; Anand *et al*., 2010). The bioactive compounds in mollusks were identified essentially as peptides, depsipeptides, sterols, sesquiterpenes, terpenes, polypropinates, nitrogenous compounds, macrolides, prostaglandins, fatty acid derivatives and alkaloids which exhibit specific types of bio-activities (Balcazar *et al*., 2006). Compounds isolated from mollusks were also used in the treatment of rheumatoid arthritis and osteoarthritis (Chellaram and Edward, 2009). Molluskan extracts also reported to exhibit antiviral and antibacterial activity against fish pathogenic bacteria (Defer *et al*., 2009).

2.4.1 Bioactive potentials of bivalves

The antioxidant defense systems are of particular interest in the case of marine invertebrates particularly bivalve mollusks. Marine bivalves are virtually untapped resource for the discovery of novel bioactivities and were reported to contain abundance of bioactive principles, which were reported to influence human health (Chellaram *et al*., 2004). Marine originated bioactive lipids have been reported to be linked in alleviating the symptoms of inflammatory conditions (Calder, 2004). Asian green mussel, *Perna viridis*, is abundant in C_{20}-C_{22} *n*-3 PUFAs, particularly EPA and DHA, which are the precursors of anti-inflammatory resolvins (E- and D-series) (King *et al*., 1990). Aerobic organisms including *P. viridis* maintain constitutive antioxidant defenses, detoxifying and scavenging the reactive oxygen species (ROS), including superoxide radical ($.O_2^-$), H_2O_2, peroxyl radical ($.RO_2$) and hydroxyl radical (.OH) that are continuously produced as a by-product of aerobic metabolism (Santovito *et al*., 2005). These compounds frequently produce structural and functional changes in lipids, proteins and other biomolecules, and are derived from the partial reduction of O_2. Bivalves were reported to repair of oxidative damage caused by ROS by increasing the expression of antioxidant molecules (Demple, 1999). The seasonality-related variations were reported for antioxidant activities (Power and Sheehan, 1996). The aquatic environment particularly intertidal zone is characterized by significant variability of temperature and salinity, and pO_2. Antioxidative indicators therefore are common biochemical markers of combating capability of mussels dwelling in the intertidal zone, against ROS. Several studies dealing with antioxidant defenses of *M. edulis* and *M. galloproincialis* demonstrated correlation between antioxidant defenses and seasonality with respect to the reproductive period and food availability (Sheehan and Power,

1999; Cancio *et al*., 1999). Dietary antioxidants, phenolic compounds and low molecular mass antioxidants (Se, Zn, vitamin E, C, & A) were found to arrest the oxidation process by ROS (Fang *et al*., 2002; Gorinstein *et al*., 2002).

2.4.2 Bioactive potentials of cephalopods

Cephalopods are considered to be a rich source of *n*-3 fatty acids that are responsible for anti-inflammatory activities (Russo and Tringali, 1983). Cephalopods were reported to be a goodt source of arachidonic acid along with sufficient *n*-3 PUFAs. PUFAs consisting of 20 carbon atoms (e.g. 20:3*n*-6, 20:4*n*-6 and 20:5*n*-3) were reported in various cephalopods including squid and octopus, which have a wide range of physiological actions including immune and anti-inflammatory response, and were reported to have vital role in neural function and reproduction (Tocher, 1995; Miliou *et al.*, 2006). The secondary metabolites derived from number of marine animals possess antibiotic, anti-larvae, antiviral and anti-cancer activities (Simmons *et al*., 2005; Grabley *et al*., 1999). The bioactive compounds isolated from the mollusks, in particular, from the gastropods are considered to have a role in the chemical defense of the animals against their predators. Many promising lead compounds have been reported from marine mollusk having anti-inflammatory activity (Morris *et al*., 1990). Chemical composition of inks from different mollusks suggested convergent chemical defenses (Derby *et al*., 2007). Methanolic tissue extract of cephalopods in 100% concentration showed the maximum activity against human pathogens (Ramasamy *et al*., 2011). Significant antimicrobial activity was observed from the polysaccharides extracted from cuttle bone and methanolic extract of body tissue of *Sepia parshadi*, against ten human pathogenic bacteria and five fungi (Montgomery *et al*., 1992). Takaya *et al.,* (1994) investigated the antitumour activity of a peptidoglycan fraction containing 7.8 % peptide, 57 % polysaccharide and 30 % pigment from the

squid (*I. argentinus*) ink against fibrosarcoma in mice. Mimura *et al.,* (1985) reported the anti-inflammatory activity of squid ink that inhibits gastric secretion (Mimura *et al.*, 1985). An angiotensin-converting enzyme purified from squid ink causes dilation of blood vessels, resulting in lower blood pressure. Cephalopod ink has antioxidant activity in both the melanin and melanin-free fractions of ink. This antioxidant activity was found to be may be related to anti-cancer effects, as established by *in vitro* studies of various cell types and cell lines (Debry, 2014).

2.5 Compounds isolated from bivalves and cephalopods

In recent years, many bioactive compounds have been extracted from marine invertebrates, such as, sponges, tunicates, bryozoans, and mollusks (Donia *et al*., 2003; Haefner *et al*., 2003). Marine invertebrates synthesize primary and secondary metabolites. Marine bivalve mollusks (Class *Pelecypoda*) settled along the shoreline of south coast of India constitute a major share of marine fauna, and were reported to possess structurally diverse anti-stress metabolites with respect to antioxidant and anti-inflammatory properties (Chandran *et al.*, 2009). These animals usually produce structurally diverse substances to form a chemical defensive system, representing a different source of bioactive compounds from marine organisms. Of the few bio-evaluated for various bioactivities, some showed interesting biological activity with respect to antioxidant and anti-inflammatory properties (Kamboj, 1999). Polar bivalves developed a battery of antioxidant defenses, which comprise of enzymes and low-molecular weight scavengers. Past studies have reported that polar bivalves are characterized by high antioxidant defenses (Lionel *et al*., 2005).

Cephalopods are commonly available throughout the world. These are important as a food resource as well as in scientific research (Ngoile, 1987). Among the invertebrates, the discovered bioactive compounds in mollusks were identified essentially as peptides, depsipeptides, sterols, sesquiterpenes, terpenes, polypropionates, nitrogenous compounds, macrolides, prostaglandins and fatty acid derivatives which presented specific types of activities (Balcazar *et al*., 2006). The specific group of bioactive compounds derived and characterized from mollusks have been described under the following sub-sections.

2.5.1 Polypropionates

The most prominent source of marine polypropionates are mollusks. Marine polypropionates not only play defensive roles in mollusks, but also showed antimicrobial, antiviral, and cytotoxic activities. Among a variety of polypropionates, a new and two previously known metabolites possessing a polypropionate carbon skeleton have been isolated from the marine gastropod mollusk *Siphonaria lessoni*, collected at Chilean coasts (Rovirosa *et al*., 2006). *Elysoidean sacoglossan* and *Placobranchus ocellatus* from Indian coast were found to be rich sources of two new ɤ-pyrone propionates, possessing a bicyclo-octane ring together with the known propionates 9,10-deoxy-tridachione, photodeoxytridachione (Ireland and Scheuer, 1979) , tridachiahydropyrone B and C (Fu *et al*., 2000) and iso- 9,10-deoxy-tridachione. Earlier Gavagnin *et al*., (1999) reported photodeoxy-tridachione from the same mollusk of Pacific region. These compounds are antioxidants and photodeoxy-tridachione has shown activity in an ichthyotoxicity assay at 5 ppm (Gavagnin *et al*., 1994). Siphonarienal was isolated from *Siphonaria grisea*, whereas other unusual pyrone-containing metabolite as siphonarin A was also isolated from *S. zelandica*.

Chemical studies on marine pulmonata of the Onchidiidae family focused on polypropionates and onchidione as the main metabolite, was characterized from the first collection of the Chinese marine pulmonate *Onchidium* sp, which was present both in the mucus and in the mantle (Carbone *et al*., 2009). Study on the second collection of the mollusk led to the isolation of onchidiol, 4-*epi*-onchidiol and related alcohols of onchidione (Wang *et al*., 2012).

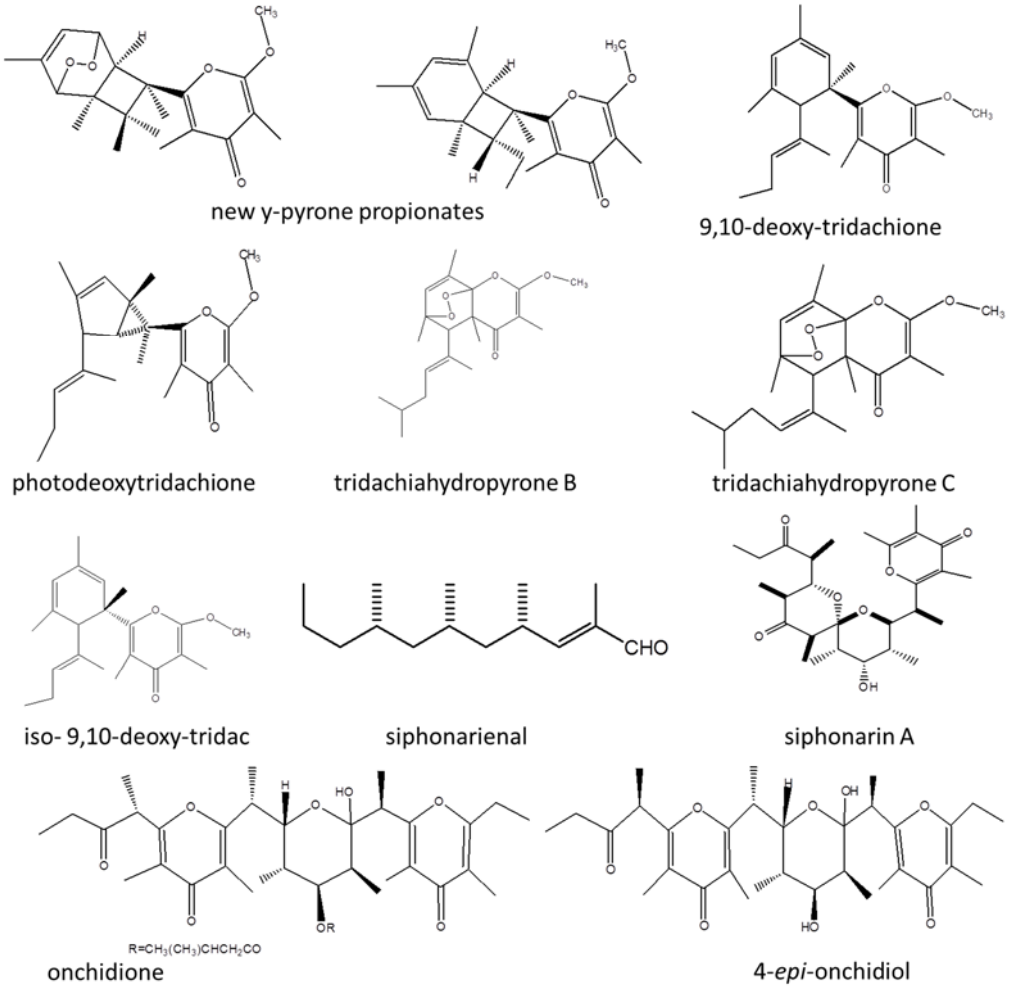


Fig 2.8 Examples of marine polypropionates isolated from mollusks

2.5.2 Nitrogen-containing compounds

The nitrogen-containing compounds of marine mollusks were found to consist of phidianidines A and B obtained from *Phidiana militaris*, belonging to the Glaucidae family of mollusks (Carbone *et al*., 2011). The phidianidines were tested in a panel of tumor cell lines and non-tumor cell lines. These componds showed a strong and selective activity against tumor cell lines in nanomolar range. The chemical study of an actinocyclidae nudibranch resulted in the isolation of actisonitrile (found in the mantle of *Actinocyclus papillatus*) and a lipid based 1,3-propanediol ether skeleton. The compound exhibited high cytotoxicity against tumor and nontumor mammalian cell lines in *in vitro* assays (Manzo *et al*., 2011).

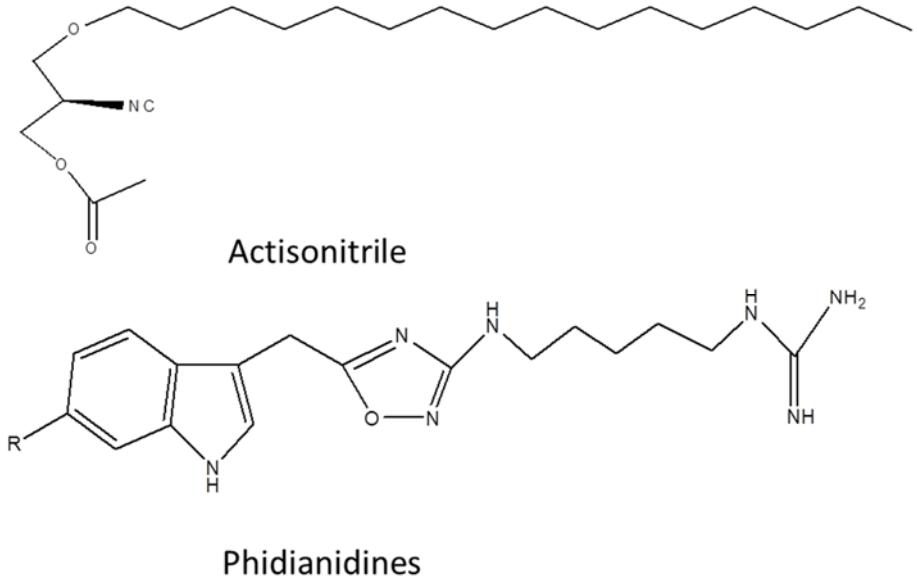


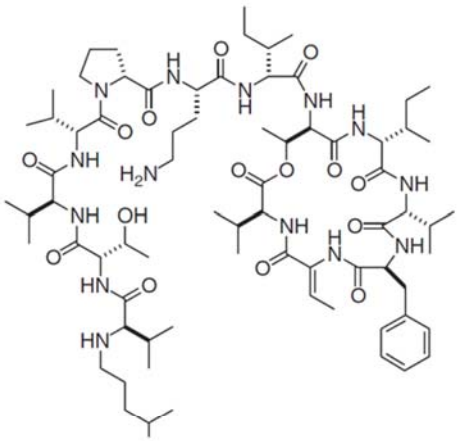
Fig 2.9 Examples of nitrogen-containing compounds isolated from mollusks

2.5.3 Cyclic peptides

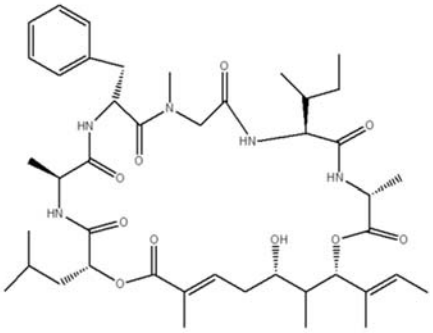
Kahalalide F, a cyclic depsipeptide isolated from the herbivorous marine mollusk, *Elysia rufescens*, has shown to be effective against cancer cell lines with

strong multidrug resistance and against cell lines resistant to topoisomerase II inhibitors. *In vivo* models have also confirmed anticancer activity in various solid tumor models (Pardo *et al*., 2008; Provencio *et al*., 2009).

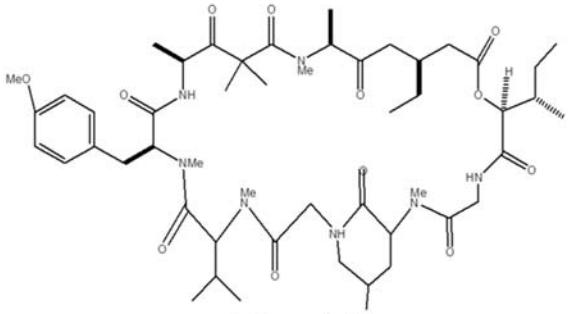
Dolastatin which is a cyclic depsipeptide isolated from *D. auricularia* was found to stabilize actin filaments as in the case of jaspakinolide/jaspamide (Umehara *et al*., 2012). Dolastatins and kahalalides were reported to be sequestered by the mollusks from the cyanobacterial diets. Kulokekahilide-2, a 26-membered cyclodepsipeptide, was isolated from Hawaiian marine mollusk, which possesses potent cytotoxicity in mammalian tumor cells (Umehara *et al*., 2012).



Kahalalide F



Kulokekahilide-2



Dolastatin 11

Fig 2.10 Examples of cyclic peptides isolated from mollusks

2.5.4 Alkaloids

Indole alkaloids from marine invertebrates have reported to possess potential anti-inflammatory properties. Manoalide, pseudopterosins, topsentins, scytonemin and debromohymenialdisine were isolated from mollusks and were found to exhibit anti-inflammatory activity (Chellaram and Edward, 2009a). Pseudopterosins have anti-inflammatory and analgesic activities, with a mechanism of action different from the common non-steroidal anti-inflammatory drugs. Commercially, pseudopterosins are found in the skin creams as topical anti-inflammatory agents (Kohl and Kerr, 2003). An alkaloid lamellarin D (LAM-D) was first isolated from the prosobranch mollusks of the genus *Lamellaria,* and subsequently obtained from *Didemnid ascidians* (Andersen *et al*., 1985). Lamellarin was reported to exhibit cytotoxicity against many different tumor cell lines and is cytotoxic (Reddy *et al*., 1999). An isoquinoline alkaloid, jorumycin was isolated from the nudibranch *J. funebris* collected off the Indian coasts (Fontana *et al*., 2000). Irvalec® and Zalypsis®, are the derivatives of jorumicin, and formulated from the lead molecules isolated from opisthobranchs, *Elysia rufescens* and *Jorunna funebris*, respectively (Reyes *et al*., 2009). The diacetyl derivative of ecteinascidin-637, was isolated from the nudibranch *Phidiana militaris* from the south China sea (Menchaca *et al*., 2003). The study of different *Chromodoris* species from the South China Sea confirmed the already described spongiane diterpenoids and their antiproliferative activities against murine lymphoma and human epidermoid carcinoma cell lines (Miyamoto, 1996).

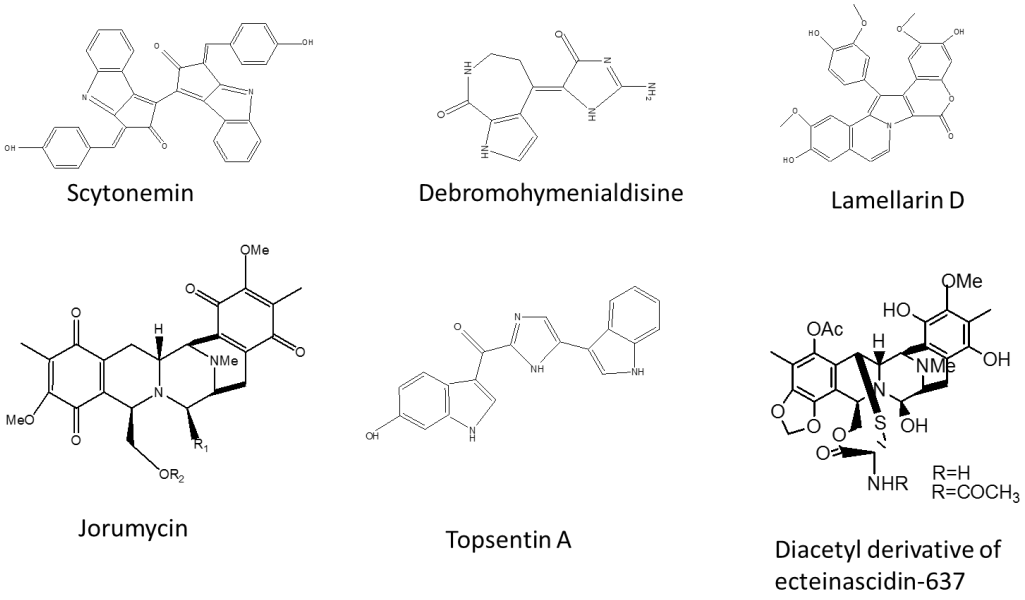


Fig 2.11 Examples of alkaloids isolated from mollusks

2.5.5 Terpenes

The study on the South China Sea nudibranch *Tritoniopsis elegans* led to the isolation of four diterpenes, tritoniopsins A-D, which displayed an unprecedented pyran ring in the cladiellane framework representing a novel cladiellane-based diterpene family. Both tritoniopsins A and B were discovered from *T. elegans* and *C. krempfi*, but the major compound in this mollusk was tritoniopsin A, which was found to possess antiproliferative activity on tumor and nontumor cell lines (Ciavatta *et al*., 2011). Two formamide-containing pupukeanane sesquiterpenoid congeners were reported from the tubercle nudi- branch *Phyllidia coelestis* (Jaisamut *et al*., 2013). Moderate to strong cytotoxicity towards tumour cell lines was observed for these terpenoids.

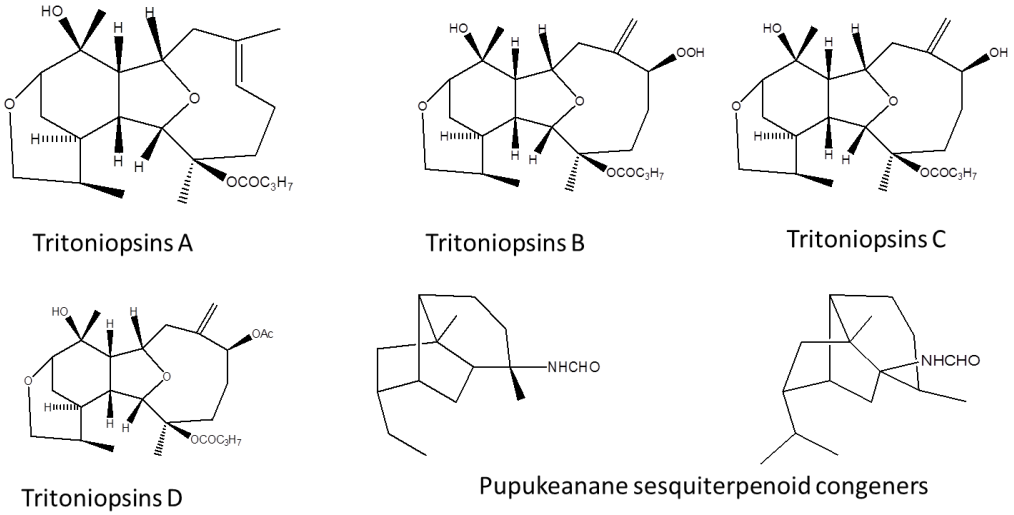


Fig 2.12 Examples of terpenes isolated from mollusks

2.5.6 Other compounds

The indole derivatives tyrindoleninone, tyrindolinone, 6-bromoisatin and 6, 6′-dibromoindirubin were isolated from the Muricidae family of marine gastropods and were reported to have anti-cancer properties (Benkendorff *et al*., 2011; Vine *et al*., 2007; Westley *et al*., 2010). Tyrindoleninone and 6-bromoisatin are indole derivatives from marine mollusk *Dicathais orbita* that were found to induce apoptosis in female reproductive cancer cell lines (Edwards *et al*., 2012). These indole compounds inhibited cell growth in solid tumour cell lines obtained from the colon and breast, and induced apoptosis and necrosis in T-cell lymphoma cells (Benkendorff *et al*., 2011).

Dolastatin-10, and Dolastatin 15, derived from the shell-less mollusk *Dolabella auricularia* (Pettit *et al*., 1987), was reported to have anti-tumour activity against breast and liver cancer in phase-I clinical trials (Tran *et al*., 1997). Two novel fatty acids pinnaic Acid and tauropinnaic acid extracted from *Pinna muricata* with phospholipase A2 (cPLA2) inhibitory activity (Uemura *et al*., 1996).

Two new molecules, 9-chloro-phorbazole D and *N*1-methyl-phorbazole A, co-occurring with the known related phorbazoles A, B and D have been isolated and characterized from *A. andersoni* mollusk. These compounds display similar *in vitro* growth inhibitory activity in the five human cancer cell lines, and it mainly relates to cytostatic effects in both human SKMEL-28 melanoma and U373 glioblastoma cells (Genoveffa *et al*., 2012). 12-Deacetoxy-12-oxo-deoxoscalarin, and a series of related scalaranes, most likely derived from dietary scalaradial, were isolated from the mollusk, thus proving the ability of *Glossodoris rufomarginata* to modify dietary molecules (Gavagnin *et al*., 2004; Fontana *et al*., 1999).

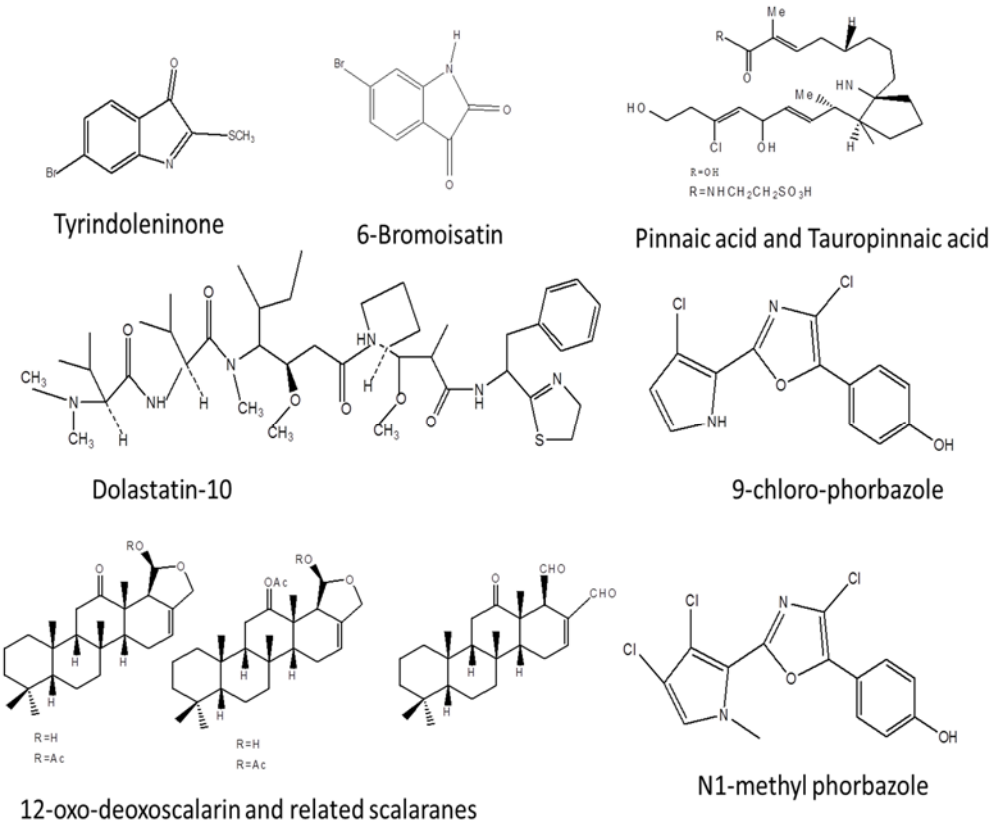


Fig 2.13 Examples of other compounds isolated from mollusks

2.5.7 Compounds isolated from bivalves

The marine mussels are not only a cheap source of protein for human consumption but also found to possess some complex bioactive compounds, which have tremendous potential in medical science. Brown mussel hydrolysate is available for human use in the Russian market with trade names Viramid and Midel as antiviral drugs. Potentially interesting HIV-virus-inhibiting compound have been isolated from the green mussel *Perna viridis,* and it has been patented (Mitra and Chatterji, 2002). Epidioxysterol (EDS) is the compound, which is responsible for inducing apoptosis was isolated from hard clam by ethyl acetate extraction (Min-Hsiung *et a*l., 2007). Zinc diprolinate isolated from black clam was found to be an inhibitor of HIV-1 replication with potential therapeutic importance (Pereira *et al*., 2000). Extracts prepared from economically important estuarine clam (*Meretrix casta*), black clam (*Villorita cyprinoides*) and mud clam (*Polymesoda erosa*) were found to possess high antiviral activity when tested with influenza virus strains type-A (A/Missisipi 1/85/H3N2) and type-B (B/Harbin 7/94) (Chatterji *et al*., 2002). *Villorita cyprinoides* (black clam) having agglutination potency on trypsin-treated rabbit erythrocytes (Learnal and Prakash, 2014). The compound, ES-285–HCl originally isolated from the clam *Spisula polynyma*, has shown selective anticancer properties against several cancer cell lines *in vitro* and against solid hepatocellular, prostate and renal tumours *in vivo* (Den Brok *et al*., 2006). Saxitoxin has been isolated from several marine organisms, such as, the Californian mussels *Mytilus californiananus*, alaskan butter clams *Saxidomus giganteus*. Saxitoxin produced effects on the cardiovascular system, and a marked hypotensive effect at a very low concentration.

The antioxidant activity detected in Japanese samples of the Pacific oyster (*Crassostrea gigas*) was attributed to 3,5-dihydroxy-4-methoxybenzyl alcohol (Watanabe *et al*., 2012). Carotenoids were isolated from *Mytilus galloprovincialis* (Black Sea, Ukraine), with absolute configurations attributed to and via comparison of ECD spectra with previously reported data (Maoka *et al*., 2011).

Bathymodiolamides were isolated from the mussel *Bathymodiolus thermophilus* collected at 1700 m depth near the hydrothermal vents on the Mid-Atlantic Ridge, and were found to inhibit tumour cell growth. Variety of bioactive fatty acid derivatives have been isolated from marine organisms, and the most unusual example is a highly chlorinated sulfolipid, isolated as a cytotoxic principle from the mussel *Mytilus galloprovincialis* (White *et al*., 2004). The ω-conotoxin peptide known as ziconotide (Prialt®) was isolated from the fish-hunting marine mollusk, *Conus magus*, and found utility as a therapeutic for severe pain. Ziconotide functions through binding as an antagonist to N-type voltage-gated calcium channels (Teichert and Olivera, 2010)

New onchidioneanalogues ilikonapyrone esters with potential cytotoxicity were reported from different mollusks belonging to *Onchidium* species. A halogenated tyrosine derivative was isolated from the marine mollusk *Buccinum undatum* (Hunt and Breuer, 1971).

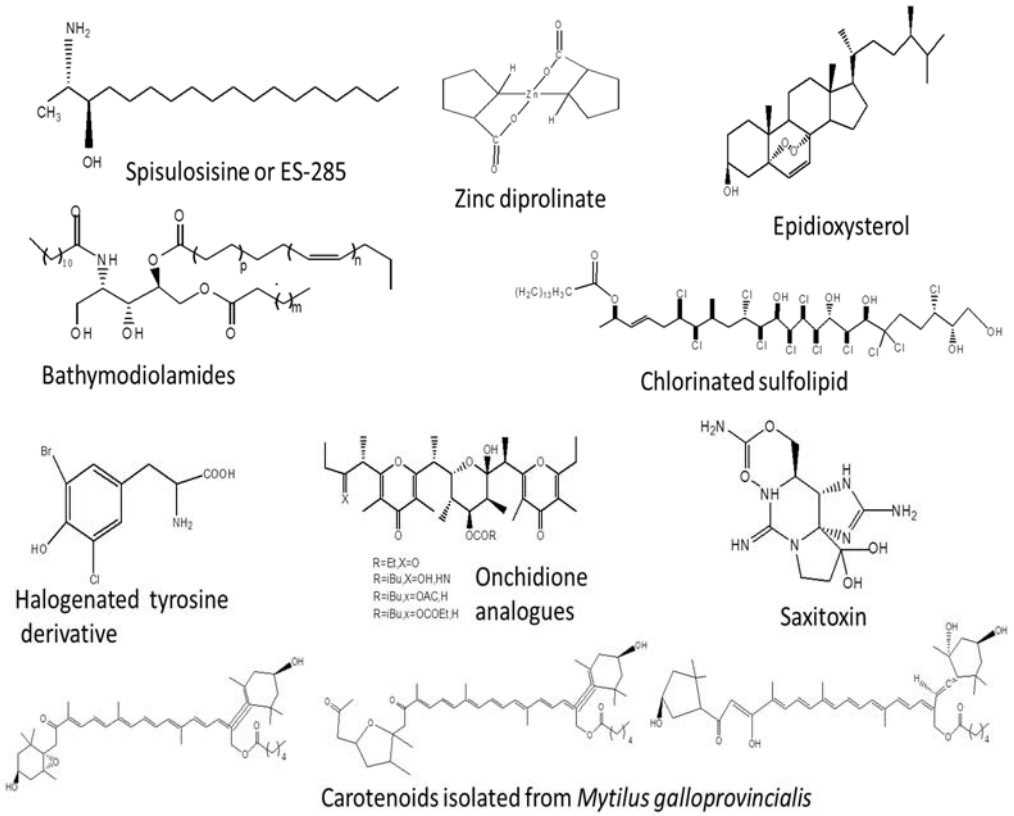


Fig 2.14 Examples of compounds isolated from bivalves

2.5.8 Compounds isolated from cephalopods

Cyclophosphamine, a well-known chemotherapeutic drug induced hemopoietic injuries, has been extracted from the squid ink (Zhong, 2009). Cyclophosphamide is commonly used to treat cancers and autoimmune diseases. Cytotoxic tyrosinase was isolated from the ink of the cuttlefish *Sepia officinalis* (Russo *et al.,* 2003). A cardioactive peptide was isolated from the brain of the common octopus, *Octopus vulgaris* (Kanda & Minakata, 2006). Baker & Murphy (Baker & Murphy, 1981) reported the occurrence of aromatic amino acids, noradrenaline and some benzoquinols from cephalopods.

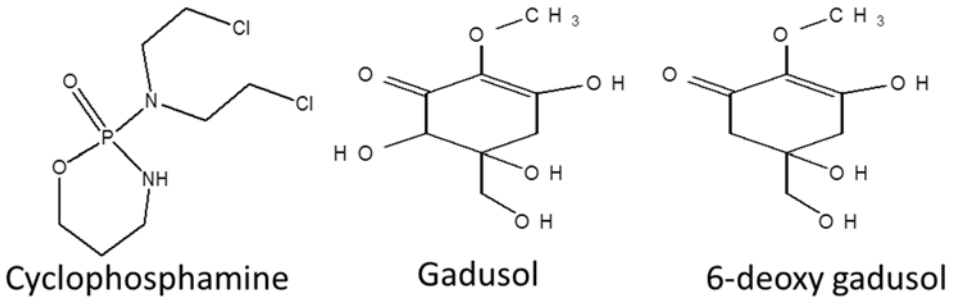


Fig 2.15 Examples of compounds isolated from cephalopods

2.5.9 Polysaccharides isolated from mollusks

Polysaccharides and polysaccharide–protein complexes have been found as common polymers in shellfish, and received considerable attention in recent years because of their anti-inflammatory, antioxidant, antitumor and immunoregulatory activities (Liu *et al*., 2008, Liu *et al*., 2007). Polysaccharides have been used for decades to stimulate the immune system, and to fight against cancer. A glycogen complex extracted from *Perna canaliculus*, was reported to pssess anti-inflammatory activity (Miller *et al*., 1993). Bioactive lipids, and specifically phospholipids are important part of the intestinal mucus (Kao, 1991), and an important regulator of epithelial function during inflammation by inhibiting cytokine tumor necrosis factor (TNF-α). Green lipped mussel from New Zealand hab been shown to contain unique *n*-3 fatty acids, which appear to act as dual inhibitor of arachidonic acid oxygenation by both cycloxygenase and lipoxygenase pathways (Tiffany *et al*., 2002), thus decreasing prostaglandin and leukotriene synthesis and downregulating the inflammatory sequence. Polysaccharides are potentially useful, and are biologically active ingredients for pharmaceutical uses due to diverse biological activities, such as, immunological, anti-radiation, anti-blood coagulation, anti-cancer, anti-human immunodeficiency virus (HIV), and hypoglycemic activities (Yoon *et al*., 2003; Yang *et al*., 2005). The activity of polysaccharides is closely related to their structure-related aspects,

such as, molecular mass, degree of substitution, degree of branching, sugar component and the structure of main chain and branches (Bohn and BeMiller, 1995). Qin (Qin, 1993) found that Misgurnus anguillicaudatus polysaccharide (MAP) played an important role in the prevention of oxidative damage in the living systems. Polysaccharide isolated from the cuttlebone of *Sepia aculeata* and *Sepia brevimana* were found to possess potential antibacterial and antifungal activity (Shanmugam *et al*., 2008).

2.6 Functional foods from marine sources

Naturally occurring bioactive extracts or single compounds are believed to benefit human health and research resulting in substantial advances in nutritional knowledge. There is also growing awareness that dietary source and form of food may affect overall health. Suitably, the role of food as an agent for improving health has been recognized, initiating the development of new classes of compound from marine environment. The added bioactive compounds are usually referred to as functional ingredients, and they are responsible for the functional bioactivities. The marine ecosystem is an untapped reservoir of biologically active compounds, which have considerable potential to supply food ingredients towards the development of new functional foods.

2.6.1 Free radicals

Oxidation is an essential process for all living organisms for the production of energy necessary for biological processes (Duan *et al*., 2006). The most common reported cellular free radicals are singlet oxygen, hydroxyl ($OH\cdot$), superoxide ($O^{2-\cdot}$) and nitric monoxide ($NO\cdot$). Also, some other molecules, such as, hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) were reported to generate free radicals in living organisms through various chemical reactions (Halliwell, 2006). Under normal circumstances, cells are able to defend

themselves against ROS induced damage with enzymatic antioxidants, such as, superoxide dismutase, catalase, glutathione peroxidase and non-enzymatic antioxidants such as vitamin E, C and glutathione. The equilibrium between the production and the scavenging of ROS may be perturbed by various biotic and abiotic stress factors, such as, salinity, UV radiation, drought, heavy metals, temperature extremes, nutrient deficiency, air pollution, herbicides and pathogen attacks.

These disturbances in equilibrium lead to sudden increase in the intracellular levels of ROS, which can cause significant damage to cell structures (Gill and Tuteja, 2010). This is associated with the onset of a variety of chronic diseases, such as, cancer and free radical induced degenerative diseases. Antioxidant molecules present in different mollusks were found to prevent cell damage from oxidative reaction in our body (Nagash *et al*., 2010). Furthermore, the radicals also play a significant role in the process of ageing and carcinogenesis (Cuzzocrea *et al*., 2001).

2.6.2 Free radicals and inflammation

Reactive oxygen species (ROS) are important mediators that initiate and propagate inflammatory responses by stimulating release of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumour necrosis factor (TNF-α) (Geronikaki and Gavalas, 2006). Inflammation is part of the complex biological response of vascular tissues to harmful stimuli, such as, pathogens, damaged cells, or irritants. The signs of acute inflammation are pain, heat, redness, swelling, and loss of function. Inflammatory diseases include rheumatoid arthritis, atherosclerosis, Alzheimer dementia, asthma, psoriasis, multiple sclerosis, and inflammatory bowel diseases (Gautam *et al*., 2009, Rang *et al*., 2012). Chronic inflammatory diseases constitute a major

cause of mortality and the World Health Organization (WHO) reported chronic noncommunicable conditions to be the leading cause of mortality in the world, representing 35 million deaths during 2005 (Tunstall, 2006).

The harmful action of the free radicals can, however, is blocked by antioxidant substances, which scavenge the free radicals and detoxify the organism. Endogenous antioxidants in the human body act through different types of mechanisms, such as, reducing the ROS generation, stopping the ROS formation chain reaction, scavenging ROS with antioxidant enzymes and reparing lesions caused by ROS via specific enzymes, such as, endonucleases, peroxidases, lipases, etc. All these antioxidant systems act differently, depending on their structure and properties, their hydrophilic or lipophilic characters, and also depending on their localization (intracellular or extracellular, in cell or organelles membrane, in the cytoplasm, etc.).

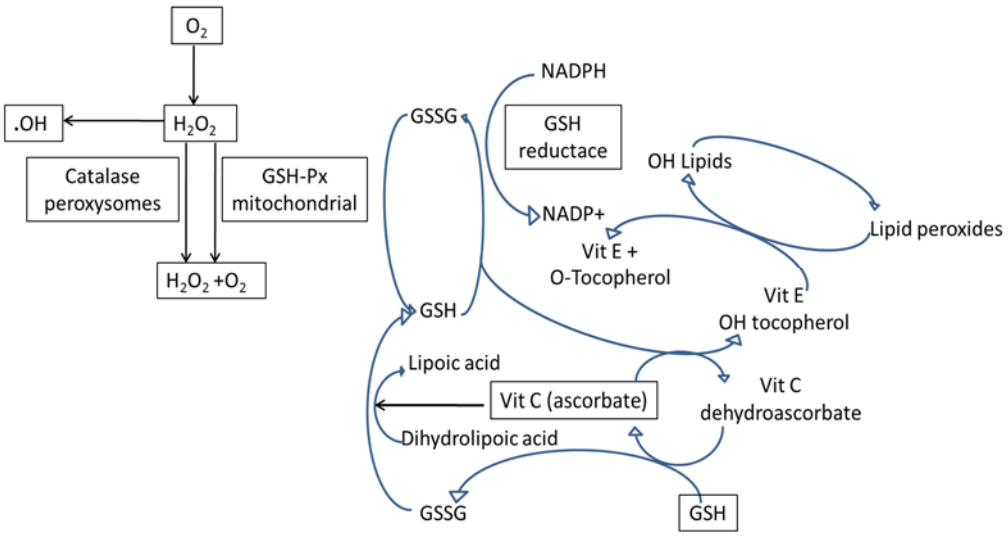


Fig.2.16 Antioxidant defenses against ROS to eliminate free radicals

The antioxidant defenses against ROS have been explained in **Fig 16**. SOD is considered a first-line defense against ROS. This enzyme is present in

nearly all cells, and converts $^{\bullet}O_2^-$ into H_2O_2. Mitochondrial and bacterial SOD contains Mn, while cytosolic SOD is a dimer containing Cu and Zn. As the H_2O_2 may still react with other ROS, it needs to be degraded by either one of the other two antioxidant enzymes, GSH-Px or catalase. GSH peroxidase is located in the mitochondria. It catalyzes the degradation of H_2O_2 by reduction, where two gluthathione (GSH) molecules are oxidized to glutathione disulfide (GSSG). Regeneration of GSH by GSH-reductase, requires NADPH, which is oxidized to $NADP^+$. Catalase, on the other hand, is localized primarily in peroxisomes, and detoxifies the H_2O_2 that diffuses from the mitochondria to the cytosol, converting it into water and molecular O_2. There are also nonenzymatic antioxidant mechanisms, which help to regenerate GSSG back into GSH (Maria-Luisa *et al*., 2013).

Current research in free radicals has confirmed that foods rich in antioxidants might play an essential role to prevent cardiovascular diseases, cancers, neurodegenerative diseases, including Parkinson's and Alzheimer's diseases, as well as inflammation and problems caused by cell and cetaceous aging (Li *et al*., 2007). Hence, consumption of antioxidants has been advocated to be useful for health as they are found to help neutralize these excess free radicals produced in the body.

2.6.3 Natural and synthetic anti-oxidants

Currently, reactive oxygen species in the food industry is being controlled or minimized by the addition of commercial synthetic antioxidants like BHA (butylated hydroxyanisol), BHT (butylated hydroxytoluene) and tBHQ (*tert*-butyl hydroquinone), which are able to reduce the free radicals causing oxidation reactions. Recently, much attention is being paid to screen natural molecules to prevent several clinical situations, such as, carcinogenicity (Kim *et al*., 2002). In

the search of new antioxidants, exploration of marine habitats has led to the discovery that marine plants and invertebrates, as potential reservoirs of antioxidants.

2.6.4 Current symptomatic treatment of inflammation

Current symptomatic treatment of inflammation is mainly by non-steroidal anti-inflammatory drugs (NSAIDs) that exert anti-inflammatory activities by inhibiting cycloxygenase (COX) enzymes, which exist as two isoforms: COX_I and COX_{II} (Jaggi *et al*., 2004). COX is a bi-functional enzyme containing a site that converts arachidonic acid (ARA) to prostaglandin G2 (PGG_2), and another site that reduces PGG_2 to prostaglandin H_2 (PGH_2) (Fernandes *et al*., 2004). Prostaglandins are potent mediators of inflammation that result in edema, pain and vasodilation.

2.6.5 Drawbacks of current treatment of inflammation

NSAIDs produce intestinal tract ulcers (with potential internal bleeding) in 10-30 percent of long-term users, and erosions of the stomach lining and intestinal tract in 30-50 percent of cases (McPhee *et al*., 2007). Selective COX_{II} inhibitors (coxibs) have come to attention in recent years, although they too exhibit side effects, most notably in relation to cardiovascular disturbances. As a result, medical researchers are looking for safer, more efficacious alternatives to both the traditional NSAIDs and the coxibs. There is a tradition of using shellfish supplements as a remedy for arthritis among indigenous people, notably in Western Mexico and throughout the South Pacific (Whitehouse *et al*., 1997). Freeze-dried extract of New Zealand green-lipped mussel *Perna canaliculus* (Seatone®) was shown to be useful to inhibit experimentally induced inflammation in the treatment of rheumatoid arthritis and osteoarthritis (McPhee *et al*., 2007).

2.6.6 Natural alternatives from marine sources

The marine world, due to its phenomenal biodiversity, is a rich natural resource of many biologically active compounds, such as, polyunsaturated fatty acids (PUFAs), sterols, proteins, polysaccharides, antioxidants, and pigments. Marine-based bioactive food ingredients can be derived from many sources, including marine plants, microorganisms, and sponges, all of which contain their own unique set of biomolecules (Rasmussen *et al*., 2007). However, these naturally occurring bioactive substances has defined health benefits on the human body. Increasing knowledge regarding the impact of diet on human health along with the state-of-the-art technologies has led to significant nutritional discoveries, product innovations, and mass production on unprecedented scale.

Research on the bioactive compounds in marine mollusks has provided interesting leads for the development of a range of complementary and alternative medicines, with potential pharmaceutical values. Mollusk-derived secondary metabolites have yielded a number of approved therapeutic drugs, as well as compounds which are still in clinical trials. Molluskan derived compounds in clinical use or final stage clinical trials include the analgesic zinconotide (Prialt®); a synthetic-conotoxin peptide from the cone snail *Conus magus* and the anti-lymphoma Brentuximab vedotin Dolstatin-10. Other novel mollusk compounds in the clinical trial include anti-tumor depsipeptide kahalalide F, isolated from the sea slug *Elysia rufescens* and anti-tumor agent ES-285, obtained from the clam *Mactromeris polynima* (Rudd and Benkendorff, 2014). Freeze-dried extract of New Zealand green-lipped mussel *Perna canaliculus* (Seatone®) was shown to be useful to inhibit experimentally induced inflammation in the treatment of rheumatoid arthritis and osteoarthritis (McPhee *et al*., 2007). Lyprinol, a commercially available preparation of *P.*

canaliculus, is the mussel oil obtained by CO_2 supercritical fluid extraction, which showed potential anti-inflammatory activity (Whitehouse *et al*., 1997). The coastline of South Indian subcontinent is bestowed with large assemblage of *P. viridis,* although they have not been explored in detail regarding their potential to be used as nutritional supplement. Considering the importance of this species, the present study is envisaged to prepare a freeze-dried concentrate from *P. viridis* collected from the South West coast of India.

Fig. 2.17 *P. viridis* along the southwestern coast of Kerala

However, the lipidic ingredients including LC-PUFAs in the freeze-dried concentrate are susceptible to peroxidation (or oxidation) due to the presence of olefinic bonds in their structure resulting in low molecular weight aldehydes, ketones, and deleterious free radical species under shelf, and are major risk factors for cardiovascular and inflammatory diseases. This very important phenomenon is

often overlooked by the producers and consumers because we cannot visualize their effect immediately, though these undesirable oxidation products and *trans*-fatty acids will lead to a cascade of immunosystem compromising diseases in long run. It is, therefore, imperative to arrest the oxidation of these fatty acids by the addition of antioxidants, and to retain its nutritional properties for a longer time period in shelf. In recent years, the use of antioxidants of natural origin is considerably enhanced by the concern about the adverse side effects of these popularly used synthetic antioxidants and other analogues. Hence we focused to explore the natural alternatives to arrest the degradation of vital nutrients with respect to long chain polyunsaturated fatty acids (PUFAs) of the *P. viridis* concentrate. Keeping these facts as background information, the present study has attempted to prepare functional foods from marine derived bioactive compounds for the prevention and treatment of anti-inflammatory and chronic diseases.

Chapter 3

NUTRITIONAL IMPORTANCE OF *PERNA VIRIDIS, CRASSOSTREA MADRASENSIS* AND *OCTOPUS DOLFFUSI*

Contents

Background of the study

Mollusks are widely distributed throughout the world, and have many representatives in the marine and estuarine ecosystems, namely, bivalve mollusks and cephalopods. The estuarine representatives are subjected to both diurnal and seasonal changes in the hydrological parameters of aquatic organisms. The impact of this will be more on resident species particularly oysters, clams and mussels etc. (Ansell, 1974a.). Location specific variations in growth and survival of bivalves have been demonstrated for *Mytilus edulis* (Incze *et al*., 1980), *Mya arnaria* (Appeldoorn, 1983), *Crassostrea virginica* (Mallet and Haley, 1983), *Crassostrea gigas* (Brown and Hartwick, 1988a) and *Ostrea edulis* (Utting, 1988).

Bivalve mollusks and cephalopods are highly delicious seafood because of their nutritive value next to fin fishes and crustaceans. Bivalves in coastal area are fairly good sources of *n*-3 PUFAs, including the long-chained eicosapentaenoic acid (20:5*n*-3; EPA) and docosahexaenoic acid (22:6*n*-3; DHA) with valuable pharmaceutical and biomedical potentials (Taylor and Savage, 2006). The

consumption of bivalve mollusks in India, particularly south Malabar area has increased in the recent years in response to the greater availability under wild and cultured conditions. Bivalve mollusks were reported to contain bioactive lipids, which include fatty acids, sphingolipids, phytosterols, diacylglycerols etc. and many of these can influence human health.

The green mussel *Perna viridis* (family: Mytilidae) is a bivalve mollusk which is native of the Indian coast and throughout the Indo-Pacific and Asia-Pacific (Benson *et al*., 2001). It forms a significant fishery and contributes nearly 50 percent to the total bivalve production of the area (Laxmilatha *et al*., 2011).

The edible oyster *Crassostrea madrasensis* is a commercially important bivalve mollusk distributed all along the east and west coasts of India. This Indian backwater oyster (*Crassostrea madrasensis*) locally known as 'Kadal muringa' is found in the backwaters and estuarine regions of Kerala

Cephalopods are valued marine organisms due to their nutritional, commercial and ecological importance. Mediterranean countries are top consumers of cephalopods being only equalled or surpassed by some Asian countries (FAO, 2012). The main reasons for the increasing demand of the cephalopods in international market are due to the fact that, these species are good source of protein and essential lipids (Sinanoglou and Miniadis-Meimaroglou, 2000), and represents an alternative to the exploited fish resources. Biochemical analyses of octopus tissues could provide valuable information regarding nutritional requirements and nutritional qualities for human consumption.


A

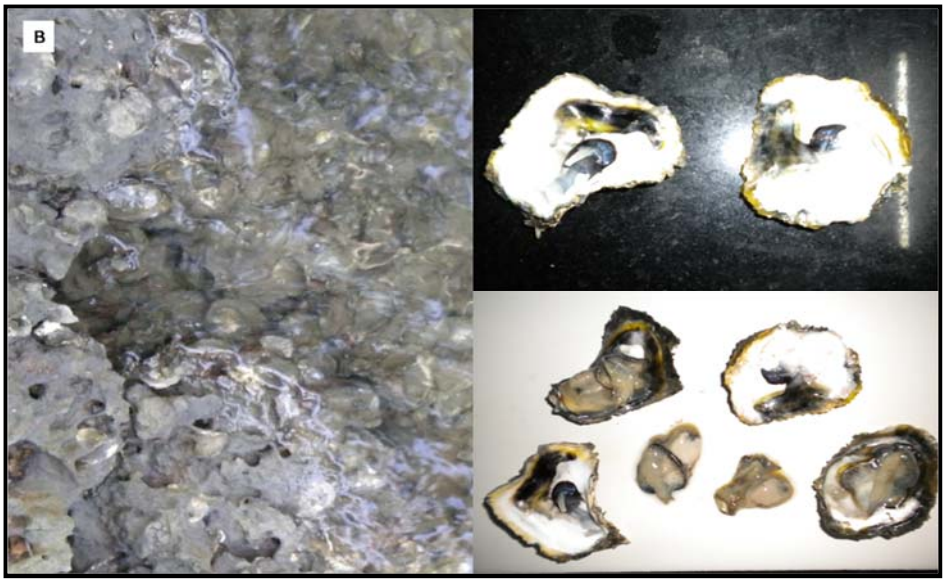

B

Fig 3.1. A: *P. viridis* samples collected from Kozhikode; **B:** Wild *C. madrasensis* samples from Cochin; **C**: *O. dolffusi* samples from Fortkochi;

The present investigation was therefore carried out to provide complete information on different nutritional qualities of *Perna viridis, Crassostrea madrasensis* and *Octopus dolffusi*. The present study also correlated the impact of seasonal and spacial variation on the essential nutritional compositions with respect to amino acids, cholesterol, proteins, lipids, and fatty acids of these mollusks harvested from south west coast of Kerala.

3.1 Methods

3.1.1 Proximate composition

The proximate composition analyses of the edible portion of the mollusks were performed as follows: Moisture was determined by oven drying at 105°C to constant weight (AOAC, 1990). Dried samples were used for

determination of crude fat, crude protein and ash contents. All analyses were done in triplicate. The crude protein was determined by the Kjeldahl method (AOAC, 1990). Crude fat was extracted from the dried tissues using Bligh and Dyer (Bligh and Dyer, 1959) method. The fat content was gravimetrically determined. Ash was determined gravimetrically in a muffle furnace by heating at 550 ºC constant weight (AOAC, 1990).

3.1.2 Lipid and fatty-acid profile of the candidate mollusks

3.1.2.1 Estimation of total lipids

The edible portion of each species (100 g) were left to extract lipids overnight in the dark in 1:1 of chloroform:methanol (2:1, v/v) and distilled water (200 mL). The lipid extracts were washed according to the Folch procedure (Folch *et al*., 1957) and the organic layer containing lipids was evaporated *in vacuo*. The lipid content was determined gravimetrically in triplicate and expressed as percent (w/w) of the edible portion.

3.1.2.2 Fatty acid profiling by gas-chromatographic analysis

The fatty acid compositions of the lipids were performed by GLC according to the established procedure (Chakraborty & Paulraj, 2008; Chakraborty & Paulraj, 2009). The fatty acid converted to fatty acid methyl esters (FAMEs) were recorded on a Perkin-Elmer (USA) AutoSystem XL gas chromatograph (HP 5890 Series II) connected with a SP 2560 (crossbond 5 percent diphenyl- 95 percent dimethyl polsiloxane) capillary column (100 m X 0.25 mm i.d., 0.50ìm film thickness, Supelco, Bellfonte, PA) using a flame ionization detector (FID) equipped with a split/splitless injector, which was used in the split (1:15) mode. The analyses were accomplished using an oven temperature ramp program: 140°C for 1 min, rising at 30°C / min to 250°C, where it was held for 1.0 min, followed by an increase of 25°C /min to 285°C,

where it was held for 2.0 min, until all peaks had appeared. The injector and detector were held at 285°C and 290°C, respectively. Nitrogen (ultra high purity > 99.99 percent) was used as the carrier gas with a pressure of 5.6 x 103 kg/m^2 and flow rate of 25 mL /min. The flow rate of hydrogen (45 mL/min) and air (450 mL/min) were maintained at a pressure of 3.5 x 104 kg/m^2. The injection volume was 0.2 µL FAMEs were identified by comparing the retention time of the samples and appropriate FAME standards and the relative percentage of the area was obtained by using the subsequent formula: Percent of total fatty acid (percent TFA) = (Area of the methyl esters / Total area of the chromatogram) X100; where. Peak areas lower than 0.1percent of the total area was not considered. Three replicate injections were performed and the mean results were expressed as percent of total fatty acids (percent TFA) ± standard deviation.

3.1.2.3 Evaluation of fatty acid based nutritional indices

The different nutritional indices with respect to fatty acids, such as, *n*-3/*n*-6, *n*-6/*n*-3, DHA/EPA, PUFA/SFA and LA/ALA ratios were calculated in order to allow comparisons with the United Kingdom department of health recommendations (HMSO, 2001). The indices of atherogenicity (AI) and thrombogenicity (TI) (Ulbricht & Southgate, 1991) have been calculated as: AI = (4 * 14:0 + 16:0 + 18:0) / (MUFA + *n*-3 PUFA + *n*-6 PUFA) ; TI = (14:0 + 16:0 + 18:0) / [(0.5 * MUFA) + (0.5 * *n*-6 PUFA) + (3 * *n*-3 PUFA) + (*n*-3 PUFA/*n*-6 PUFA). The hypercholesterolaemic (HH) ratio were determined as, HH= (18:1*n*-9 + 18:2*n*-6 + 20:4*n*-6 + 18:3*n*-3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3)/ (14:0 + 16:0) (Santos-Silva *et al*., 2002).

3.1.3 Determination of true protein and amino acids

The protein contents of the mollusks were estimated by the established method (Lowry *et al*., 1951). The absorbance of the protein aliquot was measured at 660 nm in a UV-visible spectrophotometer within 15 min against the reagent blank. The protein content of the sample was calculated from the standard curve of bovine serum albumin, and expressed as g $100g^{-1}$ edible portion. Amino acid content of the mollusks was measured using Pico-Tag method as described earlier Heinrikson and Meredith (Heinrikson and Meredith, 1984) using suitable modifications. The sample (0.1 g) was hydrolysed for 24 h at 110°C with 6 M HCl in sealed glass tubes filled with nitrogen. The hydrolyzed samples were treated with redrying reagent (MeOH 95percent: water:triethylamine, 2:2:1 v/v/v), and thereafter pre-column derivatization of hydrolysable amino acids was performed with phenylisothiocyanate (PITC, or Edman's reagent) to form phenylthiocarbamyl (PTC) amino acids. The reagent was freshly prepared, and the composition of derivatizing reagent (methanol 95percent: triethylamine: phenylisothiocyanate, 20 µL, 7:1: 1 v/v/v). The derivatized sample (PTC derivative, 20 µL) was diluted with sample diluent (20 µL, 5 mM sodium phosphate $NaHPO_4$ buffer, pH 7.4: acetonitrile 95:5 v/v) before being injected into reversed-phase binary gradient HPLC fitted with a packed column (dimethylocatadecylsilyl- bonded amorphous silica; Nova-Pak C18, 3.9 X 150 mm) maintained at 38±1°C in a column oven which connected to a Waters 2487 dual absorbance detector (ëmax 254 nm). The mobile phase eluents used were eluents A and B, whereas eluent A comprises sodium acetate trihydrate (0.14 M, 940 mL, pH 6.4) containing triethylamine (0.05percent)., mixed with acetonitrile (60 mL), and eluent B used was acetonitrile : water (60:40, v/v). A gradient elution programme, with increasing eluent B was employed for this purpose. An additional step of 100percent eluent B is used to wash the column prior to

returning to initial conditions. Standard was run prior to sample injection. Samples (PTC amino acid derivatives) were injected in triplicate, and the output was analyzed using BREEZE software (Waters Corporation, Milford, MA 01757). The quantification of amino acids was carried out by comparing the sample with the standard, and the results were expressed in g $100g^{-1}$ edible portion. The total essential amino acids (TEAA), total non-essential amino acids (TNEAA), total amino acids (TAA) and the ratio of total essential amino acid to the total non essential amino acid (EAA/NEAA) were calculated.

3.1.4 Total cholesterol content

The total cholesterol content in the edible portion of the mollusks were determined spectrophotometrically (Varian Cary, USA) as described elsewhere (Wanasundara and Shahidi, 1999) with suitable modification using o-phthalaldehyde (50 mg dL^{-1} in glacial acetic acid). The total cholesterol content of the samples were calculated from the standard curve of cholesterol, and expressed as mg/ 100g edible portion (mg/ 100g EP).

3.1.5 Estimation of minerals

The estimation of minerals was carried out by atomic absorption spectrophotometer (AAS) following the di-acid ($HNO_3/HClO_4$) digestion method with suitable modifications (Astorga-Espana *et al*., 2007). In brief, the edible portions of mollusks (2 g) were placed in digestion tubes, to which concentrated HNO_3 (7 mL) was added, and the content was kept for overnight digestion in a fume hood until no brown fumes appeared. The digestion was continued over the sand bath with HClO4 (6 mL) until the color of the solution became pale yellow to colorless. The solution was thereafter cooled and filtered through Whatman No. 1 filter paper. The filtrate was diluted with distilled water (50 mL) to be injected in atomic absorption spectrophotometer

Se, continuous flow hydride generator coupled with atomic absorption spectrometer was used. Phosphorus content was analyzed by alkalimetric ammonium molybdophosphate method as described in AOAC official method 964.06 (AOAC, 2005). The Macro minerals (Na, K, Ca and P) are represented in mg $100g^{-1}$ edible portion and the micro minerals (Fe, Mn, and Zn) are represented in mg $100g^{-1}$ edible portion

3.1.6 Estimation of vitamins

The estimation of fat soluble vitamins (A, D_3, E and K_1) in the edible portions of the mollusks was carried out using a modified method of Salo-Vaananen *et al*. (Salo- Vaananen *et al*., 2000). The stock solutions of vitamin standards were prepared °C (1, 10, 25, 50, & 100 ppm) to construct the standard curve by HPLC. All the stock solutions were stored at -20°C except vitamin D_3 where the stock solutions were stored at 4°C. The lipids were extracted using the procedure described in 3.1.2.1, which was hydrolyzed, with KOH/MeOH (0.5N, 2 mL) at 60°C for 30 min to furnish the hydrolyzed mixture, which (2 mL) was thereafter extracted with petroleum ether (12 mL), and washed with distilled water (2 x 8mL) to make it alkali-free. The nonsaponifiable matter (8 mL) was concentrated using a rotary evaporator (50°C), reconstituted in MeOH, and filtered through nylon acrodisc syringe filter (0.2 µm) to be injected (20 µL) in HPLC equipped with a C18 column (Phenomenex, 250 mm length, 4.6 mm I.D., 5ìm) in a column oven (32°C). The run time was 45 min, and the eluents were detected at 265 nm (UV-VIS detector) using the gradient programme as follows: 20percent MeOH upto 3 min, which was increased to 100percent in next 5 min and held for 37 min. The flow rate was 1 mL/min. The vitamins A, D_3, E, K_1 were expressed as µg $100g^{-1}$ edible portion. The water soluble vitamin, ascorbic acid, was determined based upon the quantitative discoloration of 2, 6-dichlorophenol

indophenol titrimetric method as described AOAC (AOAC, 2005). The vitamin C was expressed as μg $100g^{-1}$ edible portion.

3.1.7 Statistical analyses

Statistical evaluation was carried out with the Statistical Program for Social Sciences 13.0 (SPSS Inc, Chicago, USA, ver. 13.0). Descriptive statistics were calculated for all the studied variables. Analyses were carried out in triplicate, and the means of all parameters were examined for significance (p = 0.05) by analysis of variance (ANOVA). The Pearson correlation test was used to assess correlations between means. The level of significance for all analyses was $P \leq 0.05$.

3A. Nutrient profiling of green mussel (*Perna viridis* L.) from southwest coast of India

General

The green mussel, *P. viridis*, is a bivalve mollusk (family *Mytilidae*) which is found in the coastal sea beds in the Arabian Gulf in south Malabar waters along the west coast of Peninsular India. In India, two species of mussels (green mussel *P. viridis* and brown mussel *P. indica*) support a traditional sustenance fishery in Malabar and Konkan areas of Penninsular India. India has risen to one among the top ten cultured mussel producing nations in Asia, with an annual production of 10,060 tonnes (Kripa & Mohammed, 2008). The consumption of bivalve mollusks in India, particularly south Malabar area has increased in the recent years in response to the higher availability from wild and cultured conditions.

The potential health benefits related to marine mussel consumption are due to the presence of inexpensive source of protein/amino acid with high biological value and polyunsaturated fatty acids (PUFAs) especially long-chain *n*-3 PUFAs containing mainly eicosapentaenoic acid (20:5*n*-3; EPA) and docosahexaenoic acid (22:6*n*-3; DHA) which have valuable pharmaceutical and biomedical potential (McLean & Bulling, 2005) Proteins, lipids, minerals and fatty acids, contribute to the nutritional value and organoleptic characteristics of *P. viridis*. In addition, it contributes to the intake of essential minerals and trace metals and certain vitamins (Astorga *et al*., 2007).Recently, after the importance of *P. viridis* as a potential health food had been realized, studies on its biochemical composition began to receive considerable attention. It is anticipated that the determination of the essential nutritional composition of wild *P. viridis vis-à-vis* cultured one will provide the necessary

information concerning the nutrient value of this seafood for both to the consumers and to the researchers working on nutrient tables.

Among the different parameters of quality, biometric measurements and chiefly meat yield are important aspects of mussel marketability. Biochemical changes in the mussel from different sites and growth conditions may result from fluctuations of environmental parameters, *viz*., temperature, salinity, oxygen levels, and to the physiological status of the animals, depending on food availability, gametogenic cycle, and spawning (Livingstone, 2001). Earlier studies indicated the influence of environmental and nutritional conditions on the composition of bivalves (Khan *et al*., 2006; Murphy *et al*., 2002).

No reports have yet been published about the essential nutritional composition of the green mussel under wild and cultured condition. The data will provide useful information for food industries and mussel aquaculture. Therefore, considering the promising perspective for the utilization of *P. viridis*, and the need for knowledge of its biochemical composition, this work explores the influence of the growth conditions on the essential nutritional compositions of *P. viridis* harvested from different sites in south western coast of India.

3A.1 Samples collection and processing

3A.1.1 Samples collection

P. viridis (green mussel) samples (wild and cultured) were collected from two different sites (Cochin and Kozhikode) along the southwest coast of India. The sample collection sites of green mussel (*P. viridis*) (wild and cultured) from two different sites (Cochin and Kozhikode) are depicted in **Fig.3A.1.** The first one is a relatively clean site on intertidal rocky shore of the Sattar island at Cochin (UST: 1141160E, 221200N) (Lat: $9^0$58'N; Long: $76^0$16'E) in the southwestern coast of India, predominantly influenced by oceanic water from the Arabian Sea.

During the same period *P. viridis* samples were collected from Elathur (Lat: 11054'11.6"N; 75^0 12'. 21.8''E), located about 10 km north of Kozhikode city and is bounded by the Arabian Sea on the west and the Korapuzha River (Elathur River) on the north along the Southwestern coast of India. **Fig 3A.1** illustrates the sampling locations of *P. viridis*. *P. viridis* from both sites were collected at comparable depths (2 m below the surface) to avoid confounding effects of depth on the fatty acid composition. Cultured mussels were stripped from the 1/4" rope, at each site and transported for analyses following the protocol. Gender differences of the representative samples (n = 3 at each determination) were not treated. All live samples thus collected were immediately placed on ice for transportation, in isothermic boxes. Upon return, these samples were cleaned of fouling organisms and placed in filtered (1.0 mm) seawater at the ambient temperature The tissues of mussels from the two sites were combined, minced, and the samples were stored in low temperature (-20°C) until further use for biochemical characterization.

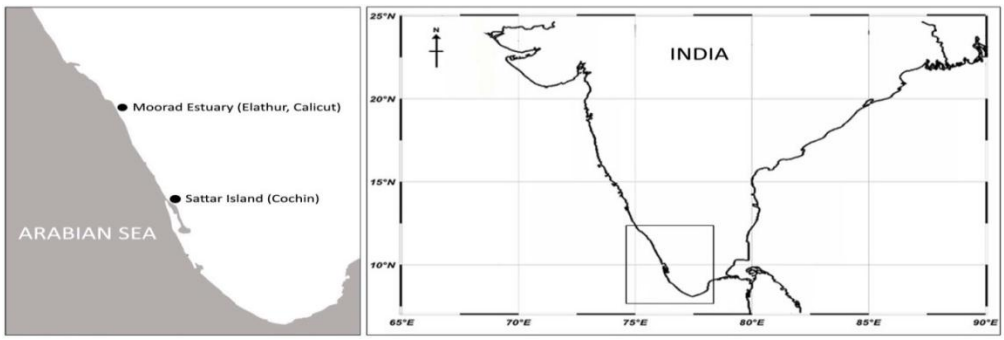


Fig.3A.1 Sample collection site of green mussel (*P. viridis*) (wild and cultured) from two different sites (Cochin and Kozhikode) along the Southwestern coast of India. Sattar island at Cochin (UST: 1141160E, 221200N) (Lat: $9^0$58'N; Long: $76^0$16'E) and Elathur (Lat: 11054'11.6"N; 75^0 12'.21.8''E) were selected as respective collection sites of *P. viridis*.

3A.1.2 Biometric parameters, condition indices, and meat yield of *P. viridis*

P. viridis thus collected were measured for their biometrical parameters, *viz.*, length, width, and thickness. Length (maximum measure along the anterior-

posterior axis), width (maximum lateral axis), and thickness (depth of the maximum longitudinal axis) of 235 randomly selected mussels, were measured using a vernier calliper. The mussels were then weighed, opened by cutting the adductor muscle with a scalpel, and the wet meat and shell weight were noted. The tissues were oven dried for 48 h at 60°C, and the dry weight determined. Condition indices are used to characterize the apparent health and quality of a biological entity. Three condition indices (CI) were taken into account in this study are as follows: (1) Economic CI (Imai and Sakai, 1961) as thickness X [0.5 (length+width)]$^{-1}$; (2) Booth's CI (Booth, 1983) flesh weight X total weight^{-1}. (3) Ecophysiological CI (Walne, 1976) as dry flesh weight / dry shell weight. The dry weight of the shells was determined by oven-drying at 80^{o}C for 12 h. In brief, the whole weight of mussels was obtained on an electronic balance, before the meat was sucked. The dimensions of shells were measured using a vernier calliper, and weighed. The meat from each mussel was weighed to obtain wet weight and dry weight, the latter after desiccation kept in a freeze-dryer for 24 h at −100°C.

3A.2 Results and discussion

The present study provided a detailed biochemical profile of the green mussel, *Perna viridis* collected from the wild and cultured conditions, for the first time, from two prominent mussel beds from different geographical regions of the south-western coast of India (Cochin and Kozhikode). Considering the production trend of edible bivalves, where the contribution of cultured *P. viridis* showing an increasing trend, information on the food qualities of cultured with reference to the wild ones is of importance. Differential growth conditions (wild and cultured) of *P. viridis* were therefore studied to understand their effect on nutritional indicators. This is essentially to develop the *P. viridis* nutritional biomarkers with respect to the collection area and growth condition for use in human food and nutrition.

3A.2.1 Biometric characteristics, percent meat yield and condition index (CI)

The commercial quality and physiological state of bivalve mollusks are adequately described by Condition Index (CI), a parameter of economic relevance reflecting the ecophysiological conditions and the health of *P. viridis*. The wild samples collected from Kozhikode showed significantly higher (P<0.05) Imai and Sakai's CI than cultured samples (3.1). The Booth's CI registered maximum value for cultured samples, collected from Kozhikode. A low value of CI for both wild and cultured samples collected from Cochin indicates that a major biological effort has been expended, either as maintenance energy under poor environmental conditions or disease, or in the production and release of gametes during spawning. The values obtained in this study indicated that cultured samples from Kozhikode were in good condition than Cochin samples. In Goa region of south-western coast of India, *P. viridis* were reported to grow faster on ropes compared to those in natural beds (Qasim *et al*., 1977).Wild samples collected from Cochin exhibited significantly higher (P< 0.05) meat yield than recorded in cultured samples. The variations of percent meat and CI of *P. viridis* were statistically significant (P<0.05). CI and meat content of green mussels were registered to be affected by a variety of environmental and endogenous factors, *viz.*, water temperature, salinity, food availability, and gametogenic cycle of animals (Okumus and Stirling, 1998). However, the parameters of meat yield and condition index from wild and cultured *P. viridis* are within the range as reported earlier (Okumus and Stirling, 1998), and, therefore, appeared to be in good condition for consumption. Principle component analyses (PCA) were carried out to gain an overview of the similarities and differences among the various parameters of wild and cultured *P. viridis* collected from different geographical locations (Cochin and Kozhikode). The major nutritional and biochemical parameters of *P. viridis vis-*

à-vis different sampling locations/growth conditions were included as variables in a PCA model and illustrated under **Fig 3A.2**. The loading plot obtained by performing PCA on conditional indices viz., Imai and Sakai (IS), Walne, Booth and meat yield, which were included as variables, were shown in **Fig 3A.2**. The formation of group A which comprises conditional indices of Walne, meat yield (wild from Cochin and cultured from Kozhikode) and Booth (cultured from Cochin and wild from Kozhikode) showed a high positive correlation with PC 1 (64.56%). PC 2 describes the remaining variability (35.44%). Correlation between various nutritional and antioxidative parameters of wild and cultured *P. viridis* from two geographical locations by loading plot diagrams (A-F) of various components (PC-1 and PC-2) in rotated space.

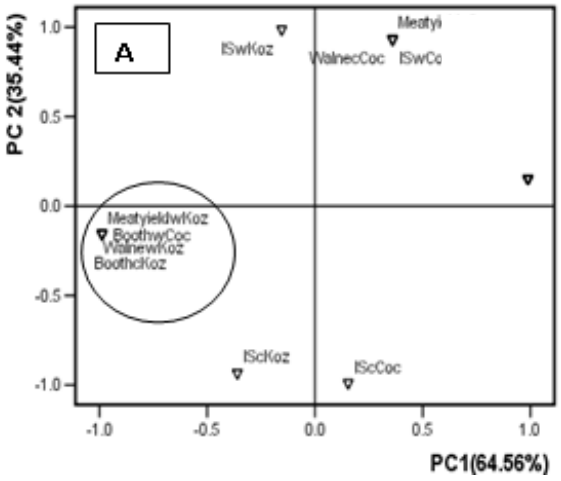


Fig 3A.2 The loading plot diagram (in PCA plot) showing the correlation of various condition indices (Imai and Sakai (IS), Walne, and Booth) *vis-à-vis* meat yields. Here, cCoc and cKoz signify cultured *P. viridis* from Cochin and Kozhikode, respectively, whereas wCoc and wKoz imply wild *P. viridis* from Cochin and Kozhikode, respectively.

3A.2.2 Proximate composition

The proximate compositions of mussels are characterized by phases of accumulation and depletion of food reserves, reflecting the stage of gonadal development, and availability of food. There was no marked variation in the

contents with respect to fat, crude fibre, and ash from different regions of the experimental area, as well as growth conditions (cultured and wild). The crude protein content varied from 7.14 -13.1%; the minimum of 7.14% was recorded in Cochin (wild) and maximum of 13.1% in samples collected from Kozhikode (wild) (**Table 3A.1**). Proximate composition analyses in *Mytilus galloprovincialis* from different Spanish origins revealed moisture content as 79-83g $100g^{-1}$, lipid 1.4-2.1 g $100g^{-1}$, and protein 6.5-10 g $100g^{-1}$ (Fuentes *et al*., 2009). An inverse relationship between moisture and protein content was apparent between Kozhikode and Cochin samples. Crude fat and ash recorded significant difference ($P<0.05$) between wild and cultured samples, and their fluctuations were also coincident with those of the crude protein. There appeared to be no significant differences in acid insoluble ash content of cultured samples collected from Cochin and Kozhikode ($P>0.05$). A positive correlation between CI and biochemical constituents of bivalves has been reported in different mollusk species (Orban *et al*., 2004).

Table 3A.1 Biometric measurements, condition index, meat yields (percent), proximate, lipid, protein, cholesterol, and mineral compositions of *P. viridis* collected from Cochin and Kozhikode

	Cochin		Kozhikode	
	wild	cultured	wild	cultured
Length (cm)	6.00 ± 0.01^{a}	8.10 ± 0.03^{b}	9.40 ± 0.07^{b}	6.33 ± 0.01^{a}
Width (cm)	2.59 ± 0.03^{a}	3.14 ± 0.03^{b}	4.10 ± 0.01^{c}	2.40 ± 0.14^{a}
Thickness (cm)	2.88 ± 0.09^{a}	3.96 ± 0.04^{b}	5.10 ± 0.01^{c}	1.90 ± 0.04^{d}
Meat yield (%)	28.4 ± 0.03^{a}	24.5 ± 0.04^{b}	27.9 ± 0.03^{a}	34.0 ± 0.01^{c}
Condition indices				
Imai and Sakai	0.63 ± 0.02^{a}	0.65 ± 0.05^{a}	0.86 ± 0.01^{b}	0.60 ± 0.02^{a}
Booth	0.28 ± 0.01^{a}	0.24 ± 0.04^{b}	0.28 ± 0.04^{a}	0.34 ± 0.02^{c}
Walne	0.06 ± 0.02^{a}	0.07 ± 0.01^{a}	0.06 ± 0.01^{a}	0.08 ± 0.01^{a}
Proximate composition (g $100g^{-1}$ wet sample)				
Dry matter	10.68 ± 0.01^{a}	17.86 ± 0.01^{b}	20.6 ± 0.02^{c}	16.8 ± 0.01^{b}

Moisture	89.32 ± 0.52[a]	82.14 ± 0.61[b]	79.3±0.60[b]	83.1±0.84[b]
Crude protein	7.14 ± 0.07[a]	12.02 ± 0.05[b]	13.1±0.18[b]	11.9±0.04[b]
Crude fat	1.27 ± 0.04[a]	1.90 ± 0.02[b]	1.96±0.02[b]	1.72±0.03[b]
Crude ash	1.42 ± 0.03[a]	1.71 ± 0.002[b]	1.65±0.02[b]	0.99±0.008[d]
Crude fiber	0.012 ± 0.002[a]	0.014 ± 0.001[b]	0.08±0.01[c]	0.07±0.007[d]
Carbohydrates (soluble)	0.84 ± 0.12[a]	2.22 ± 0.072[b]	3.78±0.31[c]	2.08±0.05[b]
Acid insoluble ash	0.61 ± 0.01[a]	0.09 ± 0.002[b]	0.13±0.01[c]	0.07±0.01[b]
Lipid (%)	1.47± 0.10[a]	2.63±0 .19[b]	2.00±0.13[c]	1.70±0.16[d]
Cholesterol (mg 100g-1)	96.6±0.78[a]	65.1±0.32[b]	58.61±0.42[c]	36.28±0.42[d]
Protein (mg 100g-1)	183.06±3.9[a]	236.6±5.38[b]	103.9±0.67[c]	220.3±1.83[d]
Macronutrients (mg 100g^{-1} wet sample)				
Na	1967.25± 14.32[a]	1942.00±26.05[a]	2136.5±1.59[b]	1195±2.12[c]
K	1450.50±84.73[a]	1563.25±87.24[b]	1532.2±3.80[b]	697.5±1.96[c]
Ca	287.00±13.41[a]	427.65±36.28[b]	280.92±0.53[a]	1095±9.2[d]
Mg	64.33±2.05[a]	353.58±8.49[b]	69.2±0.72[a]	515±1.45[c]
Micronutrients (mg 100^{g-1} wet sample)				
Cr	ND	0.17± 0.02[a]	0.365±0.07[b]	ND
Zn	1.55± 0.11[a]	2.57± 0.19[b]	3.03±0.01[c]	3.19±0.01[c]
Mn	ND	0.84± 0.05[b]	0.27±0.09[b]	ND
Cu	0.05± 0.01[a]	0.12± 0.01[b]	0.26±0.06[c]	0.22±0.01[c]
Fe	6.10± 0.08[a]	4.48± 0.25[b]	10.19±0.81[c]	4.61±0.07[b]
Se	0.04± 0.00[a]	0.03± 0.001[b]	0.04±0.01[a]	ND
Fat soluble vitamins				
Retinol (A)/IU	11.89±1.16[a]	12.71± 0.85[a]	8.20±0.15[b]	5.31±0.08[c]
Cholecalciferol (D_3)/IU	410.0±17.1[a]	442.0 ± 6.3[b]	352.5±4.3[c]	412.8±2.5[a]
Tocopherol (E)/IU	0.15±0.01[a]	0.14± 0.02[a]	0.13±0.01[a]	0.12±0.01[a]
Phylloquinone (K_1) (μg100g^{-1})	2.72±0.18[a]	2.65± 0.21[a]	1.26±0.02[b]	2.2±0.14[c]
Water soluble Vitamins				
Ascorbic acid (C)/IU	12.14±0.95[a]	9.28± 0.56[b]	12.74±0.15[a]	9.89±0.08[b]

All samples were analyzed in triplicate (n=3) from a pooled samples (20 numbers), and expressed as mean ± standard deviation. Means followed by the same letter within the same row are not significantly different, and different letters indicate significant differences (P<0.05). ND: non detectable.

3A.2.3 Protein, lipid and total cholesterol contents of *P. viridis*

Bivalve mollusks were reported to provide an inexpensive source of lipid and protein with high biological value (Astorga *et al*., 2007). The cultured samples collected from both the sites were found to possess the

significantly higher protein content (P<0.05) than their wild counterparts (**Table 3A.1**). The variations in protein contents appeared to be due to the differential availability of food (microalgae), which were reported to be major food sources of filter feeders *viz.*, bivalve mollusk *P. viridis* (Fernández-Reiriz *et al*., 1989). *P. viridis* from both locations and growth conditions were found to be rich in proteins with a low calorie count; they may therefore form an essential part of a healthy diet.

The cultured *P. viridis* harvested from Cochin registered higher lipid content (> 2. % than wild samples (<1.5%) (**Table 3A.1**). It is generally accepted that water temperature and differences in salinities are principal environmental factors affecting growth and gonadal development of marine bivalves (Pazos *et al*., 1996), which is a major reason for the differences in lipid content. The marginal differences in the lipid content of tissues of *P. viridis* apparently depend on the variation in the availability of food, temperature and microclimatic conditions. The higher relative percentage of diatoms (*Thalassiosira subtilis*, *Nitzschia seriata*, *Baciallria paradoxa*, and *Biddulphia mobilensis*) and dinoflagellates in their diet (*Ceratium furca*, *C. tripos*, *Gonyaulax spinifera*, and *Peridinium biconicum*) with high lipid content appeared to contribute to the lipid pool of *P. viridis.* The total lipid content of *P. viridis* in this study are in good agreement with earlier studies with *Mytilus edulis* and *Perna canaliculus* (Murphy *et al*., 2002), in which the total lipid content varied from 1.06 -1.97% (wet weight).

Wild samples recorded significantly higher cholesterol content than cultured samples (P<0.05) both in Kozhikode as well as Cochin, respectively. Though significant differences in cholesterol contents were observed between wild and cultured samples collected from Cochin and Kozhikode, the values are well within the limits, and considered to be safe for consumption. Plankton is

the predominant diet of mussels, which contains various sterols, and they can be incorporated into mussel tissues, and some sterols *viz.*, cholesterol can be synthesized from plankton cholesterol precursors (Li *et al*., 2007). Earlier studies reported cholesterol as predominant sterol present in mussels (Li *et al*., 2007). Cholesterol was the major sterol in *Mytilus edulis* (30%) and *Perna canaliculus* (29%) (Murphy *et al*., 2002). The loading plot of cholesterol was analyzed by PCA (**Fig 3A.3**). Cholesterol of cultured origin from both sampling locations (Cochin and Kozhikode) is the dominant features in PC 1 (74.2%).

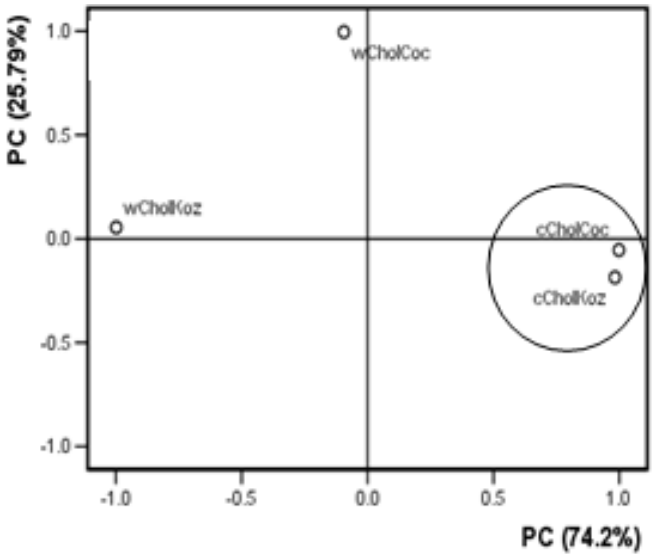


Fig 3A.3 The loading plot diagram showing the correlation of cholesterol content in cultured and wild (wCholKoz, wCholCoc, cCholCoc and cCholKoz) *P. viridis*.

3A.2.4 Mineral composition

Minerals are nutrients that are conserved by the body and play significant role in metabolism in the human body. The present study revealed that Ca and Mg were higher than those reported in the literature for bivalve mollusks (Astorga *et al*., 2007). The cultured green mussels registered

significantly higher content of the macronutrients (Ca, Mg) than wild samples from both experimental locations (P<0.05). Cultured samples collected from Kozhikode recorded significantly higher Mg content than cultured Cochin samples (P<0.05). Ca and Mg are extremely important minerals that are often out of balance in persons with arteriosclerosis and thyroid diseases. Imbalances of these minerals can result in irregular heart rate. A normal person needs a Ca/Mg ratio of about 2:1. Alterations in the metabolism of Ca and Mg have been implicated in the pathogenesis of primary hypertension (Kisters *et al*., 2004). The cultured samples from Kozhikode was found to be superior to another in terms of the Ca/Mg ratio (2.13), and therefore may be referred to be an ideal diet for good health. The cultured samples from Kozhikode registered significantly lower (P< 0.05) Na than other samples, and therefore, rated high as health food. Reports linking excessive Na intake to the genesis of hypertension and other related diseases like changes in vascular reactivity, and changes in the renin–angiotensin system are well-known (Santos *et al*., 2006).The micronutrient element, Zn was found to be significantly higher in samples collected from Kozhikode than Cochin. The cultured *P. viridis* was found to possess higher content of Zn than either the wild samples from both locations. Zn was found to be the second most abundant trace element in *P. viridis* (after Fe), and its role in the pathophysiology of disease is stimulating a great deal of interest (Coudray *et al*., 2006). Copper (Cu), which is also receiving growing attention for its role in alleviating oxidative stress, was found to be higher in samples collected from Kozhikode (~0.2 mg $100g^{-1}$).Se, the antioxidant mineral element was found to vary between 30-40 µg $100g^{-1}$ this species is a valuable natural resource to combat free-radical induced disorders in the body. The variation of mineral content appeared to be due to the influence of several factors including

food availability and differences in metal regulation. In addition, the differences in the mineral concentrations of the surrounding seawater could also influence their levels in *P. viridis* (Astorga *et al*., 2007).

3A.2.5 Vitamin and unsaponifiables

The contents of fat soluble vitamins (A, D_3, E & K_1) and water soluble vitamin (vitamin C) were recorded in **Table 3A.1**. All trans-retinol underwent highly insignificant fluctuations reaching maxima in cultured samples and minima in wild samples from Cochin. The levels of α-tocopherol, a vitamin with anti-oxidant properties, in *P. viridis* were low, and showed insignificant fluctuations between wild and cultured samples collected from the experimental locations ($P>0.05$). Significant differences were apparent in phylloquinone (K_1) content between wild and cultured samples collected from Kozhikode ($P<0.05$). Similarly, significant differences in cholecalciferol (D_3) content were realized between the wild and cultured samples collected from two different locations ($P<0.05$). The Vit C The loading plot of vitamins (**Fig 3A.4**) indicates that the vitamin E variables of both wild and cultured origin from Kozhikode are the dominant features in PC 1 accounting for 53.2% of the total variability. The highest loadings on the second component (PC 2) were defined by vitamin D_3 of both growth conditions (wild and cultured) of *P. viridis* from Cochin, representing 46.8% of the total variance. The values of *trans* retinol content are within the limits to impart their beneficial effects. Vitamin D precursors constitute a large proportion of the unsaponifiable fraction of mollusk lipids. Among minor unsaponifiable components, HPLC analyses showed that oxygenated carotenoids, characterized by typical absorption spectra, were prevalent over the less polar, late-eluting, α-carotene and β-carotene. Due to the lack of suitable standard compounds, the early-eluting xanthophylls were not identified.

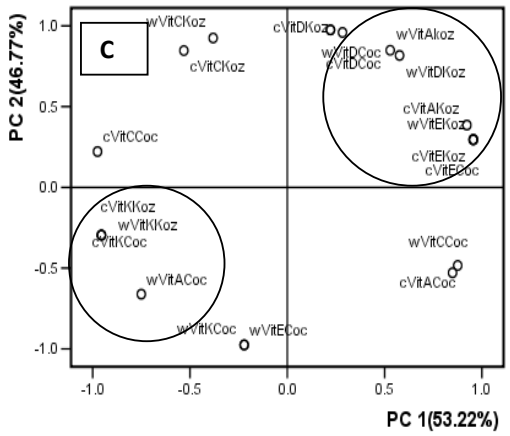


Fig 3A.4: The loading plot diagram of fat soluble and water soluble vitamins A, D_3, E, K_1, C collected from Cochin and Kozhikode

3A.2.6 Amino acid composition

A total of seventeen amino acids was identified and quantified in the samples of cultured and cultured *P. viridis* collected from two different locations is shown in **Table 3A.2**. The cultured samples were found to possess higher essential amino acids than wild samples, and present in quantities required for balanced nutrition. It is to be noted that the cultured samples collected from Kozhikode exhibited significantly higher essential amino acid content than wild samples (P<0.05). Among the wild samples from Kozhikode, the most abundant essential amino acid was found to be phenylalanine followed by arginine and leucine. The study indicated that Kozhikode samples have significantly higher methionine content (1.15-0.93g $100g^{-1}$) than Cochin (0.26-0.43g $100g^{-1}$) samples. In general, the amino acid lysine is absent in cereals and this amino acid constitutes a major share to the total essential amino acid pool in cultured *P. viridis* from both experimental locations. Threonine, another essential amino acid deficient in cereals, was found to be more in *P. viridis* from Kozhikode as compared to the samples collected from Cochin. This was supported by earlier works of Wesselinova (Wesselinova, 2000), who reported that the amounts and

types of amino acids in fish muscle were affected by location. It is, therefore, anticipated that cultured *P. viridis* is a good source to supplement the deficient amino acids in cereals. Iwasaki and Harada (Iwasaki and Harada, 1985) similarly reported lysine as one of the major amino acids in the muscles from marine origin. Among the non-essential amino acids in Kozhikode samples, negatively charged amino acid glutamate was observed to be the predominant. Glycine was found to constitute a major share, thereby signifying the potential capability of this species to withstand salinity and adverse stress. The results obtained from this study showed that cultured *P. viridis* have well-balanced and high-quality protein source in the respect of EAA/NEAA ratio. Any ratio of EAA/NEAA amino acids higher than 1.0 is considered to be excellent, and therefore it can be concluded that, *P. viridis* collected from Kozhikode is a good sources of well-balanced proteins and high-quality protein source in respect of EAA/NEAA ratio. The percentages of the major amino acids of *P. viridis* from different growth conditions (wild and cultured) and geographical locations were included as variables in a principal component analysis (PCA) and given as a loading plot in (**Fig 3A.5**). The parameters included in the loading plot are aspartic acid, glutamic acid, lysine, arginine, ΣEAA, ΣNEAA, ΣTAA and EAA/NEAA ratio (**Fig 3A.5**). The highest loadings on the first component (PC 1) were defined by variables *viz.*, glutamic acid, EAA/NEAA ratio of both growth conditions (wild and cultured) of *P. viridis* from the different geographical locations (Kozhikode and Cochin), lysine, aspartic acid, and TAA from *P. viridis* of wild and cultured origin from Kozhikode representing 73.3 % of the total variability. Lysine, EAA of cultured origin from Cochin dominates in PC 2 explaining 26.7 % of the total variance of the dataset.

Table 3A.2 Amino acid composition (g $100g^{-1}$ wet tissue) of wild and cultured *P. viridis* collected from Cochin and Kozhikode.

Amino acids	Cochin	Kozhikode

	wild	cultured	wild	cultured
Essential amino acids (EAA)				
His	0.38±0.01[a]	0.29± 0.01[b]	0.76±0.01[c]	0.12± 0.05[d]
Arg	1.70±0.01[a]	2.45± 0.06[b]	1.88±0.01[c]	4.44±0.09[d]
Thr*	0.61 ±0.06[a]	0.83± 0.02[b]	1.53±0.04[c]	1.66±0.02[d]
Val*	0.59 ±0.04[a]	0.62± 0.04[a]	0.96±0.03[b]	1.55±0.06[c]
Met*	0.25± 0.04[a]	0.43± 0.01[b]	1.14±0.07[c]	0.92±0.03[d]
Ile*	0.52± 0.05[a]	0.44± 0.01[b]	0.84±0.02[c]	1.45±0.05[d]
Leu*	1.16± 0.04[a]	1.28 ± 0.07[b]	1.82±0.01[c]	2.84±0.01[d]
Phe*	0.63± 0.06[a]	0.54± 0.01[b]	2.02±0.02[c]	1.39±0.03[d]
Lys*	0.91± 0.01[a]	1.74 ± 0.06[b]	1.64±0.03[c]	2.03±0.09[d]
EAA	**6.75 [a]**	**8.54[b]**	**12.59[c]**	**16.36[d]**
Non-essential amino acids (NEAA)				
Asp	1.79±0.03[a]	2.43± 0.01[b]	2.23±0.04[c]	3.86±0.02[d]
Glu	2.42±0.06[a]	3.05± 0.05[b]	3.08±0.03[b]	6.35±0.03[c]
Ser	0.92± 0.05[a]	1.36± 0.05[b]	2.06±0.07[c]	2.23±0.06[d]
Gly	1.85±0.03$_a$	2.01±0.07[b]	1.66±0.05[c]	4.24±0.04[d]
Ala	0.87±0.01[a]	1.31±0.08[b]	1.10±0.08[c]	2.45±0.07[d]
Pro	0.77±0.05[a]	0.47±0.03[b]	1.36±0.05[c]	1.64±0.00[d]
Tyr	0.30±0.07[a]	0.41± 0.08[b]	1.42±0.02[c]	0.87±0.01[d]
Cys	0.03± 0.01[a]	0.03± 0.01[a]	0.25±0.01[b]	0.39±0.04[c]
NEAA	**8.95[a]**	**11.07[b]**	**13.16[c]**	**22.03[d]**
EAA/ NEAA	**0.75[a]**	**0.77[a]**	**0.95[b]**	**0.74[a]**

Reversed-phase binary gradient high performance liquid chromatograph (HPLC, Waters RP PICO.TAG amino acid analysis system), fitted with a packed column (dimethylocatadecylsilyl bonded amorphous silica; Nova-Pak C_{18}, 3.9 X 150 mm maintained at 38±1°C in a column oven) was used to analyse hydrolysable amino acids by their UV absorbance (λ_{max} 254 nm). The mobile phase eluents used were A and B, eluent A comprises sodium acetate trihydrate (MeCOONa, 0.14 M, 940 mL, pH 6.4) containing TEA (Me_3N, 0.05percent), mixed with CH_3CN (60 mL); and eluent B used was acetonitrile:water (60:40, v/v). Data are means of duplicate analysis of pooled homogenates. *Note*. Tryptophan was not determined, *Essential amino acid for humans.

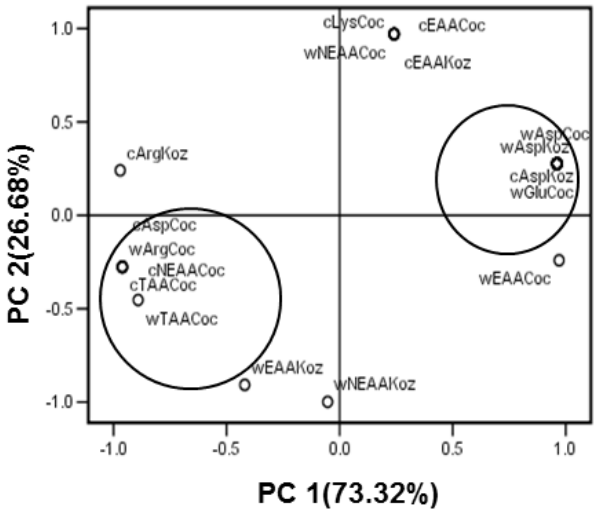


Fig 3A.5: The loading plot of amino acids (asp, glu, lys, arg) *vis-à-vis* ΣEAA, ΣNEAA, ΣTAA and EAA/NEAA ratio.

3A.2.7 Fatty acid composition

The percentage compositions of fatty acids in *P. viridis*, collected from different sites were given in **Table 3A.3**. The different groups of fatty acids are illustrated in the following section.

3A.2.7.1 Saturated fatty acids (SFAs)

Cultured samples collected from Kozhikode recorded significantly lower (P<0.05) total SFAs than wild samples. Mussel fatty acid profiles usually contain about ~30-40% SFA (Alkanani *et al*., 2007), a level which was found in the present study. The predominant SFA was recorded to be 16:0 (21-28% of total fatty acids, TFA), with a maximum recorded in samples collected from Cochin (≥28% TFA) and minimum in those from Kozhikode (~21% TFA). Palmitic acid (16:0) is the major SFA in mussels (Alkanani *et al.*, 2007; Orban *et al*., 2002) and appeared to be insignificant, between the samples collected from cultured or wild condition (**Table 3A.3**) (P>0.05).SFAs are used for

energy storage, and therefore, their concentration increases during periods of enhanced feeding activity (Gockse *et al*., 2004). Growth of mussels in the presence of readily available non phytoplanktonic organic material can too result in the accumulation of a higher proportion of SFAs (Freites *et al*., 2002).

3A.2.7.2 Monounsaturated fatty acids (MUFAs)

The wild samples collected from Cochin recorded significantly higher total MUFAs than cultured samples ($P<0.05$) (**Table 3A.3**). However, no significant differences were apparent between wild and cultured samples harvested from Kozhikode ($P>0.05$). MUFA was found to be highly dependent on 18:1*n*-9, the primary constituent that contributed a major share to total MUFA pool, registering maxima in wild samples (16-18% TFA), and minima (14-15% TFA) in cultured samples. The total content of MUFAs was found to be highly dependent on 18:1*n*-9, the primary constituent registered maximum in wild samples. The earlier studies reported that the fatty acid 16:1*n*-7 is believed to be a diatom marker (Alkanani *et al.*, 2007) whereas 18:1*n*-9 is not restricted to a single phytoplankton group. This further supports the fact that a food ingested by *P. viridis* by filter feeding is directly reflected in their fatty acid composition. In an earlier study, Orban *et al* (Orban *et al*., 2002) too did not observe consistent fluctuations of the MUFAs, particularly 16:1*n*-7.The other MUFA detected as16:1*n*-7, which is abundant in diatoms, and significant differences were apparent between those of wild and cultured samples ($P<0.05$). The share of this fatty acid was found to be lower than those reported in literature (16:1*n*-7, 11-14%) (Alkanani *et al*., 2007).

3A.2.7.3 Polyunsaturated fatty acids (PUFAs)

PUFAs are considered as single most important nutritional indicator dictating the quality of *P. viridis*. The prevalence of PUFAs, amounting to about 24-34% of total fatty acids, over the MUFAs (18-24%) is evident in *P. viridis* harvested from both locations and growth conditions. These results were in accordance in mussels from different studies (Orban *et al*., 2004). Although other authors (Orban *et al*., 2002) have reported that the PUFA in mussels predominated over the SFAs, a wide range of variation in the percentages of these compounds in these mollusks has been detected, ranging from 29-48% for PUFA, 16–32% for MUFAs, and 23–45% for SFAs. The wild samples collected from Cochin showed insignificant differences in total PUFA content than cultured samples ($P>0.05$) (**Table 3A.3**). Interestingly, the samples collected from Kozhikode registered significantly higher total PUFA content (31-35%) than those from Cochin (24-25%, $P>0.05$). However, it is of note that a level of >20% PUFA with LC-fatty acids are a source of balanced nutrition, and, therefore, the samples from Cochin too satisfies the requirement of balanced nutrition. The marginal variability in fatty acid composition is due to the fact that the lipid levels and composition of marine bivalves depend on the biochemical and environmental conditions of seed development, environmental conditions, including the phytoplankton resources available (Fuentes *et al*., 2009).

Table 3A.3 Fatty acid composition of *P. viridis* collected from two different growth conditions (wild and cultured) and locations (Cochin and Kozhikode) of south west coast of India.

Fatty acids	Fatty acids (% total fatty acids, TFA)			
	Cochin		Kozhikode	
	wild	cultured	wild	cultured
Saturated fatty acids				
16:0	28±0.02[a]	28.3±0.09[a]	21.25±1.4[b]	20.68±0.04[b]
17:0	1.32±0.02[a]	1.37±0.06[a]	0.12±0.08[b]	0.06±0.12[c]
18:0	8.84±0.76[a]	8.54±0.15[a]	4.83±0.42[b]	1.56±0.58[c]
20:0	0.22±0.41[a]	0.32±0.36[b]	1.09±0.01[c]	0.72±0.04[d]
22:0	ND	0.04±0.02[a]	2.11±0.01[b]	2.26±0.02[b]
24.0	1.18±0.01[a]	1.16±0.01[a]	2.68±0.02[b]	1.83±0.07[c]
∑ SFA	39.56[a]	39.73[a]	34.15[c]	29.61[d]
Monounsaturated fatty acids				
14:1*n*-7	0.22±0.01[a]	0.84±0.01[b]	1.57±0.01[c]	1.4±0.02[d]
16:1*n*-7	3.26±0.05[a]	2.95±0.07[b]	1.21±0.18[c]	1.39±0.14[d]
18:1*n*-9	17.6±0.01[a]	13.7±0.04[b]	15.49±0.3[c]	14.91±0.49[d]
22:1*n*-9	ND	ND	2.47±0.04[a]	3.74±0.15[b]
24:1*n*-9	0.55±0.15[a]	0.42±0.26[b]	3.38±0.05[c]	2.14±0.03[d]
∑ MUFA	21.63[a]	17.91[b]	24.03[c]	23.57[c]
Polyunsaturated fatty acids				
18:2*n*-6	1.54±0.02[a]	1.03±0.01[b]	1.09±0.83[b]	1.17±0.04[c]
18:3*n*-6	0.55±0.01[a]	0.46±0.01[b]	0.85±0.001[c]	1.03±0.15[d]
18:3*n*-3	1.68±0.15[a]	1.48±0.16[b]	1.03±0.82[c]	0.86±0.01[d]
18:4*n*-3	0.11±0.01[a]	0.98±0.02[b]	0.3±0.05[c]	0.42±0.05[d]
18:4*n*-6	1.01±0.08[a]	1.12±0.17[b]	1.15±0.03[b]	1.98±0.01[c]
20:2*n*-6	0.09±0.03[a]	1.05±0.04[b]	0.12±0.08[c]	0.39±0.08[d]
20:3*n*-6	0.77±0.1[a]	0.7±0.02[a]	0.54±0.06[b]	1.92±0.04[c]
20:4*n*-6	1.32±0.03[a]	0.26±0.05[b]	1.19±0.02[c]	0.86±0.18[d]
20:3*n*-3	0.21±0.01[a]	0.2±0.02[a]	0.25±0.09[a]	0.27±0.03[a]
20:5*n*-3	7.95±0.46[a]	7.38±0.65[a]	12.68±0.05[b]	12.84±0.05[b]
22:5*n*-3	0.55±0.11[a]	0.42±0.06[b]	2.41±0.06[c]	3.01±0.03[d]
22:6*n*-3	9.6±0.05[a]	8.96±0.06[a]	9.6±0.16[a]	9.87±0.03[a]
∑ PUFA	25.38[a]	24.04[a]	31.13[c]	34.58[d]
∑ C_{18}PUFA	4.89[a]	5.07[a]	4.42[b]	5.46[c]
∑ C_{20}PUFA	10.34[a]	9.59[b]	14.70[c]	16.24[d]
∑ *n*-3	20.1[a]	19.42[a]	26.27[b]	27.27[b]
∑ *n*-6	5.28[a]	4.62[b]	4.94[a]	7.35[c]
n-3/*n*-6	3.81[a]	4.20[b]	5.30[c]	3.70[d]
∑ PUFA/∑ SFA	0.64[a]	0.61[a]	0.91[b]	1.17[c]
22:6*n*-3/20:5*n*-3	1.21[a]	1.21[a]	0.76[b]	0.77[b]

ΣSFA: total saturated fatty acids; ΣMUFA: total monounsaturated fatty acids; ΣPUFA: total polyunsaturated fatty acids; Data presented as mean values of three samples (mean ± SD). These values do not total 100percent because minor fatty acids are not reported. ND implies non detectable (or fatty acids present below 0.05percent). *a,b,c,d values for each sample with different letters in the same row are significantly different at $P<0.05$.

3A.2.7.4 *n*-3 polyunsaturated fatty acids (*n*-3 PUFAs)

The PUFA composition of *P. viridis* was characterized by the predominance of *n*-3 PUFAs (**Fig 3A.6)** particularly 20:5*n*-3(EPA) and 22:6*n*-3(DHA), which constitute >80% of total PUFAs. The total content of *n*-3 PUFAs was recorded to be significantly higher (26-27%) in *P. viridis* collected from Kozhikode than those collected from Cochin (19-20%) ($P<0.05$). Presence of higher amount of 20:5*n*-3 and 22:6*n*-3 in Kozhikode samples than Cochin samples largely reflects the composition of the planktonic diet (De Moreno *et al*., 1980). EPA content was found to be significantly higher in samples collected from Kozhikode than those recorded in Cochin samples, and no significant differences ($P>0.05$) were apparent between wild and cultured samples (**Fig 3A.7**). No significant differences ($P>0.05$)were realized in the share of DHA to the total fatty acid pool as registered in the samples collected from Cochin and Kozhikode under two different growth conditions (**Fig 3A.7**). It is to be noted that 20:5*n*-3 content of >5% is good to provide a balanced nutrition with respect to fatty acids, and thereby indicating the *P. viridis* collected from Cochin is equally good as Kozhikode with respect to fatty acid composition. Kharlamenko *et al* (Kharlamenko *et al*., 1995) reported a high 20:5*n*-3 content is a fatty acid marker characteristic of diatoms. The PUFA composition also concurs with numerous studies of mussels and other bivalve species (Alkanani *et al*., 2007; Orban *et al*,. 2002).Bivalves are filter feeders with diets consisting largely of phytoplanktons that contain a high proportion of long-chain *n*-3 PUFAs, such as 22:6*n*-3 and 20:5*n*-3 (Li *et al*., 2007; Murphy *et al*., 2002).Phytoplankton is the most important food resource for mollusks, and can be considered to be the most important source of unsaturated fatty acids, *viz*., C20:5 and C22:6 (De Moreno *et al*., 1976). Therefore, the quantitative and qualitative availability of food influences the unsaturation level and composition of fatty acids (Freites *et al*., 2002), even if green mussels are able to convert 20:5 and 22:6 fattyacids, as observed by Orban *et al* (Orban *et al*., 2002). The variations of 20:5*n*-3 and 22:6*n*-3

(P<0.05), the fatty acids known to be synthesized by diatoms and dinoflagellates, may be related to the type of food ingested by *P. viridis* during their growth. The fact that lipids in mussel tissue are rich in C_{20-22} PUFAs seems to suggest that, food consists of phytoplankton elements. The New Zealand mollusk *P.canaliculus* reported to contain a similar PUFA profile, dominated by 20:5*n*-3 and 22:6*n*-3.

A significantly higher proportion of C_{18} PUFAs were recorded in cultured *P. viridis* collected from Kozhikode than in wild samples (P<0.05). A higher proportion of C_{18} PUFAs in cultured samples from both locations (>5%) than in wild (<5%) apparently indicated that they feed on the water column. This conclusion is supported by the fact that *Dunaliella* sp., a chlorophytan microalga, which is characterized by C_{18} PUFAs (Volkman *et al*., 1998), is present in the water column. Interestingly the cultured *P. viridis* recorded lower 20:4*n*-6 than those in wild counterparts. The *n*-3/*n*-6 fatty acid ratio is of *P. viridis* were found to be about 3.7-5.3, which is considerably higher than the health foods available in the market, and therefore may *P. viridis* may serve as an effective alternative to balance the higher intake of *n*-6 fatty acids. This ratio was also found to be directly proportional to the anti-inflammatory activities due to the fact that 20:5*n*-3 is a precursor to anti-inflammatory lipid mediators (E-series of resolvins, RvE1), whereas docosanoids (DHA, 22:6*n*-3) to the D-series of resolvins (RvD1) and protectins (neuroprotectin D1, NPD1). Proper balance of dietary *n*-3/*n*-6 PUFAs is integral to prevent chronic diseases including cardiovascular diseases by reducing ventricular arrhythmias, serum triacylglycerol concentrations, and atherosclerotic plaque growth. Reversing the *n*-3/*n*-6 ratio in a favourable manner is the need of the hour to retain disease-free healthy populace. An earlier study conducted in this line concluded that a 20% reduction in overall mortality and a 45% reduction in sudden death were reported in subjects with pre-existing cardiovascular diseases when given 850 mg *n*-3 fatty acids (Cordain *et al*., 2005). It is, therefore, apparent that higher

dietary intakes of *P. viridis*, with *n*-3/*n*-6 ratio of 4-5 as realized in the present study may favourably alter the ratio resulting in preventing inflammatory and autoimmune diseases. This is potentially important because the ratios of these fatty acids in the tissues are determined largely by their ratios in the diet (Cleland *et al*., 2006).

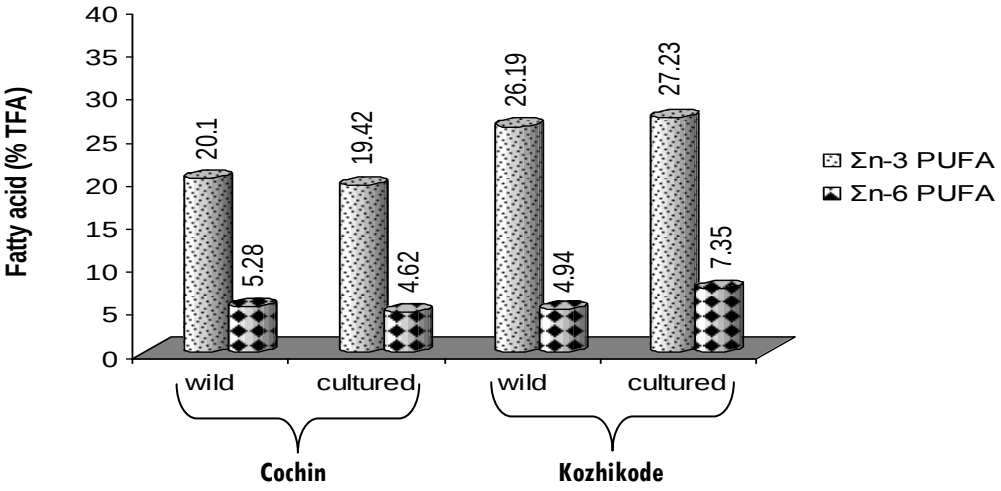


Fig 3A.6: Polyunsaturated fatty acid profiles of wild and cultured samples of *Perna viridis* collected from Cochin and Kozhikode. Differential compositions of Σ*n*-3 and Σ*n*-6 PUFAs in the representative samples.

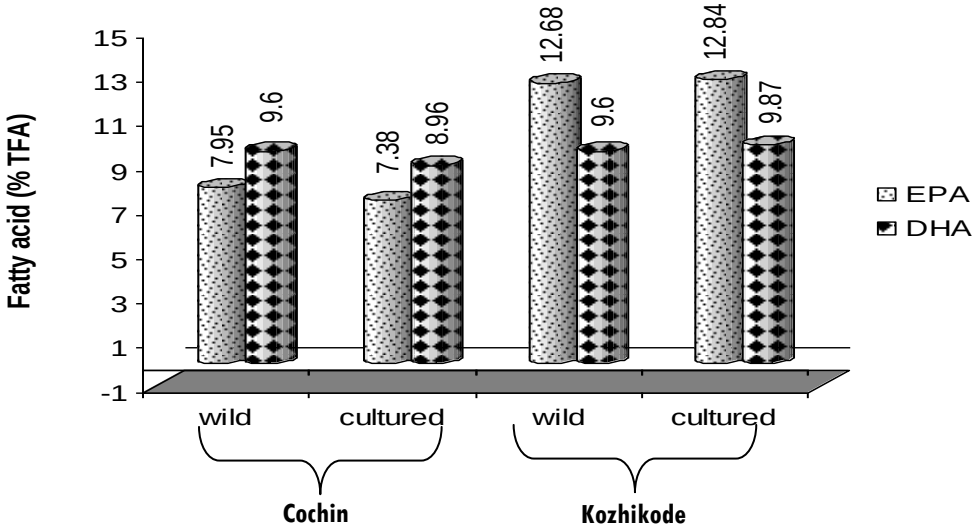


Fig 3A.7: EPA and DHA content of wild and cultured samples of *P. viridis* collected from Cochin and Kozhikode.

3A.2.7.5 *n*-6 polyunsaturated fatty acids (*n*-6 PUFAs)

The total content of *n*-6 PUFAs were recorded to be comparatively higher in cultured samples collected from Kozhikode than those collected wild (**Table 3A.3**). This is apparently due to the fatty acids 18:4*n*-6 and 20:3*n*-6, which contributed a major share to the total *n*-6 PUFA pool. Among the *n*-6 PUFAs, 20:4*n*-6, 18:2*n*-6 and 20:3*n*-6 was the dominant fatty acids. Interestingly the wild samples collected from the experimental locations recorded significantly higher 20:4*n*-6 than those collected under cultured condition ($P<0.05$). However, it is of note that the *n*-6 fatty acids, particularly 20:4*n*-6 was reported to have an important role in stress tolerance. Higher content of 20:4*n*-6 of wild *P. viridis* (>1%) than that in cultured ones (<1%) revealed the ability of the former to withstand adverse stress conditions prevailing in a wild environment. The fatty acid AA (20:4*n*-6), the 20 carbon *n*-6 PUFA was reported to be the key link between PUFAs and eicosanoid family of inflammatory mediators (series-4 leucotrienes LTB_4 and series-2 prostaglandins PGE_2) and pro-inflammatory cytokines (TNF-α) in a cascade of metabolic processes involving 5-lipoxygenase (5-LOX) and cyclooxygenase-2 (COX-2)(Calder, 2006) and PUFAs. A family of anti-inflammatory mediators such as resolvins derived from *n*-3 LC-PUFAs viz., EPA and DHA produced via the COX-2 reaction, and are considered to be anti-inflammatory (Calder, 2006).

3A.2.7.6 Ratios of different families of PUFAs and their correlation analyses

Low levels of *n*-6 (5-7%) and high levels of *n*-3 PUFAs (19-27%) in *P. viridis*, were apparent in the present study (**Table 3A.3**). The average *n*-3/*n*-6 ratio was recorded as ~4 in samples collected from Cochin, and 4-5 in

Kozhikode samples (**Table 3A.3**). No significant differences (P>0.05) in the ratio between ∑PUFA/∑SFA were realized in the *P. viridis* collected from Cochin under wild or cultured conditions. The higher ∑PUFA/∑SFA ratio in cultured samples collected from Kozhikode was mainly contributed by *n*-3 fatty acids particularly 20:5*n*-3, 22:5*n*-3, and 22:6*n*-3 (**Table 3A.3**). The loading plot which includes ΣPUFA, Σ*n*3, Σ*n*6, EPA, and DHA when analysed by PCA were shown in **Fig. 3A.8**. The first principal component (PC 1) accounted for 68.3% of the variability in the data set. The second principal component (PC 2) accounted for 31.7% of the variance in the data set. This revealed a high correlation between ΣPUFA and an important *n*-3 fatty acid, DHA of green mussel collected from Cochin, despite different growth conditions. The higher negative loadings of the PC 1 are for variables *n*-6, lipid of wild origin from Cochin, DHA of wild origin from Kozhikode, and EPA of cultured origin from Kozhikode, which are grouped as C, highlighting its inverse relationship with other components.

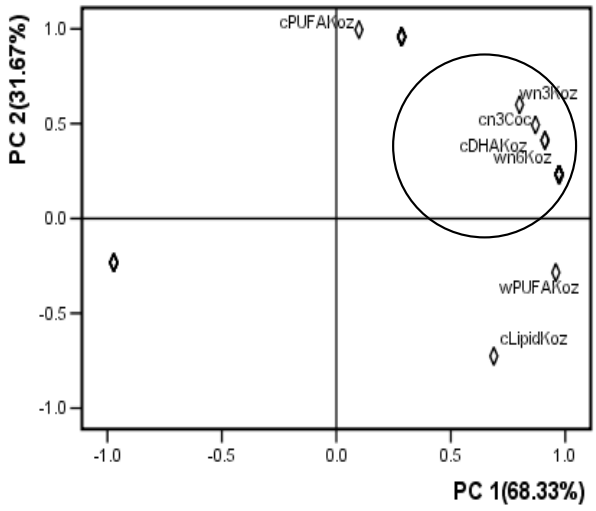


Fig.3A.8: Loading plot diagrams showing the correlation of fatty acids (ΣPUFA, Σ*n*-3, Σ*n*-6, EPA, DHA) and Σcarotenoids of *P. viridis*.

The present study provides insights in different biochemical and fatty acid variation of *P. viridis* collected from two different geographical locations on the south-west coast of India (Cochin and Kozhikode), and under different growth conditions (cultured and wild). No significant differences in different nutritional and biochemical parameters under observation were apparent between *P. viridis* collected from different locations of the south-western coast of India, and the cultured samples were found to equal or sometimes better than their wild counterparts in terms of this food quality indicators. The higher PUFA content of the samples collected from this mussel harvesting area also contribute to their potential to exhibit anti-inflammatory activities. *P. viridis*, by virtue of realizing a balanced EAA/NEAA ratio with considerable amount of lysine and threonine, is a well-balanced and high-quality protein source, and a candidate species to supplement these amino acids of cereals. The optimum and balanced quantities of vitamins, mineral nutrients, and low cholesterol contents added to the good qualities of this particular species, and therefore, proved to be a desirable item in the human diet in the south-western region of India.

3B) Nutrient profiling of edible oysters (*Crassostrea madrasensis* L.) from Southwest coast of India

General

Oysters are one of the most valued seafood as they constitute a rich source of fatty acids, amino acids, and minerals, which are essential for providing a balanced diet (Nagabhushanam and Bidarkar, 1978).The edible oyster, *Crassostrea madrasensis*, is a bivalve mollusk (family *Ostreidae*); found in the coastal sea beds in the Arabian Gulf in south Malabar waters along the west coast of Peninsular India. The oyster fishery of Kerala is

limited to backwaters like Dharmodam, Kayamkulam, Ashtamudi, Paravoor, Kadalundi where *C. madrasensis* is the most prominent species (Harikumar *et al*., 2007), but the culture operations are possible only for six months during the pre-monsoon period (Appukuttan, 2007).

Considering the promising perspective for the utilization of *C. madrasensis*, as a potential health food, studies on its biochemical composition began to receive considerable attention. This work anticipated the influence of the growth conditions on the essential nutritional compositions of *C. madrasensis* harvested from south western coast of India. No reports have yet been published about the essential nutritional composition of this species under wild and cultured condition. The present study also directed to elucidate the effect of various climatological parameters on the nutritional profile of *C. madrasensis.* The data will provide useful information for seafood industries and oyster aquaculture. The integration with the nutritional composition and climatological factors was attempted to focus for a more holistic assessment of the overall biological significance of wild and cultured *C. madrasensis*.

3B.1 Sample collection and processing

3B.1.1 Collection of wild and cultured *C. madrasensis* samples

The samples (wild and cultured) were collected on April - May (pre monsoon season) from a relatively clean site in intertidal rocky shore of Sattar island at Cochin, south west coast of India (UST: 1141160E, 221200N) (Lat: $9^0$58'N; Long: $76^0$16'E), which is predominantly influenced by oceanic water from the Arabian Sea.

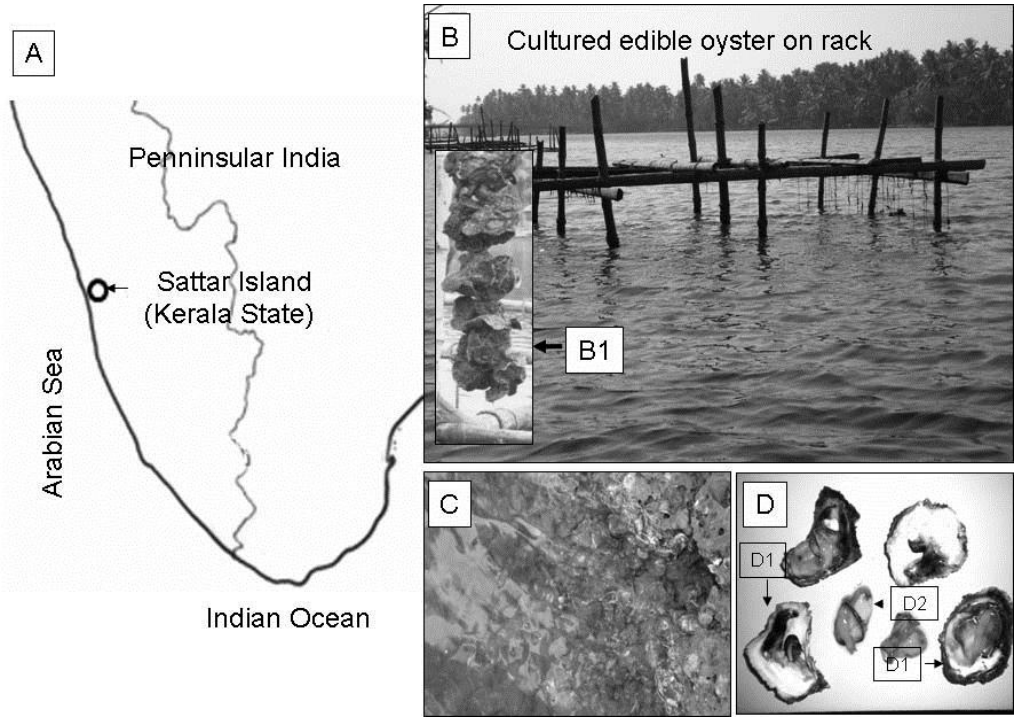


Fig 3B.1: Sampling locations of *C. madrasensis* (A) Sample collection site of edible oyster (*C. madrasensis*) (wild and cultured) along the Southwestern coast of India. Sattar island at Cochin (UST: 1141160E, 221200N) (Lat: $9^0$58'N; Long: $76^0$16'E) was selected as the collection sites of *C. madrasensis*; (B) A site of culture of *C. madrasensis* by rack method; (B1) Cultured *C. madrasensis* on rack; (C) Wild *C. madrasensis*; (D) *C. madrasensis*; (D1) Shell with meat and (D2) Meat portion of *C. madrasensis*.

Fig.3B.1 illustrates the sampling location of *C. madrasensis*. Sattar island region was selected considering the abundance of *C.madrasensis* and preferable environmental parameters for successful development of commercial bivalve culture and this region is a natural bed of this particular species. Peak water temperature was observed in pre-monsoon season (before May) with an average monthly temperatures of 30.0°C (±2.18) measured with thermistor temperature recorders. The samples were collected from comparable depths (2 m below the

surface) to avoid confounding effects of depth on the fatty acid composition. Cultured oysters were stripped from the 1/4" rope, at each site. All live samples thus collected were immediately and transported to the laboratory after proper washing.

3B.1.2 Determination of biometric parameters, condition indices, and meat yield of *C. madrasensis*

The samples of *C. madrasensis* were cleaned of fouling organisms and then measured for their biometrical parameters, *viz.*, length, width, and thickness to determine the condition indices as described in section 3.1.2.2.1

3B.2 Results and discussion

The present study provided a detailed biochemical profile of oysters, *Crassostrea madrasensis* collected from the wild and cultured conditions, from the most prominent oyster bed along the south-western coast of India (Sattar island). The samples of *C. madrasensis* were collected in May as there is a drop of salinity after May due to the monsoon. The periods of faster growth from April to May (pre-monsoon season) coincided with high chlorophyll-a levels. The chlorophyll-a peaks indicate an increase in primary production by May, associated with the increase in water temperature (Sasikumar *et al*., 2007).It is reasonable to assume that October-May period, when the environmental conditions are relatively stable, should be favorable for oyster culture (Purushan *et al*., 1983).Therefore this particular period has been chosen to collect both the wild and cultured samples of this target species.

3B.2.1 Morphometric characteristics of *C. madrasensis* in wild and cultured conditions

Biometric measurements, condition indices and meat yield (percent) of wild & cultured *C. madrasensis* samples are given in **Table 3B.1**.The current study indicated that there was substantial variation in the condition index of wild and cultured oysters. CI and meat content of mollusks were registered to be affected by a variety of environmental and endogenous factors, *viz.*, water temperature, salinity, food availability, and gametogenic cycle of animals (Okumus and Stirling 1998). There was no significant difference in CI of Walne and Booth between wild and cultured oysters. A significant difference (P<0.05) in meat yield was recorded between oysters in different growth conditions, wild and cultured, with higher values were observed for cultured oysters. Positive correlations between meat yield and chlorophyll-a levels in cultured oysters (**Fig 3B.2**, $r^2 = 0.88$) and negative correlation with wild oysters ($r^2 = 0.13$) indicated that, food availability and relatively high stocking densities are the important factors governing the increased growth of cultured oysters. The decreased growth rate of wild *C. madrasensis* may be due to some environmental factors such as disturbances in sites, competition for space and slow water movement (Rajagopal *et al.*, 2002). Maximum values of CI and meat yield was due to the seasonal blooms in Sattar island waters.

Table 3B.1 Biometric measurements, condition indices, meat yield (percent), cholesterol, vitamins and mineral compositions of wild & cultured *C. madrasensis*.

	Wild	Cultured
Meat Yield	12.16±0.09[a]	12.98±0.08[b]
Length (cm)	10.5±0.12[a]	10.7±0.31[a]
Thickness (cm)	3.90±0.06[a]	3.80±0.02[a]
Width (cm)	8.5±0.06[a]	8.6±0.01[a]
Condition indices		
Imai and Sakai	0.41±0.01[a]	0.39±0.02[a]
Booth	0.12±0.01[a]	0.13±0.02[a]
Walne	0.02±0[a]	0.03±0.01[a]
Moisture(%)	81.2±0.67[a]	82.0±1.27[a]
Cholesterol (mg $100g^{-1}$)	34.5±0.96[a]	43.3±0.72[b]
Fat soluble Vitamins		
Retinol (A)/IU	5.35±0.16[a]	4.26±0.08[a]
Cholecalciferol (D2)/IU	439.9±1.33[a]	459.2±0.8[b]
Tocopherol (E)/IU	0.20±0.00[a]	0.15±0.02[b]
Phylloquinone (K_1)(µg $100g^{-1}$)	1.50±0.01[a]	0.95±0.01[b]
Water soluble Vitamins		
Ascorbic acid (C)/IU	9.76±0.30[a]	9.78±0.03[a]
Macronutrients (mg $100g^{-1}$ wet sample)		
Na	185.6±3.93[a]	146.5±4.73[b]
K	265.4±4.48[a]	515.4±2.21[b]
Ca	174.7±1.36[a]	120.8±0.16[b]
P	319.1±1.53[a]	530.2±4.97[b]
Micronutrients (mg $100g^{-1}$ wet sample)		
Fe	3.14±0.01[a]	4.23±0.01[b]
Mn	ND	ND
Zn	3.66±0.16[a]	4.48±0.14[b]
Se	0.04±0.00[a]	0.03±0.00[a]
Na/K	0.5815[a]	0.2725[b]
Ca + P	493.9[a]	643.46[b]
Σ micro nutrients	6.85[a]	9.10[b]

All samples were analysed in triplicate (n=3) from pooled samples (30 numbers), and expressed as mean ± standard deviation. Means followed by the same letter within the same row are not significantly different, and different letters indicate significant differences ($P<0.05$). ND: non detectable.

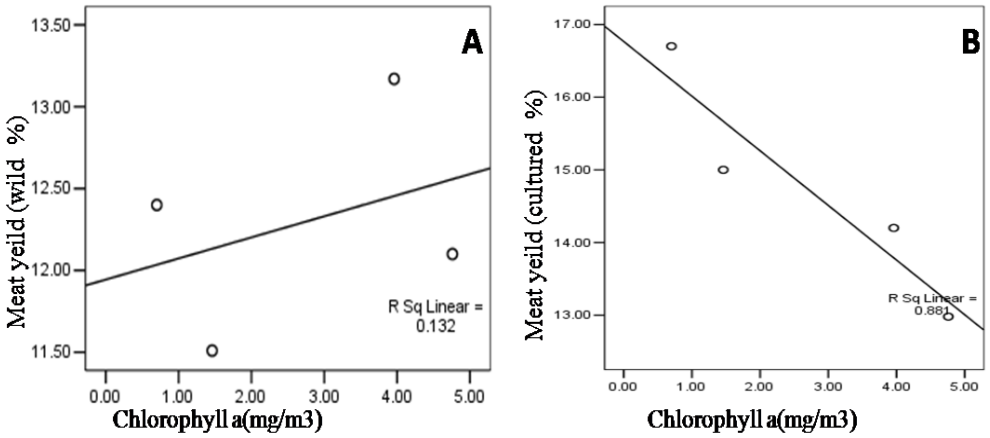


Fig.3B.2 Correlation between various nutritional indices and chrophyll-a of wild and cultured oysters collected from Cochin. (A) Correlation plot (negative correlation) between meat yield and chlorophyll-a levels in wild oysters ($r^2 = 0.13$). (B) Correlation plot (positive correlation) between meat yield and chlorophyll-a levels in cultured oysters ($r^2 = 0.88$).

3B.2.2 Lipid content and fatty acid composition of *C. madrasensis* in different growth conditions

Lipid content and fatty acid composition of *C. madrasensis* were recorded in **Table 3B.2**.No significant difference in lipid content was observed among wild and cultured oysters (P>0.05). The fatty acid and lipid composition of marine bivalves depend on the biochemical and environmental conditions of seed development, environmental conditions, including the phytoplankton resources available (Fernandez-Reiriz *et al*., 1989).

Table 3B.2 Lipid and fatty acid composition of *C. madrasensis* collected from two different growth conditions (wild and cultured).

	Wild	Cultured
Lipid (%)	7.04±0.38[a]	5.63±0.01[b]
Saturated fatty acids		
C 12:0	0.14±0.02[a]	0.2±0.03[b]
C 14:0	4.36±0.09[a]	7.84±0.37[b]
C 15:0	0.92±0.30[a]	1.28±0.27[b]

C 16:0	19.9±0.10[a]	23.7±0.28[b]
C 17:0	6.95±0.08[a]	3.83±0.51[b]
C 18:0	5.81±0.07[a]	7.63±0.37[b]
C 20:0	2.12±0.70[a]	0.28±0.01[b]
C 22:0	0.43±0.02[a]	0.2±0.01[b]
C 24:0	0.04±0.01[a]	0.85±0.56[b]
∑ SFA	40.7±0.60[a]	45.9±0.67[b]
Monounsaturated fatty acids		
C14:1 *n*-7	0.54±0.02[a]	1.20±0.13[b]
C16:1 *n*-7	3.50±0.20[a]	5.38±0.35[b]
C18:1 *n*-7	ND	ND
C18:1 *n*-9	4.14±0.20[a]	5.79±0.43[b]
C20:1 *n*-9	0.43±0.08[a]	0.21±0.01[b]
C22:1 *n*-9	4.58±0.16[a]	6.35±0.20[b]
C24:1	0.04±0.01[a]	0.26±0.06[b]
∑ MUFA	13.2±0.05[a]	19.2±0.50[b]
Polyunsaturated fatty acids		
C 18:2*n*-6	ND	ND
C 18:3*n*-6	ND	ND
C 18:3*n*-3	4.27±0.34[a]	4.42±0.10[a]
C 18:4*n*-3	1.85±0.10[a]	1.28±0.04[b]
C 18:4*n*-6	0.68±0.32[a]	0.73±0.11[a]
C 20:2*n*-6	ND	ND
C 20:3*n*-6	1.88±0.11[a]	0.72±0.19[b]
C 20:4*n*-6	3.32±0.04[a]	2.23±0.06[b]
C 20:3*n*-3	1.52±0.09[a]	1.57±0.23[b]
C 20:5*n*-3	9.38±0.07[a]	6.76±0.20[b]
C 22:5*n*-3	1.33±0.08[a]	0.59±0.02[b]
C 22:6*n*-3	11.5±0.10[a]	6.48±0.15[b]
Σ PUFA	35.8±0.08[a]	24.8±0.78[b]
∑ *n*-3	21.7±0.25[a]	14.5±0.47[a]
∑ *n*-6	12.7±0.29[a]	10.2±0.31[b]
∑ C_{18}PUFA	6.80±0.08[a]	6.44±0.06[a]
∑ C_{20}PUFA	15.6±0.78[a]	11.2±0.57[b]
n-3/*n*-6	1.70±0.14[a]	1.42±0.02[b]
n-6/*n*-3	0.59±0.02[a]	0.70±0.01[a]
∑ PUFA/ ∑ SFA	0.81±0.09[a]	0.55±0.02[a]

DHA/EPA	1.18±0.11[a]	0.96±0.03[b]
AI	2.53	3.56
TI	0.53	0.8
HH	1.35	0.83

ΣSFA: total saturated fatty acids; ΣMUFA: total monounsaturated fatty acids; ΣPUFA: total polyunsaturated fatty acids AI: Atherogenic index; TI: thrombogenic index; HH: hypocholesterolaemic/ hypercholesterolaemic ratio;Data presented as mean values of three samples (mean ± SD). These values do not total 100percent because minor fatty acids are not reported. ND implies non detectable (or fatty acids present below 0.05percent). *ab values for each sample with different letters in the same fraction are significantly different at $P<0.05$. Within a row, values not sharing the same superscript are significantly different ($P<0.05$). Data for each species are the mean of nine individual animals, representing three animals from each of three sites.

3B.2.2.1 Saturated fatty acids (SFAs)

Cultured samples recorded significantly lower ($P<0.05$) total SFAs than wild samples **(Table 3B.2)**. Palmiticacid (C16:0) was the primary SFA found in both wild and cultured oysters contributing 40% to the total SFA content in wild and 45.9% to the total SFA content in cultured samples. The presence of readily available non-phytoplanktonic organic material in the cultured condition resulted in the accumulation of higher proportion of SFAs (Freites *et al*., 2002)

3B.2.2.2 Monounsaturated fatty acids (MUFAs)

The predominant MUFA was found to be C18:1*n*-9, was significantly ($P<0.05$) greater in cultured oysters than wild ones. Significantly greater MUFA content was observed for cultured samples than wild samples($P<0.05$).The greater amount of MUFA content in cultured oysters may probably due to the greater content of monoenoic fatty acids in the food of the cultured oysters. The total MUFA in wild samples ranged from 13.2% in wild and 19.2% in cultured samples. The second abundant MUFA detected was 16:1*n*-7, a diatom marker, registered significantly greater values ($P<0.05$) in cultured samples ($P<0.05$). Significantly great levels of 16:1*n*-7 and 18:1*n*-

9 was registered in phytoplanktons (diatoms and dinoflagellates) and this further supports the fact that foods ingested by bivalves by filter feeding are directly reflected in their fatty acid composition.

3B.2.2.3 Polyunsaturated fatty acids (PUFAs) and ratios of different families of PUFAs

PUFAs are very important biochemical indicators of bivalves contributing to their nutritional quality. A wide range of variation in the percentages of PUFA compounds in this mollusk has been detected between wild and cultured ones, 35%t for wild and 25% for cultured ones. The wild samples recorded significantly greater PUFA content ($P<0.05$) than cultured samples. The total content of *n*-6 PUFAs and *n-3* PUFAs (EPA and DHA) were recorded to be significantly greater ($P<0.05$) in wild oyster samples. Wild oysters recorded positive correlations between chlorophyll-a and PUFA composition ($r^2 = 0.997$) and maximum values of C_{18} PUFA and C_{20} PUFA indicated that, besides gametogenic cycle influences, the blooming of different planktonic populations may affect the lipid profile of those organisms, like bivalves, relying on plankton as the main food source (Albentosa *et al*., 1996).

Docosahexaenoic acid (DHA, C22:6*n*-3) and EPA were the prominent PUFAs in wild and cultured oysters followed by 18:3*n*-3 and 20:4*n*-6.Diatoms contain considerable amounts of 20:5*n*-3 (EPA) and 22:6*n*-3 (DHA), respectively. Therefore, the ratio of DHA: EPA can be used as an indicator of the predominant phytoplankton class assimilated by the mussels. The DHA/EPA ratio was also insignificant among wild and cultured oysters ($P>$ 0.05). Ratios > 1 indicate a dominance of dinoflagellates in the diet, while ratios < 1 reflect a dominance of diatoms (Budge & Parrish, 1998). A good correlation was observed between EPA, DHA and chlorophyll-a concentration in wild samples ($r^2 = 0.96$) ($r^2 = 0.99$) respectively. Various algal species

including dinoflagellates, cryptomonads, and certain thraustochytrids as well as zooplankton are known to contain elevated levels of DHA (Murphy *et al*., 2002). The *n*-3/*n*-6 ratio was 1.70 in wild samples and 1.42 in cultured samples. Also, the *n*-6/*n*-3 ratio was 0.59 in wild samples and 0.70 in cultured samples. No significant differences were observed in both this ratio among wild and cultured oysters ($P>0.05$). The UK Department of health recommends an ideal ratio of *n*-6/n-3 of 4.0 at maximum (HMSO, 1994) and the values greater than 4.0 are considered harmful to health and may promote cardiovascular diseases. Among *n*-6 PUFAs, 20:4*n*-6, 18:2*n*-6 and 20:3*n*-6 was the dominant fatty acids. The total content of *n*-6 PUFAs presumably associated with a high 18:2*n*-6 intake which is probably contributed to by a microbial diet (Abad, *et al*., 1995).

The atherogenic and thrombogenic indexes were found to be greater in the cultured samples compared with wild samples **(Fig.3B.3)**, which gives an indication of the attitude of a composite diet or a single food to protect from atherosclerosis and platelets aggregation. The greater *n*-3 fatty acid content and consequently the greater *n*-3/*n*-6 fatty acid ratio in the wild samples apparently contributed to lower atherogenic and thrombogenic indices. It has been reported that due to the anti-atherogenic and anti-thrombogenic properties, the *n*-3 PUFAs play a major role to protect human beings from atherosclerosis and platelets aggregation (Barrento *et al*., 2010). The ideal HH ratio noted in the oysters also contributed towards its qualities to be judged as desirable from the consumer health perspective.

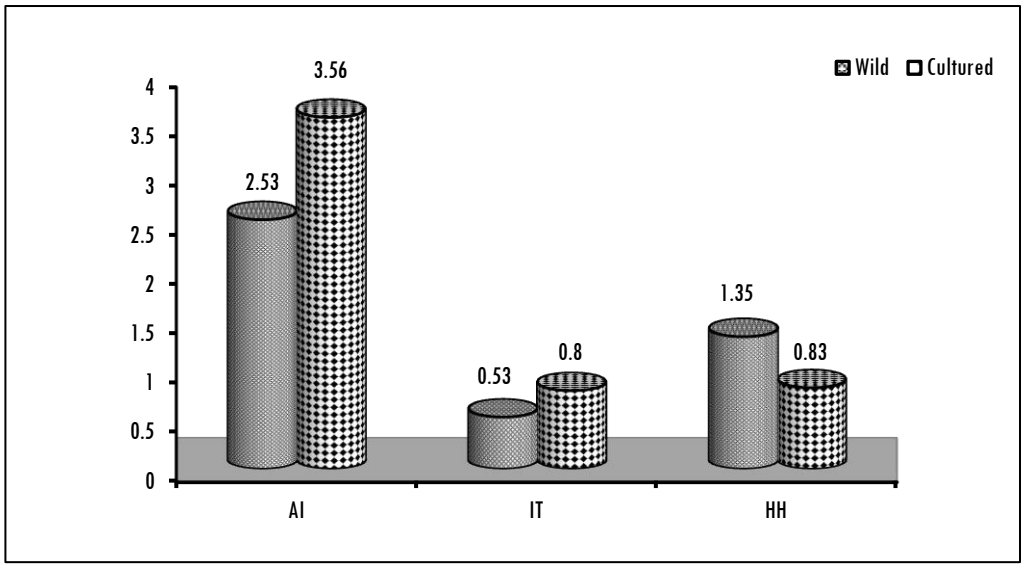


Fig.3B.3 Differential compositions of health indices of wild and cultured oysters collected from Cochin.

3B.2.3 Total cholesterol composition

The cultured samples exhibited significantly, greater cholesterol content (43.3 mg $100g^{-1}$) than wild samples (34.5 mg $100g^{-1}$) (**Table 3B.1**). The cholesterol content of wild samples showed very good positive correlation with PUFA content (**Fig 3B.4**, $r^2 = 0.83$). Cholesterol generally ranges from around 2-90% of total sterols in various mollusks. Plankton is the predominant diet, which contains various sterols, and they can be incorporated into its tissues, and some sterols *viz.*, cholesterol can be synthesized from plankton cholesterol precursors (McLean & Bulling, 2005).The wild samples were found to possess greater essential amino acids, than cultured samples, although the difference appeared to be insignificant and present in quantities required for balanced nutrition.

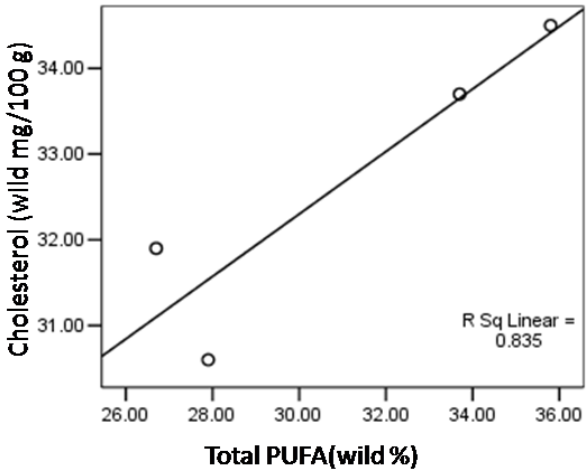


Fig.3B.4. Correlation plot between cholesterol (mg $100g^{-1}$) and total PUFA (percent) in wild oysters collected from cochin.

3B.2.4 Amino acid composition of cultured and wild *C. madrasensis*

Seventeen amino acids (expressed as g $100g^{-1}$ protein) were identified and quantified in both the wild and cultured oysters (**Table 3B.3**). Wild samples exhibited significantly greater ($P<0.05$) amount of total essential (ΣEAA) and non-essential (ΣNEAA) amino acid content than cultured samples. The most abundant essential amino acid (EAA) was found to be arginine in both wild and cultured (2.30 -3.31 g $100g^{-1}$ protein) oysters, followed by leucine and lysine. Among non-essential amino acids (NEAA), glutamic acid constitutes major share followed by glycine in both wild (2.91 g $100g^{-1}$ protein) and cultured (2.20 g $100g^{-1}$ protein) oysters. The total amino acid content (ΣAA) was found to be significantly greater ($P<0.05$) for wild oysters (~ 20 g $100g^{-1}$ protein) compared to their cultured counterparts (~ 13 g $100g^{-1}$ protein). The richness of amino acids in wild oysters was also been related to the maximum ripeness (Dridi *et al*., 2007).Alanine, glycine and arginine, which are normally contained at a relatively high level in marine mollusks, are crucial in energy metabolism by

maintaining glycolysis through the formation of opines under hypoxic conditions(Lei *et al*., 2013).

Table 3B.3 Amino acid composition (g $100g^{-1}$ protein) of *C. madrasensis* collected from two different growth conditions (wild and cultured) along the south west coast of India.

	Wild	Cultured
Essential amino acids (E)		
His*	0.55±0.01^a	0.38±0.07^b
Arg*	3.31±0.03^a	2.30±0.04^b
Thr*	0.77±0.09^a	0.57±0.02^b
Val*	0.65±0.08^a	0.50±0.03^b
Met*	0.48±0.02^a	0.26±0.09^b
Ile*	0.62±0.03^a	0.39±0.01^b
Leu*	1.56±0.01^a	1.06±0.04^b
Phe*	0.87±0.09^a	0.51±0.02^b
Lys*	1.46±0.02^a	0.90±0.04^b
Non-essential amino acids (NE)		
Glu	2.91±0.02^a	2.20±0.11^b
Ser	1.50±0.04^a	0.92±0.07^b
Gly	2.73±0.07^a	1.26±0.04^b
Ala	1.42±0.04^a	1.06±0.04^b
Pro	0.85±0.02^a	0.65±0.01^b
Tyr	0.44±0.02^a	0.47±0.04^a
Cys	0.40±0.06^a	0.06±0.04^b
EAA	10.27±2.05^a	6.87±0.11^b
NEAA	10.25±0.01^a	6.62±0.41^b
EAA/NEAA	1.0±0.01^a	1.04±0.02^a

Reversed-phase binary gradient high performance liquid chromatograph (HPLC, Waters RP PICO.TAG amino acid analysis system), fitted with a analyse hydrolysable amino acids by their UV absorbance (λ_{max} 254 nm). The mobile phase eluents used were A and B, eluent A comprises sodium acetate trihydrate (MeCOONa, 0.14 M, 940 mL, pH 6.4) containing TEA (Me_3N, 0.05percent), mixed with CH_3CN (60 mL); and eluent B used was acetonitrile:water (60:40, v/v). Data are means of duplicate analysis of pooled homogenates. *Note*. Tryptophan was not determined, *Essential amino acid for humans.

The slight difference in the ΣEAA and ΣNEAA among wild and cultured oysters appeared to be due to the different environmental and nutritional conditions. This demonstrates the potential capability of *C. madrasensis*, growing in wild condition, to withstand salinity and adverse stress conditions during summer, because glycine or its conjugate (glycine betaine) that was earlier reported to have unique osmolytic property (Eklund *et al*., 2005) and helps to protect the cells during summer against osmotic injury. Any ratio of EAA/NEAA amino acids greater than 1.0 is considered to be excellent, and therefore it can be concluded that, *C. madrasensis* are good sources of well-balanced proteins and high-quality protein.

3B.2.5 Vitamin and unsaponifiable composition of maricultured and wild *C. madrasensis*

The fat soluble vitamins A, D_3, E, K and water soluble vitamin C were recorded in **Table 3B.1**. The cultured samples exhibited significantly greater ($P<0.05$) content of cholecalciferol than wild counterparts .The levels of α-tocopherol, a vitamin with anti-oxidant properties, (0.20 IU in wild & 0.15 in cultured) showed significant difference ($P<0.05$) among wild and cultured samples. As only plants synthesize α-tocopherol, algae, either direct or indirectly, are the ultimate source of this compound for oysters. Phylloquinone (K_1) registered significantly high values ($P<0.05$) in wild samples than cultured ones. The values of *trans* retinol content are within the limits to impart their beneficial effects. Vitamin D precursors constitute a large proportion of the unsaponifiable fraction of mollusk lipids. Among minor unsaponifiable components, HPLC analyses showed that oxygenated carotenoids, characterized by typical absorption spectra, were prevalent over the less polar, late-eluting, α-carotene and β-carotene. Due to the lack of suitable standard compounds, the early-eluting xanthophylls were not identified

3B.2.6 Mineral composition of cultured and wild *C. madrasensis*

Minerals are nutrients that are conserved by the body and play significant role in metabolism in the human body. **Table 3B.1** shows the trace mineral composition (mg $100g^{-1}$ wet edible tissue) of cultured and wild *C. madrasensis*. The comparison between cultured and wild oysters revealed that there was significant greater ($P<0.05$) concentrations of Na and Ca in wild oysters (Na = 185.6 mg $100g^{-1}$; Ca = 174.7 mg $100g^{-1}$) and K and P in cultured oysters (K = 515.4 mg $100g^{-1}$; P = 530.2 mg $100g^{-1}$). The cultured samples recorded lower Na and Ca than wild samples, and therefore rated high as a health food. The results in the present study agree with other studies, which reported mussels to be a good source of Ca, Zn and Fe (Astorga *et al*., 2007).In general, the present study revealed that cultured *C. madrasensis* samples exhibited greater micronutrient contents than wild samples. Se, the antioxidant element was found to be 0.04 mg/ 100g wet sample for wild and 0.03 mg $100g^{-1}$ wet sample for cultured samples. The concentration of trace minerals in oysters is influenced by a number of factors such as seasonal and biological differences (size, age, sex and sexual maturity), food source and environment water chemistry, salinity, temperature and contaminants; (Lal, 1995). The variation of mineral content between wild and cultured oysters appeared to be due to the influence of several factors including food availability and differences in metal regulation (Astorga *et al*., 2007). In addition, the differences in the mineral concentrations of the surrounding seawater could also influence their levels in bivalves (Peaz *et al*., 1995).

No significant difference was observed between wild and cultured collected from southwestern coast of India and the minor differences in biochemical indicators could be attributed to the differential feeding patterns of

oysters grown under wild and cultured conditions. Growth conditions play vital role in physiological mechanisms of oysters guiding fatty acid metabolism. High levels of PUFA (21.7-36.4%) including *n*-3 PUFAs (12.5-23.2 %), low levels of *n*-6 PUFA (maximum of 7% of total fatty acids) observed in both wild and cultured oysters. The ratios of essential/non-essential amino acids (EAA/NEAA) indicated that both oyster tissues are good sources of well-balanced proteins. The cholesterol content in wild oysters is low (<35 mg $100g^{-1}$) compared to cultured samples (>40 mg $100g^{-1}$).The well optimised contents added to the good qualities of this particular species, therefore proved to be a desirable item in the human diet in this region of India.

3.C Nutrient profiling of *Octopus dolffusi* collected from south west coast of Kerala

General

Cephalopods are considered as the most active and specialized class of mollusks. Octopus is one of the main cephalopod species, representing about 8.8 percent of cephalopod world catches. Octopus has received more attention in recent years due to its high nutritional and market value, excellent palatability and increasing demand throughout different regions of the world. Cephalopods are carnivorous, active predators, and the shallow water species are characterized by rapid growth because they have very high feeding rates and most part of the elements can be incorporated in the diet. Due to their rapid growth and market value, the culture of cephalopods is an area of increasing interest (Walsh *et al*., 2002; García García *et al*., 2004; Nabhitabhata *et al*., 2005; Sykes *et al*., 2006; Cerezo Valverde *et al*., 2008; Rosas *et al*., 2007, 2008).

Octopus and cephalopods in general are characterized by low lipid contents, with relatively large phospholipid and sterol fractions, and triacyglycerides as minor components (Nash *et al*., 1978; Hayashi and Yamamoto, 1987; Navarro and Villanueva, 2000). The lipid of cephalopods contain a high percentage of PUFA, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are particularly high in paralarval and juvenile cephalopods (Navarro and Villanueva, 2000, 2003; Hamasaki and Takeuchi, 2001; Moxica *et al*., 2002; Okumura *et al*., 2005; Almansa *et al*., 2006; Kurihara *et al*., 2006; Seixas *et al*., 2008). The high levels of EPA and DHA in marine lipids imply the presence of a strong antioxidant system in marine animal tissues. Dietary supplementation with EPA and DHA is expected to attenuate inflammatory responses by reducing the content of arachidonate or inhibiting eicosanoid production (Ramesha and Pickett, 1986). Cephalopods contain low level of lipid with large amounts of polyunsaturated fatty acids (Sinanoglou and Miniadis-Meimaroglou, 1998).

From the nutritional point of view, less attention has been paid to the nutritional profile of cephalopods. Recently, octopus is finding more acceptances because of its extraordinary nutritional qualities. Therefore, proper understanding about the biochemical constituents of this species has become a primary requirement for the nutritionists and dieticians. Biochemical changes in the octopus in different seasons may result from fluctuations of environmental parameters, *viz*., temperature, salinity, oxygen levels, and to the physiological status of the animals, depending on food availability, gametogenic cycle, and spawning. The nutritional compositions vary among species due to geographical differences of fishing grounds and cause difficulty to recommend a suitable preventive diet. Therefore, considering the promising

perspective for the utilization of octopus, and the need for knowledge of its chemical composition, this work determined the seasonal variations (winter and postmonsoon) of essential nutritional compositions with respect to amino acids, cholesterol, proteins, lipids, and fatty acids of *P. viridis* harvested from south western coast of India to assess possible seasonal differences in nutritional composition.

3C.1. Sample collection and processing

3C.1.1 Collection of *O. dolffusi* samples

Octopus dolffusi samples were collected from the Fort Kochi, area of Kerala along the South-west coast of India in different seasons as winter (January-February) and post-monsoon (August-September). The samples were cleaned and the edible tissues were minced for analyses. Although, age and sex differences in nutritional composition could occur, we regarded the octopus samples as a whole food source, which was representative of the market, and thus totally used by the local population, without any age or sex differences.

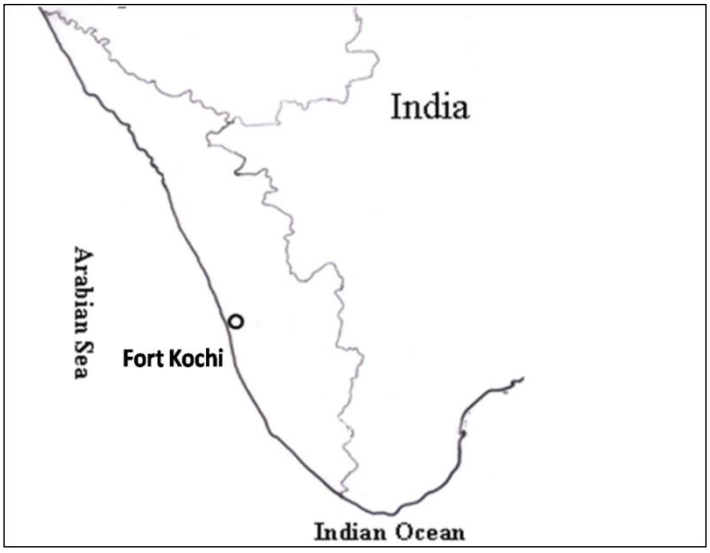


Fig.3C.1 Sample collection site of *O. dolffusi,* along the Southwestern coast of India.

3C.1.2 Biometric parameters and meat yield of *O. dolffusi*

O. dolffusi samples were measured for their biometrical parameters, *viz.*, length, width, and thickness. Length, width and thickness of randomly selected samples were measured using a vernier caliper. The samples were then weighed and the tissues were oven dried for 48 h at 60°C, and the dry weight was also determined.

3C.2 Result and discussion

3C.2.1 Biometric characteristics and percent meat yield of *O. dolffusi*

The octopus samples collected in winter season recorded significantly greater ($P< 0.05$) meat yield than samples collected during postmonsoon (**Table 3C.1**). The length and weight of octopus collected in postmonsoon season is significantly higher compared to octopus collected in winter season The size and weight of cephalopods are dependent on growth stage, temperature, salinity, oxygen, light, food, competition, social interaction and sex (Forsythe *et al.*, 2002; Okuzumi and Fujii, 2000).

Table 3C.1 Biometric parameters, meat yields (percent), proximate, lipid, protein, cholesterol, and mineral compositions of *O. dolffusi* collected from Cochin

	Winter	Postmonsoon
Length (cm)	25.6±0.05[a]	34.8±0.25[b]
Weight (gm)	13.8 ±0.92[a]	15.6±0.56[b]
Meat yield (%)	21.5±0.65[a]	25.4±0.32[b]
Proximate composition (g 100g^{-1} wet sample)		
Dry matter		
Moisture	80.1±0.84[a]	85.2±1.43[b]
Crude protein	12.42±0.22[a]	10.11±0.04[b]
Crude fat	2.46±0.17[a]	2.41±0.08[a]
Crude ash	1.01±0.04[a]	0.96±0.04[a]

Crude fiber	0.03±0.01[a]	0.02±0.00[a]
Lipid (%)	4.56±0.17[a]	5.16±0.13[b]
Cholesterol (mg $100g^{-1}$)	98.6±0.47[a]	105.3±0.29[b]
Protein (mg $100g^{-1}$)	215.1±2.56[a]	197.6±6.10[b]
Macronutrients (mg $100g^{-1}$ wet sample)		
Na	283.±1.46[a]	197.7±0.95[b]
K	518.±4.35[a]	376.5±2.46[b]
Ca	4.72±0.28[a]	4.52±0.24[a]
Mg	19.3±0.57[a]	18.56±0.16[a]
P	310.±1.64[a]	301.3±1.56[b]
Na/K	0.54±0.02[a]	0.49±0.00[a]
Ca+P	315.±1.32[a]	294.5±1.84[b]
Micronutrients (mg 100^{g-1} wet sample)		
Cr	0.15±0.01[a]	0.12±0.01[a]
Zn	0.72±0.01[a]	0.65±0.00[a]
Mn	0.2±0.01[a]	0.1±0.00[a]
Cu	0.22±0.01[a]	0.16±0.01[b]
Fe	5.69±0.54[a]	4.91±0.05[b]
Se	0.37±0.01[a]	0.31±0.02[a]
Fat soluble Vitamins		
Retinol (A)/IU	0.32±0.01[a]	0.37±0.06[a]
Cholecalciferol (D_3)/IU	410.1±0.81[a]	394.5±2.57[b]
Tocopherol (E)/IU	0.17±0.06[a]	0.16±0.01[a]
Phylloquinone (K_1) (µg $100g^{-1}$)	1.14±0.02[a]	1.21±0.15[a]
Water soluble Vitamins		
Ascorbic acid (C)/IU	0.76±0.25[a]	0.84±0.13[b]

All samples were analyzed in triplicate (n=3) from a pooled samples (20 numbers), and expressed as mean ± standard deviation. Means followed by the same letter within the same row are not significantly different, and different letters indicate significant differences (P<0.05). ND: non detectable

3C.2.2 Inter seasonal variability in proximate composition of *O. dolffusi*

The seasonal variations in the proximate compositions of octopus collected in two different seasons were shown in **Table 3C.1**.The proximate compositions of octopus recorded ~12% crude protein and 2.5% crude fat in winter season whereas in post-monsoon season the values were 10% and 2% respectively. The results of protein analysis indicated no significant variation throughout the seasons. Moisture content of octopus significantly fluctuated (P<0.01) across the season. There was no marked variation in the crude fibre and ash content of octopus samples. These results were in accordance with the results of other cephalopod species of Mediterranean origin, which revealed moisture content as 78-83g $100g^{-1}$, and crude ash as 1.2-2.1 g $100g^{-1}$ (Yesim *et al*., 2008).

3C.2.3 Inter seasonal variability in protein and total cholesterol contents of *O. dolffusi*

The octopus samples collected during the winter season were found to possess significantly greater protein and cholesterol content (P<0.05) than samples collected in post-monsoon season. The level of cholesterol in octopus samples collected in winter and post-monsoon season were 98 mg $100g^{-1}$ and 105 mg $100g^{-1}$, respectively **(Table 3C.1)**. Okuzumi and Fujii (Okuzumi and Fujii, 2000) reported that the cholesterol content in several cephalopods species as, 123 mg $100g^{-1}$ in cuttlefish, 180 mg $100g^{-1}$ in squid and139 mg $100g^{-1}$ in octopus. The protein content of octopus samples recorded 215.1 mg $100g^{-1}$ in winter season and 197.6 mg $100g^{-1}$ during post-monsoon season. Contrary to fish and crustaceans, the cephalopods exclusively use protein for both growth and energy supply. Cephalopods are composed mainly of protein

(75-85% dry weight) (Iwasaki and Harada, 1985; Boucaud-Camou, 1990). The main reason for the increasing demand in cephalopods is that, they are a good source of balanced protein with essential lipids (Sinanoglou and Miniadis-Meimaroglou 2000).

3C.2.4 Inter seasonal variability in lipid content and fatty acid composition of *O. dolffusi*

The lipid content and fatty acid composition of octopus were recorded in **Table 3C.2**. Significant differences in lipid content were observed among octopus samples collected during different seasons ($P>0.05$). Lipid content of octopus increased in post-monsoon season (5.2 %) compared to that during the winter (4.6 %). The direct mobilization of muscle protein provides metabolic energy during periods of starvation and the direct use of protein as an energy reserve may account for the lack of major glycogen or lipid reserves in cephalopod tissues (Storey and Storey, 1983; O'Dor *et al*., 1984). The major factor affecting the lipid content in cephalopods was the abundance of food (Ackman, 1995; Robards *et al*., 1999; Rosa *et al*., 2002). The high lipid content in post-monsoon season might be due to the availability of more nutrients in monsoon season than in other seasons. It has also been indicated that the lipid content of fish changes due to species, gender, geographical origin and season (Rasoarahona *et al*., 2005). The low level of lipids in cephalopod was due to the poor absorption of lipids (O'Dor *et al*., 1984) and consequent poor utilization by the animal's metabolism (Ballantyne *et al*., 1981).

Table 3C.2 Fatty acid composition of *O. dolffusi* collected in winter and postmonsoon season

Fattyacids(percent total fatty acids, (TFA))	Winter	Postmonsoon
Saturated fatty acids		
C12:0	1.92±0.05[a]	2.85±0.03[b]
C14:0	2.32±0.01[a]	2.91±0.02[b]
C15:0	1.18±0.54[a]	1.23±0.61[a]
C16:0	15.9±0.02[a]	16.7±0.01[b]
C17:0	0.46±0.32[a]	1.23±0.24[b]
C18:0	8.59±0.11[a]	6.46±0.09[b]
C22:0	0.36±0.01[a]	0.58±0.01[b]
C24:0	1.03±0.01[a]	1.99±0.01[b]
∑ SFA	31.8±1.07[a]	34.0±1.02[b]
Monounsaturated fatty acids		
C16:1 n7	2.48±0.05[a]	2.69±0.08[b]
C18:1n7	4.17±0.01[a]	4.15±0.02[a]
C18:1 n9	10.1±0.74[a]	9.58±0.29[b]
C20:1 n9	9.62±0.03[a]	8.49±0.01[b]
C22:1 n9	3.12±0.05[a]	4.19±0.02[b]
C24:1	0.57±0.01[a]	0.67±0.01[b]
∑ MUFA	30.1±0.89[a]	29.7±0.34[b]
Polyunsaturated fatty acids		
C18:2*n*-6	2.16±0.18[a]	2.24±0.08[b]
C18:3*n*-6	2.94±0.13[a]	2.65±0.02[b]
C18:3*n*-3	1.11±0.01[a]	1.55±0.03[b]
C18:4*n*-3	0.75±0.01[a]	1.61±0.01[b]
C20:2*n*-6	1.16±0.01[a]	0.25±0.01[b]
C20:3*n*-6	1.38±0.02[a]	0.11±0.01[b]
C20:4*n*-6	3.35±0.14[a]	4.68±0.05[b]
C20:5*n*-3	6.93±0.21[a]	5.94±0.06[b]
C22:5*n*-3	1.92±0.05[a]	2.84±0.03[b]
C22:6*n*-3	15.7±0.54[a]	13.3±0.07[b]
Σ PUFA	37.4±1.3[a]	35.1±1.12[b]
∑ C_{18}PUFA	6.96±0.12[a]	8.05±0.02[b]
∑ C_{20}PUFA	12.8±0.09[a]	10.9±0.11[b]
∑ *n*-3	26.4±0.35[a]	25.2±0.42[b]
∑ *n*-6	10.9±0.21[a]	9.93±0.06[b]
n-3/*n*-6	2.41±0.05[a]	2.54±0.01[b]
∑ PUFA/∑ SFA	1.17±0.02[a]	1.03±0.01[b]
22:6*n*-3/20:5*n*-3	2.27±0.04[a]	2.24±0.08[a]
AI	1.59±0.00[a]	1.60±0.00[a]
TI	0.42±0.00[a]	0.43±0.00[a]
HH	2.26±0.64[a]	2.04±0.16[a]

ΣSFA: total saturated fatty acids; ΣMUFA: total monounsaturated fatty acids; ΣPUFA: total polyunsaturated fatty acids AI: Atherogenic index; TI: thrombogenic index; HH: hypocholesterolaemic/ hypercholesterolaemic ratio; Data presented as mean values of three samples (mean ± SD). These values do not total 100percent because minor fatty acids are not reported. ND implies non detectable (or fatty acids present below 0.05percent). *a,b values for each sample with different letters in the same row are significantly different at $P<0.05$.

3C.2.4.1 Saturated fatty acids (SFAs)

The total saturated fatty acid composition of octopus recorded 31.8% in winter season and 34 % in post-monsoon season (**Table 3C.2**). The highest proportions of saturated fatty acids in cephalopods were due to myristic acid (C14:0, 1–3%), palmitic acid (C16:0, 15.5–25.2%), heptadecanoic acid (C17:0, 1.05–2.6%), stearic acid (C18:0, 4.32–10%).The results of fatty acid analysis in octopus are within the range of previous studies.(Ozyurt *et al*., 2006; Reale *et al*., 2006; Rosa, Pereira, & Nunes, 2005; Zlatanos *et al*., 2006). Palmitic acid was the abundant fatty acid in marine lipids, and recorded 16% in the samples. Palmitic acid was found to be 20% of the total fatty acids in the cephalopod tissues (Passi *et al*., 2002; Phillips *et al*., 2002). Studies have shown that SFA was positively associated with the total cholesterol levels and coronary heart diseases (Caggiula and Mustad, 1997).

3C.2.4.2 Monounsaturated fatty acids (MUFAs)

MUFA are believed to reduce the incidence of cardiovascular diseases by decreasing low density lipoprotein (LDL) cholesterol in blood (Appel *et al*., 2005; Chow, 2008; Kris-Etherton, 1999). No significant difference was observed between the MUFA content of octopus samples collected in two different seasons ($P<0.05$). Octopus samples revealed 30 % of MUFA content in both seasons **(Table 3C.2)**. Consumption of cephalopods would contribute the intake of MUFA (Ozogul *et al*., 2008). Inclusion of higher dietary MUFA has been suggested by NCEP (2001) due to their beneficial effects on cardiovascular heart disease (Kalogeropoulus *et al*., 2004; Keys *et al*., 1986). The predominant MUFAs were oleic acid (C18:1*n*-9) and cis-11-eicosenoic acid (C20:1), which were found to be greater in the samples collected during winter than in post-monsoon season. It was also observed that the proportion of these fatty acids changed significantly throughout the seasons (Ozogul *et*

al., 2008). The variation of fatty acid composition depends on species, food availability, age, geographical area, season and salinity (Ozogul and Ozogul, 2005; Ozyurt *et al*., 2006).

3C.2.4.3 Polyunsaturated fatty acids (PUFAs) and ratios of different families of PUFAs

PUFA are the predominant fatty acids of octopus, and these long-chains fatty acids are beneficial in preventing health problems but with their own specialized and distinctive functions. Significant differences in total PUFA content was observed between the samples collected during different seasons ($P>0.05$). The samples collected in winter season (37.4 %) recorded greater total PUFA content than in the post-monsoon samples **(Table 3C.2)**. The variation of fatty acid composition depends on species, food availability, age, geographical area, season and salinity (Ozogul and Ozogul, 2005; Ozyurt *et al*., 2006). Cephalopods contain large amounts of polyunsaturated fatty acids (52.9–56.3% of total fatty acids) (Sinanoglou and Miniadis-Meimaroglou, 1998). EPA (20:5*n*−3) and DHA (22:6*n*−3) were the most abundant fatty acids found in the lipids of *O. vulgaris*, as has been reported for many cephalopod species (Jangaard and Ackman, 1965; Culkin and Morris, 1970; Nash *et al*., 1978). The major polyunsaturated fatty acids identified in octopus are eicosapentaenoic acid and docosahexaenoic acid. The highest EPA and DHA proportions were obtained for octopus samples collected in winter (16% and 7% respectively) whereas post-monsoon samples recorded only 13% and 6% respectivelyin that order **(Fig.3C.2)**. These fatty acids are the precursors of the eicosanoids and are necessary for maintaining the impermeability barrier of the skin, and are also involved in cholesterol transport and metabolism (Tarley *et al*., 2004; Osman *et al*., 2001). The most characteristic PUFAs were found to be docosahexaenoic acid (20% and 36%) and eicosapentaenoic acid (8% and 18%), in the octopus

species (Culkin and Morris, 1970; Gibson, 1983; Sinanoglou and Miniadis-Meimaroglou,1998). Polyunsaturated fatty acids with 20 carbon atoms (e.g. 20:3*n*-6, 20:4*n*-6 and 20:5*n*-3) are known precursors of eicasonoids, which have a wide range of physiological actions, such as, assisting in blood clotting, the immune response, the inflammatory response, cardiovascular tone, renal function, neural function, and reproduction (Tocher, 1995). Dietary supplementation with EPA and DHA is expected to attenuate inflammatory responses by reducing arachidonic acid content or inhibiting eicosanoid production (Ramesha and Pickett, 1986). Cephalopods contained small amounts of fat and high amount of EPA and DHA as reported earlier (Kalogeropoulus *et al*., 2004; Ozyurt *et al*., 2006; Reale *et al*., 2006; Zlatanos *et al*., 2006). DHA was known to be an important constituent of the building blocks of the biological membranes in the brain and retina affecting cognitive and visual functions (Chow, 2008; Connor, 2000). EPA is more efficient in reducing cellular inflammation preventing depression disorders (Martins, 2009), and in the reduction of serum triacylglycerols (formerly known as triglycerides). The EPA and arachidonic acid (AA) concentration in the octopus indicated an inverse relationship, where decreased EPA levels resulted in respective increased AA levels.

No significant difference was observed between the *n*-3/*n*-6 and DHA/EPA content of octopus samples collected during two different seasons ($P<0.05$). The recommended *n*-3/*n*-6 ratio was reported to be greater than 1.0 (Chow, 2008). The fatty acid composition of several species of teleosts, cephalopods and crustaceans were studied by Passi *et al* (Passi *et al*., 2002), and found that the edible muscle had an *n*−3/*n*−6 ratio of greater than 1, confirming the importance of octopus as an ideal dietary source of *n*-3 PUFA for humans. Samples collected in winter season exhibited greater *n*-3 PUFA content required

for maintaining the structural and functional integrity of cell and more importantly for the development and survival of fast growing cephalopods (Navarro and Villanueva, 2000). Cardiovascular mortality was noted to be inversely proportionate to the intake of *n*-3 fatty acids (Kafatos *et al*., 1997). Moreover, they play an important role in the neurodevelopment of infants and in fat glycemic control (Caponio *et al*., 2011).

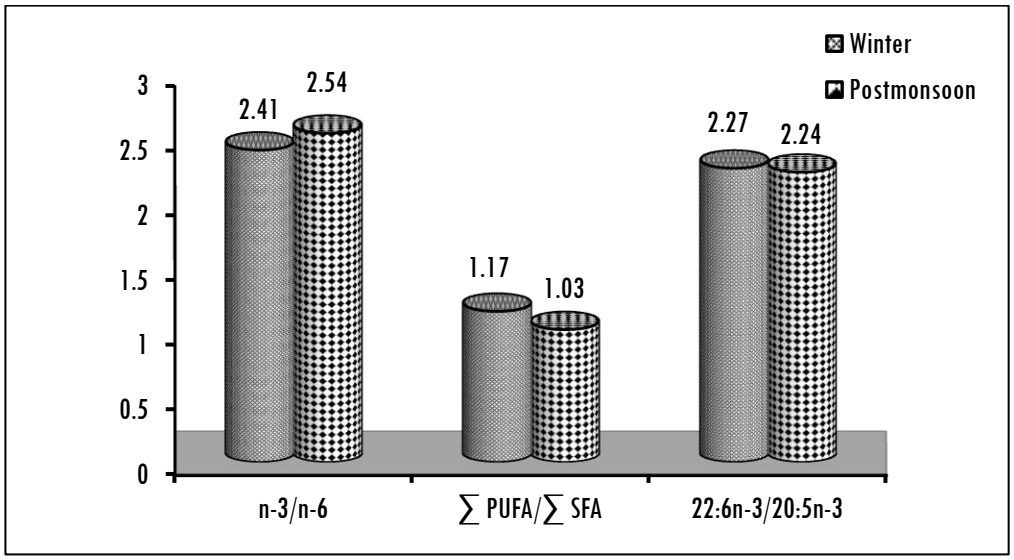


Fig.3C.2. Differential compositions of *n*-3/*n*-6 PUFAs, ∑ PUFA/∑ SFA, 22:6*n*-3/20:5*n*-3 of *O. dolffusi* collected during winter and post monsoon season.

The changes in the fatty acid composition of total lipids observed in the octopus reflected to those that occurred mainly in the composition of phospholipids. No significant difference was observed in atherogenic and thrombogenic indices for samples collected during winter and post-monsoon season. The greater PUFA content and consequently the higher PUFA/SFA fatty acid ratio were apparent in the samples collected in winter, contributed to lower atherogenic and thrombogenic indices.

3C.2.5 Amino acid composition of *O. dolffusi*

Samples collected during winter exhibited greater amount of total essential (ΣEAA) amino acids than those collected in pre-monsoon season (**Table 3C.3**). The amino acid content could vary among organisms due to geographical differences, species, age and physiological condition (Capillas *et al*., 2002). The abundant essential amino acids (EAA) were found to be arginine and proline in winter as well as post-monsoon samples. Arginine and proline were reported as the main substrates amino acid catabolism for energy in the cephalopods (Hochachka and Fields, 1983). Arginine (1.6g $100g^{-1}$) and proline (1.5g $100g^{-1}$) were found to be significantly greater in the samples collected in post-monsoon season. This is because the arginine available is condensed with glucose-derived pyruvate to form octopine, the main anaerobic end product that accumulates in adult cephalopods during periods of exercise and stress (Hochachka *et al*., 1977; Storey and Storey, 1978). Proline is involved in oxidative metabolism in cephalopods and during exercise (Villanueva *et al*., 2004). The ability of cephalopods to absorb amino acids by the integument fluids also exists in other mollusks (Manahan, 1990) and might represent a phylogenetic remnant for this group of exclusively carnivorous animals. The non-essential amino acid recorded greater values in post-monsoon season. Proline and alanine (non-essential amino acid) in the diet must be reinforced, given the fact that both amino acid represent one of the most important energy resources in cephalopods with a production not sufficient to cover catabolic demands (Lee, 1994).

Table 3C.3 Amino acid composition (mg $100g^{-1}$ wet tissue) of *O.dolffusi* collected in winter and post-monsoon season

Amino-acids	Winter	Post-monsoon
Essential amino acids (E)		
His	0.79±0.01[a]	0.84±0.02[b]
Arg	1.72±0.16[a]	1.59±0.01[b]
Thr*	0.58±0.01[a]	0.74±0.05[b]
Val*	0.89±0.01[a]	0.81±0.07[a]
Met*	0.66±0.01[a]	0.94±0.05[b]
Ile*	0.77±0.02[a]	0.82±0.03[a]
Leu*	1.41±0.04[a]	1.35±0.12[b]
Phe*	0.82±0.02[a]	0.98±0.02[b]
Lys*	1.04±0.05[a]	1.16±0.09[b]
Σ EAA	8.68±0.33[a]	9.23±0.46[b]
Non-essential amino acids (NE)		
Glu	0.86±0.09[a]	0.95±0.09[b]
Ser	0.89±0.06[a]	0.76±0.05[b]
Gly	0.74±0.04[a]	0.82±0.07[b]
Ala	1.01±0.03[a]	1.31±0.06[b]
Pro	1.55±0.01[a]	1.51±0.02[a]
Tyr	0.73±0.02[a]	0.76±0.07[a]
Cys	0.23±0.01[a]	0.37±0.01[b]
Σ NEAA	6.01±0.01[a]	6.48±0.37[b]
Σ EAA/ Σ NEAA	1.44±0.01[a]	1.42±0.01[a]
TAA	14.6±0.28[a]	15.71±0.83[b]

Reversed-phase binary gradient high performance liquid chromatograph (HPLC, Waters RP PICO.TAG amino acid analysis system), fitted with a packed column (dimethylocatadecylsilyl bonded amorphous silica; Nova-Pak C_{18}, 3.9 X 150 mm maintained at 38±1°C in a column oven) was used to analyse hydrolysable amino acids by their UV absorbance (λ_{max} 254 nm). The mobile phase eluents used were A and B, eluent A comprises sodium acetate trihydrate (MeCOONa, 0.14 M, 940 mL, pH 6.4) containing TEA (Me_3N, 0.05percent), mixed with CH_3CN (60 mL); and eluent B used was acetonitrile:water (60:40, v/v). Data are means of duplicate analysis of pooled homogenates. *Note*. Tryptophan was not determined, *Essential amino acid for humans.

3C.2.6 Vitamin and unsaponifiables of *O. dolffusi*

The fat soluble vitamins A, D_3, E, K and water soluble vitamin C were recorded in **Table 3C.1**. Among the vitamins, A and E play a major roles in growth, reproduction and embryonic development. Vitamin A is present in many forms including the free alcohol form of retinol. Vitamin E, being the most bioactive component, is present in two structural types: as tocopherols and tocotrienols, and the levels of α-tocopherol (0.16 IU) were found to be greater in samples collected during winter season, and showed no significant difference (P>0.05) among samples collected in various seasons. The α-tocopherol is a lipid-soluble antioxidant, known for its ability to protect sensitive molecules, such as, unsaturated fatty acids against oxidation, by its ability to neutralize free radicals and reduce lipid peroxidation (Pazos *et al*., 2005) The α-tocopherol concentration exhibited proportional content with *n-3* PUFAs as also supported by earlier studies (Chow, 2008). Phylloquinone (K_1) and cholecalciferol (D_3) registered significantly high values (P<0.05) in samples collected in winter than during the post-monsoon season. Seasonal variation in retinol and tocopherol contents was observed in squid *Watasenia scintillans*, especially in relation to spawning period (Motoe *et al*., 1997).

3C.2.7 Mineral composition of *O. dolffusi*

Minerals are required for the maintenance of normal metabolic and physiological functions of living organisms. The absorption of minerals from seawater also could take place by osmotic uptake through the gills and the body surface as the cephalopods live in the hypoosmotic environment (Nurjanah *et al*., 2012). **Table 3C.1** showed the trace mineral composition (mg $100g^{-1}$ wet edible tissue) of *O. dolffusi*. The major functions of essential elements in the body include the formation of skeletal structure, maintenance of colloidal systems, as well as regulation of acid base equilibrium. They are important components of hormones, enzymes and structural proteins (Lall, 2002; Villanueva and Bustamante, 2006). Zn is involved in numerous protein functions, such as, the carbonic anhydrase and is efficiently absorbed in the samples collected during winter (0.72 mg $100g^{-1}$) than those in post-monsoon

(0.65 mg $100g^{-1}$). Sodium (283.1 mg $100g^{-1}$ and potassium (518.2 mg $100g^{-1}$) concentrations were found to be greater in the octopus samples collected during winter. These elements and are required for maintaining homeostasis and the acid-base balance. No significant difference was apparent in the contents of P and Ca which are required for the formation of skeletal structure of the body (Lall, 2002). High Na/K ratio (0.54) was recorded in the edible tissues of the samples collected during the post-monsoon season. The values of Ca+P ratio obtained in the samples varied 295 – 315 mg $100g^{-1}$. Minerals were reported to be absorbed by the digestive gland of octopus, they swallow massive quantities of sea water during feeding (Wells and Wells, 1989). Se, the antioxidant element was found to be greater in the samples collected during the winter season as compared to that in post-monsoon season.

The present work elucidated the importance of *O. dolffusi* as balanced sources of amino acid, fatty acid, mineral and vitamin. *O. dolffusi* is a fairly good source of well-balanced proteins with good ratio of essential to nonessential amino acids. The balanced nutritional profile of *O. dolffusi*, makes it one of the most suitable and healthy forms of human food.

3.2 Conclusions

Octopus was found to be the good protein source with low in fat content, and considered as the most interesting inhabitants of the seas. Greater levels of EPA and DHA in the lipids implied the presence of antioxidant system in the edible muscle of *O. dolffusi*. Greater levels of PUFA including *n*-3 PUFAs, important in the human diet for their platelet anti-aggregating and blood pressure-reducing properties, lesser levels of *n*-6 PUFA, and relatively high *n*-3/*n*-6 PUFA ratio values characterized *P. viridis.* The higher PUFA content also contributed to their potential to exhibit anti-inflammatory activities, and therefore, proved to be a highly desirable item for use as food and nutrceutical supplement.

Chapter 4

BIOACTIVE POTENTIALS WITH RESPECT TO ANTI-OXIDATIVE AND ANTI-INFLAMMATORY PROPERTIES OF *PERNA VIRIDIS, CRASSOSTREA MADRASENSIS* AND *OCTOPUS DOLFFUSI*

Contents

Background of the study

Free radicals are continuously produced in our body due to the utilization of oxygen which results in the generation of a series of reactive oxygen species (ROS) and non-free radical species. Reactive oxygen species are highly reactive and can easily react with the vital biological molecules including proteins, lipids, lipoproteins and DNA. These free radicals are naturally scavenged by antioxidant mechanism in mammalians. However uncontrolled generation of free radicals is associated with lipid and protein peroxidation, resulting in cell structural damage, tissue injury, or gene mutation which ultimately lead to a various pathophysiological disorders, such as, arthritis, diabetes, inflammation, cancer and genotoxicity (Kourounakis *et al*., 1999). The human body constantly quenches excessive oxidants and free radicals through various scavenging mechanisms, such as, use of antioxidant enzymes and molecules. In certain circumstances, the body's natural defence mechanism becomes inefficient, which makes mandatory dietary intake of

antioxidant compounds as an alternative, suggesting that there is an inverse relationship between dietary intake of antioxidants and the incidence of diseases caused by the deficiency of these substances. The commercially available synthetic antioxidants are used to reduce the deleterious effect of oxidative-induced reactions in food and biological systems. However, the potential toxicity of these synthetic antioxidants has aroused an increased interest in identifying alternative natural and safe sources of antioxidants, and the search for natural antioxidants, especially of marine origin, has notably increased during the recent years.

Marine organisms are considered as unique biostore of active compounds, having enormous therapeutic potential, which led to the growing interest in investigation of natural products for the discovery of immuno-stimulatory activity, such as, antioxidant, anti-inflammatory and antimicrobial compounds. Among marine organisms, mollusks are widely distributed throughout the world, and have many representatives in the marine and estuarine ecosystems, namely, slugs, whelks, clams, mussels, oyster, scallops, squids and octopus. Bivalves and cephalopods are considered to be a valuable food items as they constitute rich source of antioxidants, essential for providing a balanced diet. They are also very good source of biomedically important products. Bivalves were reported to repair of oxidative damage by ROS, by increasing the expression of antioxidant molecules (Santovito *et al*., 2005). Mollusks are considered as one of the important sources to derive bioactive compounds that exhibit antimicrobial, anti-inflammatory, and antioxidant activities (Anbuselvi *et al*., 2009; Chellaram and Edward, 2009b; Benkendorff *et al*., 2011). Many bioactive compounds have been extracted, characterized and purified from the bivalves and cephalopods. Compounds isolated from mollusks were used in the treatment of rheumatoid arthritis and

osteoarthritis (Chellaram and Edward, 2009a). Cephalopods are considered to be a rich source of *n*-3 fatty acids that are responsible for anti-inflammatory activities (Russo and Tringali, 1983). Long chain fatty acids consisting of 20 carbon atoms (eg., 20:3*n*6, 20:4*n*6 and 20:5*n*3) were reported in various cephalopods including squid and octopus, which have a wide range of physiological actions including immune and anti-inflammatory responses, and were reported to have vital role in neural function and reproduction (Tocher, 1995; Miliou *et al.*, 2006). Polyunsaturated fatty acids (PUFAs) are important constituents of the phospholipids of all cell membranes. Changes in fatty acid composition of cell membranes influence inflammation, and these compositions can modify the membrane fluidity, cell signalling leading to altered gene expression, and the pattern of lipid mediator production. Cells involved in the inflammatory response are typically rich in the *n*-6 fatty acid (arachidonic acid), and eicosanoids produced from arachidonic acid have roles in inducing inflammation. Long-chain *n*-3 PUFAs also gives rise to eicosanoids, and these may have differing properties from those of arachidonic acid-derived eicosanoids. Long-chain *n*-3 PUFAs give rise to a family of anti-inflammatory mediators, termed resolvins, which are anti-inflammatory and inflammation resolving, through the pathways involving cyclooxygenase and lipoxygenase enzymes. Thus, *n*-3 PUFAs are potent anti-inflammatory agents, and have therapeutic efficacy in a variety of acute and chronic inflammatory settings.

The present study attempted to highlight the promising antioxidant and anti-inflammatory activities of bivalves and cephalopods using different *in-vitro* antioxidant assays. Antioxidants from natural sources play a paramount role in helping endogenous antioxidants to neutralize the oxidative stress. The natural antioxidants have lesser toxicity, and were proved to be effective in

scavenging free radicals and avoiding the excess ROS formation in the body (Youdim *et al*., 2001). The study designed to evaluate the antioxidant and anti-inflammatory capacities of freeze dried extract and different solvent fractions and aqueous fractions of selected species such as *Perna viridis, Crassostrea madrasensis* and *Octopus dolffusi*. This is particularly important to assess the in-build antioxidant and anti-inflammatory capacities to inhibit free radical formation and inflammation in the body, and to evaluate their potentials to isolate bioactive molecules.

4.1 Materials and methods

4.1.1 Instrumentation

A bench top refrigerated high – speed microprocessor controlled centrifuge equipped with asynchronous motor and programmable micro-controlled variable frequency controller (Superspin Plasto Crafts R-V/Fm, Mumbai, India) was used for centrifugation. Crude solvent extracts were concentrated using a rotary vacuum evaporator (Heidolph Instruments GmbH & Co. KG Schwabach, Germany). All spectrophotometric data were acquired using Varian Cary 50 UV-visible spectrophotometer (Varian Cary, USA).

4.1.2 Determination of antioxidant and anti-inflammatory potential of the aqueous and solvent fractions of the mollusks

4.1.2.1 *In vitro* antioxidant assays

4.1.2.1.1 2, 2 -Azino-bis-3-ethylbenzothiozoline-6-sulfonic acid diammonium salt (ABTS) radical scavenging activity

ABTS (2, 2′-azino-bis-3ethylbenzothiozoline-6-sulfonic acid diammonium salt) assay was performed by the method of Arnao *et al*. (Arnao *et al*., 2001) with some suitable modifications. The samples (1 mg) were allowed to react with the $ABTS^{+}$ solution (2850 mL) for 2h in dark. The absorbance was recorded at 734

nm using the spectrophotometer, and expressed relative trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) in terms of %TEAC (trolox equivalent antioxidant capacity, 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid).

Briefly, ABTS was dissolved in deionized water to 7 mM concentration, and potassium persulfate ($K_2S_2O_8$) was added to a concentration of 2.45 mM. The reaction mixture was kept at room temperature overnight (12-16 h) in dark condition. The resultant intensely colored ABTS.+ radical cation was diluted with methanol to give an absorbance of ~0.70 at 734 nm. The crude extracts (50 µL) of different concentrations (0.1 – 0.5 µg/mL) was diluted 100 times with the ABTS.+ solution to a total volume of 5 mL. Absorbance was measured spectrophotometrically at different concentrations (0.1-0.5 µg/mL) for each extract results of the assay were expressed as percent radical scavenging ability. The assay was performed in triplicate. Fresh stocks of ABTS.+ solution were prepared before analyses.

4.1.2.1.2 2, 2′-Diphenyl-1-picryl hydrazil (DPPH•) radical scavenging activity

The free radical scavenging activities of the samples were determined by 2, 2′-diphenyl -1- picryl hydrazyl (DPPH) free radical scavenging activity (Moreira *et al*., 2013). The radical scavenging activity was expressed as percent inhibition of DPPH formation using the formula: percent inhibition of DPPH free radical = {(absorbance of control- absorbance of sample) × 100}/ absorbance of control. Butylated hydroxyl toluene (BHT) was used as the standard.

Briefly, DPPH solution was prepared (80 µg/mL) in methanol, and an equal volume of sample solution (2.5 mL) and DPPH solution (2.5 mL) were added at different concentrations (0.5-1 mg/mL). The mixture was shaken

vigorously, and allowed to stand at room temperature for 30 min, and the absorbance was measured at 517 nm. The lesser absorbance of the reaction mixture indicated greater free radical scavenging activity. The percentage DPPH radical scavenging activity was calculated using the following formula: DPPH· scavenging effect (%) = 100 (A_0 - A_1/A_0), where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the standard sample or crude extract.

4.1.2.2 *In vitro* anti-inflammatory assays

4.1.2.2.1 Cycloxygenase (COX) inhibition assay

Cycloxygenase (COX-1 and COX-2) inhibition assays were performed using 2, 7-dichlorofluorescein method with suitable modifications. In brief, leuco-2, 7-dichlorofluorescein diacetate (5 mg) was hydrolyzed at room temperature in 1 M NaOH (50 µL) for 10 min followed by the addition of 1 M HCl (30 µL) to neutralize the excess of NaOH before the resulting leuco-dichlorofluorescein (1-DCF) was diluted in 0.1 M tris-buffer (pH 8). COX enzyme (COX-1 and COX-2) was diluted in 0.1 M tris-buffer (pH 8), so that a known aliquot gave an absorbance change of 0.05/min in the test reaction. The test samples (or the equivalent volume of MeOH, 20 µL) were pre-incubated with the enzymes at room temperature for 5 min in the presence of hematin. Premixed phenol, 1-DCF and arachidonic acid were added to the enzyme mixture to begin the reaction, and to give a final reaction mixture of arachidonic acid (50 µM), phenol (500 µM), 1-DCF (20 µM) and hematin (1 µM) in 1 mL final volume of 0.1 M tris-buffer (pH 8). The reaction was recorded spectrophotometrically over 1 min at 502 nm. A blank reaction mixture was analyzed in the spectrophotometer reference cell against each test reaction to account for any non-enzymatic activity attributed to the test sample. This blank consisted of the reaction mixture without the addition of the enzyme.

4.1.2.2.2 Lipoxygenase 5 (5-LOX) inhibition assay

The 5-lipoxygenase (5-LOX) inhibition assay was carried out using the principle of 1-4 diene (linoleic acid) oxidations to 1- 3-diene (Baylac and Racine, 2003) with modification. Briefly, an aliquot of the stock solution (50 µL, in DMSO and tween 20 mixture; 29:1, w/w) of each test sample was placed in a 3 mL cuvette, followed by addition of pre-warmed 0.1 M potassium phosphate buffer (2.95 mL, pH 6.3) and linoleic acid solution (48 µL). Thereafter, ice-cold buffer (potassium phosphate) (12 µL) were added with 5-LOX (100 U) before being recorded spectrophotometrically at a wavelength of 234 nm. Prior to 5-lipoxygenase (LOX) inhibition assay, two samples were prepared as mentioned above but only with the mixtures of DMSO and tween 20, to serve as controls (no enzyme inhibition).

4.1.2.3 *In vivo* anti-inflammatory assay

The *in vivo* carrageenan-induced mice paw edema experiment was carried out as previously described (Winter *et al*., 1962).Thirty female BALB/c mice (20-30 g, 6–8 weeks old) were used in this study and those were housed under normal laboratory conditions (24 – 26ºC and 60 -75% RH), under a 12h light/dark cycle by fasting with distilled water. The animals were divided into 5 groups of 6 animals each. A 1% solution of carrageenan in saline (0.1 mL/mice) was injected subcutaneously into the right hind paw 30 min after the test samples (250 mg/kg mice) had been administered orally. The control group (aspirin, 200 mg/kg mice) received normal saline as the vehicle. Paw volumes were measured at 0, 1, 2 and 4h after injection, and the thickness of the edema was measured with an electronic micrometer (aerospace; 0-25 mm range, least count: 0.001 mm). Percentages of inhibition were obtained using the formula: (Tt −To) X 100/ To, where Tt is the average thickness for

each group after treatment in different time intervals and To is the average thickness obtained for each group before any treatment (zeroth hour).

4.1.3 Statistical analysis

Statistical evaluation was carried out with the Statistical Program for Social Sciences 13.0 (SPSS Inc, Chicago, USA, ver. 13.0). Descriptive statistics were calculated for all the studied traits. Analyses were carried out in triplicate and the means of all parameters were examined for significance ($p<0.05$) by analysis of variance (ANOVA). The Pearson correlation test was used to assess correlations between means. The mean variance in the data set was detected using principal component analysis (PCA). All data were mean centered and scaled to equal unit variance prior to PCA.

4A. Antioxidative and anti-inflammatory activities of the solvent and aqueous fractions of *Perna viridis*

4A.1 Description of study area and preparation of *P. viridis* extract

Green mussel *P. viridis* (10 kg) were collected from their natural habitat at Elathur (Lat: 11054'11.6"N; Long: 750 12'.21.8''E), in Calicut district of Kerala**.** The shell-on samples of *P. viridis* were thoroughly washed in running distilled water, and the meat (3.0 kg) was manually removed without applying heat. The samples were thereafter homogenized by a grinding machine, kept overnight under -80ºC for freezing, and were freeze-dried in a laboratory freeze dryer (Alpha 1-4 LD plus, Germany) for 36 h to get the freeze-dried green mussel extract (FDPE, 213.6 g; yield 7.1 g/100g). This was then powdered and stored in vacuum packed polyethylene biohazard autoclave bags (Fisherbrand TM, Fischer Scientific) at -80 ºC until further processing.

The ground and freeze dried mussel samples (FDPE) (150 g) were extracted with MeOH (650 mL x3) at an elevated temperature (80 ºC) for 2h in a water bath. The samples were then filtered through Whatman filter paper to obtain the clarified filtrates (710 mL) and dried over Na_2SO_4 (50 g), before being concentrated (50 ºC) *in vacuo* to yield a dark brown viscous mass (MME, 28.08 g). A portion of the methanol extract (MME, 20g) was mixed with distilled water (50 mL) and partitioned with $CHCl_3$ (2 x 100 mL) and EtOAc (2 x 100 mL) to separate the components with their polarity. The extracts were dried over anhydrous Na_2SO_4 (25-30 g), concentrated *in vacuo* to yield corresponding solvent fractions MCH (8.48 g) and MEA (3.01 g).

The mussel polysaccharide was isolated from *P. viridis* by hot-water extraction followed by precipitation with ice cold (-20 ºC) ethanol. The ground fresh mussel meat (100 g) was extracted thrice with hot water (1:1 ratio at 60ºC for 2 h) and the aqueous extracts were combined, pooled and centrifuged (model superspin R-V/Fm, Plastocrafts) at 9500 rpm for 20 min to separate the water insoluble impurities from the extracts. The supernatant was concentrated (50°C) *in vacuo* to one-third volume before being precipitated with ice-cold ethanol under stirring. The polysaccharide pellets were obtained by centrifugation, re-dissolved in water (5 mL) and precipitated with ice cold ethanol. Finally, the precipitate was lyophilized in vacuum freeze dryer (model ALPHA 1–4 LD plus) to obtain crude polysaccharide (MPS) (3.01 g, yield based on raw mussel = 3.01 %).The supernatant solution left after the precipitation of polysaccharide, was concentrated to dryness under vacuum to yield a yellow mass (MPP, 3.56 g).

4A.2 Results and discussion

Marine organisms are considered as vast untapped resources of bioactive molecules having enormous therapeutic potential, which led to the

growing interest in investigation of natural products with antioxidative and anti-inflammatory compounds lead molecules. *Perna viridis*, commonly known as the Indian green mussel, is a widely distributed edible mytilid bivalve seen all along both east and west coast of India.

4A.2.1 *In vitro* anti-oxidative activity of aqueous and solvent fractions of *P. viridis*

The *in vitro* antioxidant activity of *P. viridis* was recorded under **Table 4.1**. FDPE showed a dose dependent pattern in DPPH radical scavenging activities at 0.5–1 mg/mL. The observed total antioxidant radical scavenging activity (TARSA) of the freeze dried mussel powder (at a concentration of 1 mg/mL) was 13%. Previous studies demonstrated the presence of antioxidative molecules, especially in mussels (Lemaire and Livingstone, 1993). The present results can also be compared with other reports of some other species, such as, *Mytilus edulis* (Cheung *et al*., 2002; Gaspic *et al*., 2002; Riveros*et al*., 2002; Sukhotin *et al*., 2002). The results obtained in the present study were in accordance with those recorded earlier. The ABTS scavenging effects shown by FDPE at a concentration of 0.5 mg/mL was found to be 0.2%. The results obtained in this study were in fair agreement with the previous investigations on *Mytilus galloprovincialis*,

The MEA fraction was found to be more effective in stabilizing the ABTS**.+** (23.59%, 0.5 µg/mL), and DPPH radicals (60.43%, 1 mg/mL) than MCH fraction (37.35% and 15.31%, respectively). The MEA fraction recorded significantly greater ABTS and DPPH radical scavenging capacities than MCH fraction ($P<0.05$). The extract was able to reduce stable radical DPPH to the yellow coloured diphenylpicrylhydrazine, which was noticeable by the discolouration of test samples from purple to yellow. The decrease in absorbance caused by the antioxidants in the extracts might be due to the hydrogen donating ability that resulted in the scavenging of the free radical.

Recent study on the methanolic extract of *P. viridis* showed significant radical scavenging property against DPPH radical (Jena *et al*., 2010). The antioxidant activity of *P. viridis* reassure the presence of secondary metabolites with antioxidant properties (Sreejamole *et al*., 2013).

MPP (60.43% and 23.59%) recorded significantly greater DPPH and ABTS scavenging activities than MPS fractions (37.4% and 15.3%) respectively. The higher DPPH and ABTS activity of MPP fraction revealed the presence of antioxidative lead molecules in these fractions. Earlier studies reported that the polysaccharides from mollusks had potential pharmaceutical properties, such as, anti-tumor, immunity-enhancement (Vetvicka *et al*., 2004), anti-inflammation (Mikheiskaya *et al*., 1988) and anti-aging effects. The ABTS assay was based on the generation of a blue/green $ABTS^{+}$ which is applicable to both hydrophilic and lipophilic antioxidant systems. The antioxidant activities of polysaccharides were not a function of a single factor but a combination of several factors, such as, content of sulfuric radicals, molecular weight, protein content and type of sugar (Donghong *et al*., 2013).

Table 4.1. *In vitro* anti-oxidative activity of aqueous and solvent fractions of *P. viridis.*

	Concentration (mg/mL)	FDPE	MCH	MEA	MPS	MPP
DPPH scavenging activity (% TARSA)	0.5	8.24 ± 0.14^{a}	33.14 ± 0.24^{b}	49.62 ± 0.32^{c}	26.56 ± 0.21^{d}	35.71 ± 0.24^{b}
	0.75	10.54 ± 0.08^{a}	41.72 ± 0.36^{b}	56.85 ± 0.41^{c}	32.83 ± 0.26^{d}	42.75 ± 0.35^{b}
	1	13.41 ± 0.13^{a}	46.62 ± 0.29^{b}	60.43 ± 0.26^{c}	37.35 ± 0.13^{d}	48.62 ± 0.28^{b}
ABTS radical scavenging activity (% TEAC)	0.1	0.15 ± 0.01^{a}	8.43 ± 0.02^{b}	14.51 ± 0.08^{c}	8.27 ± 0.02^{b}	11.78 ± 0.02^{d}
	0.3	0.19 ± 0.01^{a}	12.76 ± 0.09^{b}	19.27 ± 0.11^{c}	$13.44\pm0.11b^{d}$	14.13 ± 0.12^{d}
	0.5	0.21 ± 0.01^{a}	16.24 ± 0.15^{b}	23.59 ± 0.17^{c}	15.31 ± 0.04^{b}	18.94 ± 0.08^{d}

DPPH radical scavenging activity at 0.1 mg/mL was expressed in percent; ABTS radical scavenging activity at 0.1 mg/mL is expressed in percent; FDPE – Freeze dried *P. viridis* extract; MCH-Mussel chloroform fraction; MEA-Mussel ethyl acetate fraction; MPS-Mussel polysaccharide; MPP-Mussel polyphenol fraction.

4A.2.2 *In vitro* anti-inflammatory activity of aqueous and solvent fractions of *P. viridis*

Freeze dried mussel powder (5 mg/mL) showed inhibitive property against the pro-inflammatory COX-2 and 5-LOX enzymes, and the activities were found to be comparable ($P<0.05$) with the standard NSAIDs, aspirin and indomethacin. The COX-1 and COX-2 inhibition properties of the freeze dried mussel powder were found to be comparable with the activities reported for the total lipid extracts of *P. canaliculus*, *Mytilus edulis* and fish oil (McPhee *et al*., 2007). FDPE (5mg/mL) showed a comparable inhibition (48.5 and 51.9%, respectively) with aspirin and indomethacin, against pro-inflammatory COX-2 and 5-LOX enzymes. The earlier reports (McPhee *et al*., 2007) indicated that the total lipid extracts of *P. canaliculus* and *M. edulis* realized a moderate inhibition of COX-1 and COX-2 (12 and 25%; 18 and 24%, respectively, in that order at 1 µg/mL) than those reported in the present study.

MEA fraction registered significantly greater ($P<0.05$) COX-1, COX-2 and 5- LOX inhibition than MCH fraction. MEA fraction recorded greater COX-2 (51.4%) and 5-LOX (56.1%) inhibition than MCH fraction at 5 mg/mL (49.8% and 52.4%, respectively). The MEA fraction exhibited positive correlation with COX-2 and DPPH radical scavenging activity, whereas MCH fraction showed negative ($r^2=0.87$) correlation with DPPH radical scavenging activity. The IC_{50} value of COX-2 in MEA extract was found to be 4.54 mg/mL whereas it was 5.01 mg/mL for the MCH fraction. The presence of diverse groups of secondary metabolites, such as alkaloids, polyphenols, terpenes and sterols in the ethyl acetate extract of *P. viridis* was reported in an earlier literature (Sreejamole *et al*., 2013).The extract from *P. viridis* was

reported to be active against inflammatory joint diseases, influenza, herpes, HIV, and hepatitis viral strains (Mitra and Chatterji, 2004).

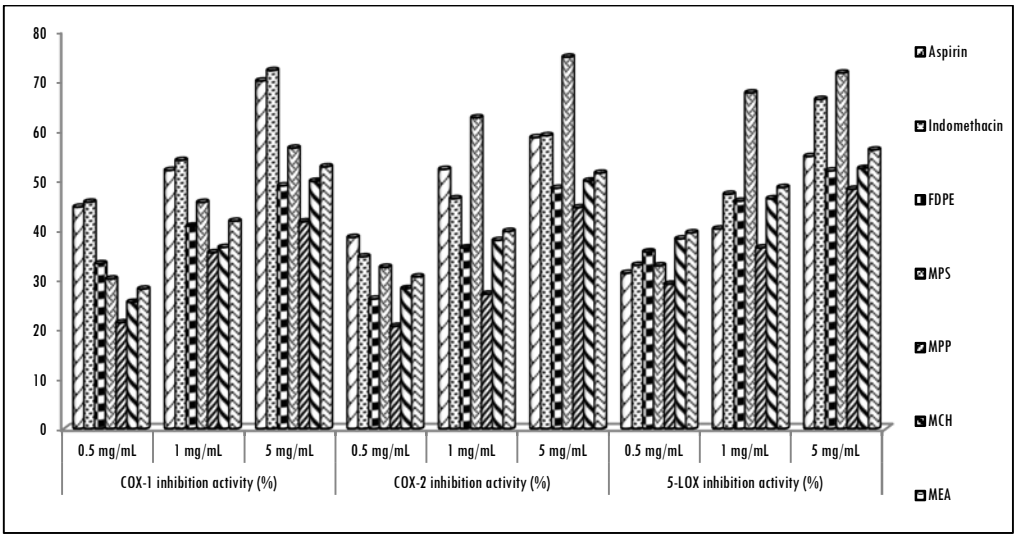


Fig 4.1 *In vitro* anti-inflammatory activity of aqueous and solvent fractions of *P. viridis*

The MPS fraction appeared as white powder, and had no absorption at 280 nm or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid. The supernatant solution left after the precipitation of polysaccharide, was evaporated to dryness under vacuum (MPP). MPS registered significantly greater COX-2 inhibition (74.8%) than synthetic drugs aspirin and indomethacin (58.6% and 59%, respectively, 5mg/mL). Also no significant differences were apparent between MPS (32.5%) and indomethacin (34.6%) at lower concentration (0.5 mg/mL). MPS registered significantly greater 5-LOX inhibition (71.6%, 5 mg/mL) than aspirin and indomethacin (54.8 and 66.3%, respectively, 5 mg/mL). A water soluble glucan was reported to possess significant anti-inflammatory and antioxidant activities (Zhang *et al.*, 2010).

4A.2.3 *In vivo* anti-inflammatory activity of aqueous and solvent fractions of *P. viridis*

Carrageenan induced paw edema is a standard and most commonly used technique to screen anti-inflammatory activity (Winter, 1962). Carrageenan is a sulfated polysaccharide that promotes acute inflammation by activating pro-inflammatory cells. Inflammation induced by carrageenan, is acute, non-immune, well-researched, and highly reproducible. It is expected that after tissue injury, an animal will display spontaneous pain behaviour. This peripheral hypersensitivity or pain perception can be explained on the basis of local release of various inflammatory mediators *i.e*., bradykinin, prostaglandins or cytokines, which can activate and sensitize the peripheral nerve endings (Baccaglini, 1983). The inflammation is characterized by increased tissue water and plasma metabolism of arachidonic acid by both COX and LOX enzyme pathway (Gamache *et al*., 1986). Different extracts demonstrated a time dependent anti-inflammatory effects in mice with carrageenan induced footpad edema. Comparison of purified compounds with reference drug (aspirin) on carrageenan-induced hind paw edema in mice were recorded in **Fig 4.2**. The FDPE have potent anti-inflammatory activity, which significantly decreased paw edema ($P<0.05$) with a maximum inhibition at 6th h. Whitehouse *et al.* investigated the anti-inflammatory activity of the oil extracted from the freeze-dried powder of the New Zealand green-lipped mussel (Lyprinol™) in adjuvant-induced polyarthritis or collagen-induced arthritis in rats (Whitehouse *et al*., 1997). Maximum inhibition of edema by MEA fraction (68.3%) was achieved on 5thh. However MCH fraction recorded maximum inhibition of edema during 6thh. The anti-inflammatory activities of aqueous/ethanol and methanol extracts of *Perna viridis* was reported in an earlier literature (Sreejamole *et al.,* 2011).

Maximum inhibition of edema by MPS was achieved during the 4th h (74.2%) whereas MPP polyphenols recorded maximum activity (68.8%) in 3rd h. The study showed that MPS effectively suppressed the edema produced by the histamine, which indicated that the polysaccharide exhibited its anti-inflammatory activities by means of either inhibiting the synthesis, release or action of inflammatory mediators *viz*., histamine, serotonin and prostaglandin's involved in the inflammatory response. The polysaccharides may be the potential therapeutic agent involved in the inflammatory disorders, controlling the initial phase of inflammation and provoking an inhibition of edema formation similar to the reference drugs (aspirin, 68. 7% in 2nd h) compounds. MPS and aspirin exhibited highest activity in 4th h (74.2% and 79.8 %, respectively) and started losing activity around 5th h (67.35% and 77.32%, respectively), which might be related to the lower level of these compounds in the blood circulation. The results obtained in the present study were in accordance with the previous reports that several glucan-type polysaccharides had potential anti-inflammatory activities (Czarnecki and Grzybek, 1995).

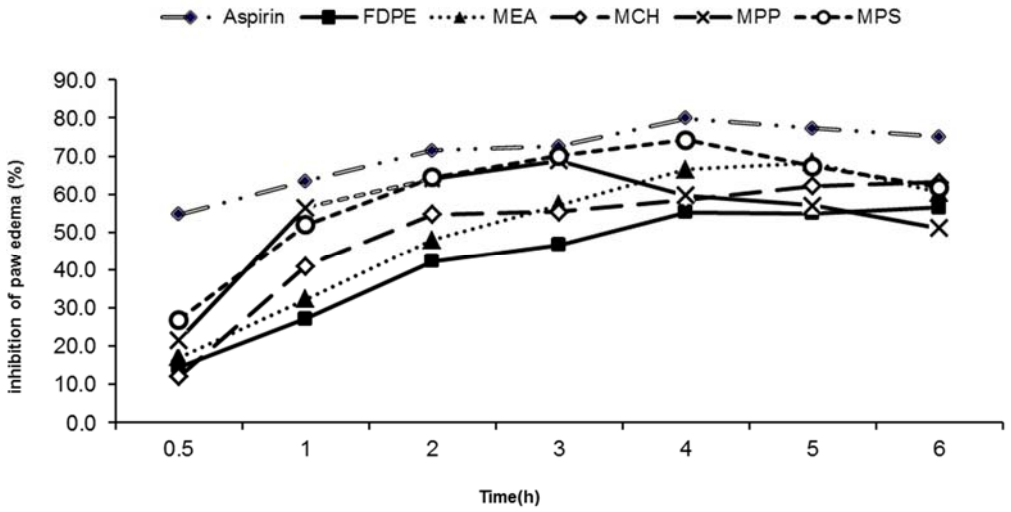


Fig 4.2 Inhibition of rat paw edema (%) after the administration of solvent and aqueous fractions of *P. viridis*

4B. Anti-oxidative and anti-inflammatory potential of the solvent and aqueous fractions of *Crassostrea madrasensis*

4B.1 Description of study area and preparation of *Crassostrea madrasensis* extract

The *C. madrasensis* samples were collected from the intertidal rocky shore of Sattar island at Cochin, south west coast of India (UST: 1141160E, 221200N) (Lat: 9058'N; Long: 76016'E), which is predominantly influenced by oceanic water from the Arabian sea. The tissues of edible oysters were combined, minced, and freeze dried to furnish freeze dried oyster powder (FDCP).

The ground and freeze dried octopus samples (150 g) were extracted with MeOH and the extracts were filtered with Whatman filter paper No.1 to obtain the clarified filtrates, before being concentrated (50 ºC) *in vacuo* to yield a dark brown viscous mass (CME, 16.98 g). The methanolic extract (CME, 50g) was partitioned with $CHCl_3$ (2 x 200 mL), and the aqueous portion was again partitioned with EtOAc (2 x 150 mL) to separate the components with varying polarity. The solvent extracts were dried over anhydrous Na_2SO_4 (45-60 g), before being concentrated *in vacuo* using a rotary vacuum evaporator (Superfit, India) to yield the corresponding solvent fractions CRCH (25.32 g) and CREA (6.01 g).

The ground fresh oyster meat (100 g) was extracted with hot water (1:1 ratio) at 60 ºC for 2 h with occasional stirring. The water extract collected by centrifugation were pooled together and concentrated *in vacuo* to one third of the original volume. The concentrated aqueous extract was precipitated with threefold volumes of ice-cold ethanol. The polysaccharide pellets were obtained by centrifugation and then re-dissolved in water (5 mL) before being

precipitated with ice-cold ethanol (10 mL). Finally, the precipitate was lyophilized in vacuum freeze dryer (model ALPHA 1–4 LD plus) to obtain crude polysaccharides (CPS) (2.85 g, yield based on raw oyster 2.85%). CPS appeared as yellow powder, and no absorption was recorded at 280 nm or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid. The supernatant solution left after the precipitation of polysaccharide, was concentrated *in vacuo* to dryness to yield a yellow mass (CRPP, 1.73 g).

4.B.2 Results and discussion

4B.2.1 *In vitro* antioxidative activity of aqueous and solvent fractions of *C. madrasensis*

Antioxidant activity of the crude ethyl acetate extract derived from the edible muscle of *C. madrasensis* was evaluated through various antioxidant assays. The radical scavenging capacities of different solvent and aqueous extracts against different test radicals can be explained by different mechanisms involved in the radical–antioxidant reactions. DPPH is a compound that possesses a nitrogen free radical, and is readily neutralised by a free radical scavenger. This assay was used to test the ability of the antioxidative compounds functioning as proton radical scavengers or hydrogen donors. The decrease in absorbance of the DPPH radical caused by antioxidants was due to the scavenging of the radical by hydrogen donation.

The DPPH radical scavenging abilities of the extracts derived from edible oyster were found to be comparable with that of ascorbic acid. The CREA fraction exhibited 55.7% DPPH radical scavenging ability and 20.83% ABTS radical scavenging ability at a concentration of 1 mg/mL. The CREA fraction exhibited significantly greater DPPH and ABTS radical scavenging abilities than CRCH fraction (**Table 4.2**, $P<0.5$). The method involving DPPH

radical scavenging activity is based on the reduction of methanolic DPPH-solution in the presence of hydrogen donating antioxidant, and the formation of the non-radical form DPPH. The CREA extract was able to reduce the stable radical DPPH to the yellow-colored diphenylpicrylhydrazine (Li *et al*., 2007). The methanolic extract of gastropod *Pleuroploca trapezium* was likewise found to exhibit good scavenging activity towards the DPPH radical with an IC_{50} value of 4021 µg/mL. The present study demonstrated that the CPS registered 35.9% DPPH and 12.2% ABTS activities whereas CRPP showed having 45.7% DPPH and 17.2% ABTS activities. The differences in DPPH scavenging activities might be due to scavenging different types of antioxidant principles extracted at varying solvent polarities. Previous studies on the radical scavenging abilities of *C. madrasensis* showed 23.5 % DPPH radical scavenging activity of the crude methanolic extract (Li *et al*., 2007).

Table 4.2. *In vitro* antioxidative activity of aqueous and solvent fractions of *C. madrasensis.*

	Concentration (mg/mL)	FDCP	CRCH	CREA	CPS	CRPP
DPPH scavenging activity (% TARSA)	0.5	6.49±0.02^a	22.62±0.13^b	34.24±0.23^c	22.94±0.12^b	28.32±0.13^d
	0.75	8.16±0.01^a	34.73±0.16^b	46.71±0.16^c	29.52±0.24^d	36.65±0.18^e
	1	10.62±0.08^a	40.65±0.21^b	55.75±0.32^c	35.85±0.17^d	45.73±0.14^e
ABTS radical scavenging activity (% TEAC)	0.1	0.11±0.01^a	8.42±0.05^b	12.52±0.05^c	7.21±0.07^d	8.57±0.06^b
	0.3	0.14±0.01^a	11.71±0.09^b	16.37±0.14^c	10.86±0.05^b	14.55±0.09^d
	0.5	0.16±0.01^a	15.14±0.12^b	20.83±0.27^c	12.24±0.06^d	17.23±0.11^e

DPPH and ABTS radical scavenging activity at 0.1 mg/mL were expressed in percent; FDPE – Freeze dried *C. madrasensis* extract; CRCH- *C. madrasensis* chloform fraction; CREA- *C. madrasensis* ethyl acetate fraction; CPS- *C. madrasensis* polysaccharide; CRPP- *C. madrasensis* polyphenol fraction.

4B.2.2 *In vitro* anti-inflammatory activity of aqueous and solvent fractions of *C. madrasensis*

The COX inhibition assay used in this study is based on the oxidation of 1-dichlorofluorescein (1-DCF) by the hydroperoxide formed in the cyclooxygenase reaction. The method determines the second peroxidase

reaction of the enzyme, which has a higher turnover than the initial oxygenase reaction (Larsen *et al*., 1996). The fractions CREA realized greater COX-2 (44.8%) and 5- LOX activities (51.3%) than CRCH fractions (42.5% and 50.1%, respectively in that order). Aspirin and indomethacin recorded significantly greater COX-1 activity than CREA and CRCH fractions (P<0.05). CPS recorded higher COX-2 (69.7%) and 5-LOX inhibitions (64.2%, 5 mg/mL) than CRPP (33.5% and 37.5%, respectively, at 5 mg/mL). CPS fraction recorded significantly greater anti-COX-2 and anti-5-LOX inhibition than aspirin and indomethacin (P<0.05).

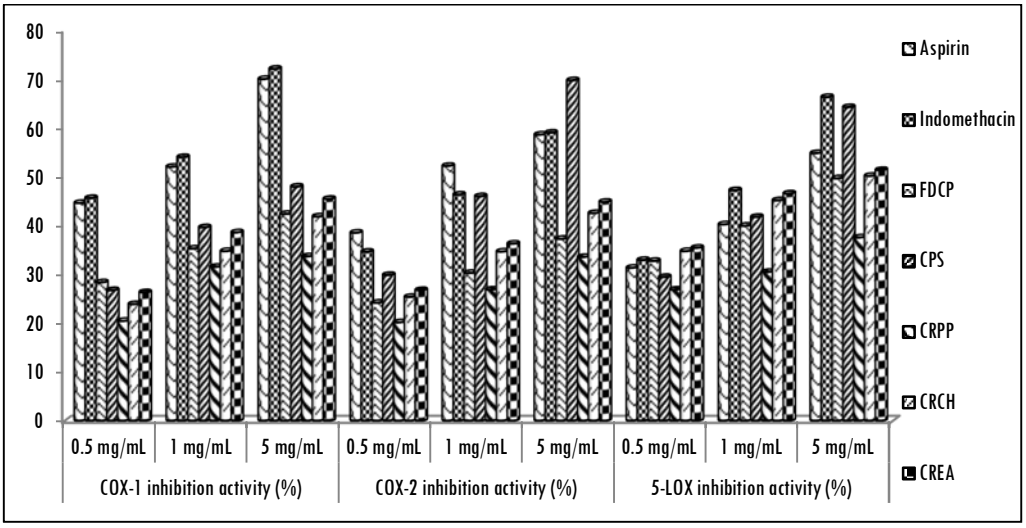


Fig 4.3 *In vitro* anti-inflammatory activity of aqueous and solvent fractions of *C. madrasensis*

4B.2.3 *In vivo* anti-inflammatory activity of aqueous and solvent fractions of *C. madrasensis*

Carrageenan-induced edema has been commonly used as an experimental animal model for acute inflammation, and is believed to be biphasic. Inflammation was induced by carrageenan injection for 1 h and thereafter treated with the crude fractions. The effect of CREA in reducing the carrageenan-induced acute inflammation was found to the similar to a comparable dose of aspirin. Inflammation was reduced by 50% compared with the untreated group within

three hours after the treatment with CREA. Maximum inhibition of edema by CREA fraction (64.9%) was achieved on 4thh whereas CRCH fraction recorded maximum inhibition (56.3 %) of edema in 5th h. These indicated that CREA was quickly absorbed and biologically available. After 5th h the inflammed pawsizes continued to shrink, showing that the treatment was pharmacologically active for a prolonged time. The *in-vivo* anti-inflammatory activity indicated higher activity of CRPP fraction (58.5%) in 3th h, and the activity was decreased to 42.3 in 6th h. It has been suggested that the early hyperemia of carrageenan induced edema result from the release of histamine and serotonin (Kulkarni *et al*., 1986), and the CRPP fraction were efficient in reducing the edema resulted from the release of histamine and serotonin. However maximum inhibition of edema by CPS was achieved during 5th h (63.3%) and CPS fractions were able to reduce the delayed phase of carrageenan induced paw edema resulted mainly from the potentiating effect of prostaglandins on mediator release, especially bradykinin.

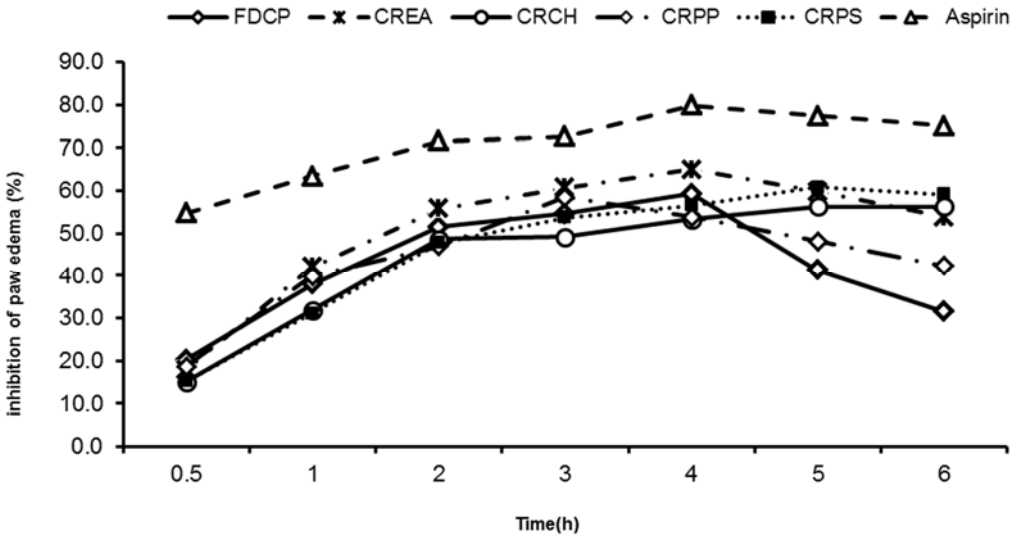


Fig 4.4 Inhibition of rat paw edema (%) after the administration of solvent and aqueous fractions of *C. madrasensis*

4.C Antioxidative and anti-inflammatory potential of the solvent and aqueous fractions of *Octopus dolffusi*

4.C.1 Describtion of study area and preparation of *O. dolffusi* extract

Octopus dolffusi samples were collected from the Fort Kochi harbor along the South-west coast of Kerala. The samples thus collected were immediately placed in ice for transportation and the samples were processed, cleaned, minced, before being stored in low temperature (-20^{o}C) until further use. The samples collected were minced and freeze dried to obtain *Octopus dolffusi* powder (FROP).

The ground and freeze dried octopus samples (100 g) were extracted with EtOAc: MeOH (1:1 v/v) at room temperature (500 mL x 2, 30ºC) and then at an elevated temperature (500 mL x 2, 50ºC) for 2 h on a water bath. The extracts were thereafter filtered and concentrated (50 ºC) *in vacuo* to yield a dark brown viscous mass (OEM, 16.98 g). The residue was further extracted with $CHCl_3$: MeOH (1:1 v/v, 200 mL x 3) for 2 h on a water bath. The extracts were filtered and concentrated *in vacuo* (Heidolph, Germany) to afford the crude solvent extract (OCM, 3.01 g).

The octopus polysaccharide was isolated from *O. dolffusi* by hot-water extraction and ethanol precipitation. The ground fresh octopus meat (100 g) was extracted thrice with hot water (1:1 ratio) at 60 ºC for 2 h. The water extracts were collected before being centrifuged (SuperspinR-V/Fm, Plastocrafts) to separate the water insoluble impurities from the extracts. The clarified supernatant (275 mL) was concentrated to one third of the original volume, and precipitated with ice-cold ethanol (95%) before being and kept at 4ºC for overnight. The precipitated polysaccharide pellets were obtained by centrifugation and redissolved in water (5 mL) before being precipitated with

ice cold ethanol (10 mL). Finally, the precipitate was lyophilized in a vacuum freeze dryer (model ALPHA 1–4 LD plus) to obtain the crude polysaccharides (OPS) (2.55 g, yield based on raw mussel 2.55 %).The OPS appeared as light pink powder, and had no absorption at 280 nm or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid. The supernatant solution left after the precipitation of polysaccharide, was concentrated *in vacuo* to obtain a highly hygroscopic light yellow powder (OPP, 1.6g).

4.C.2 Results and discussion

4C.2.1 *In vitro* antioxidative activity of aqueous and solvent fractions of *O. dolffusi*

In vitro antioxidative activities of aqueous and solvent fractions of *O. dolffusi* were recorded in **Table 4.3.** The methanol fraction (OEM) of *O. dolffusi* exhibited 31.9% DPPH radical scavenging activity. Earlier studies showed that methanolic extract of *B. spinosa* exhibited 39.4% DPPH radical scavenging activity at 10 mg/mL whereas the BHT and ascorbic acid showed 63.7 and 59.8% respectively (Premanand *et al.,* 2010). The DPPH and ABTS activities of the OCM fraction was 35.2 % and13.8%, respectively, (1 mg/mL) and that of OEM fraction was 56.9% and 20.8%, respectively, at that concentration. OEM fraction recorded significantly greater DPPH and ABTS radical scavenging activities than OCM fraction ($P<0.05$). These results could be compared with the ethyl acetate extract of other mollusks *L. duvauceli*. The DPPH and ABTS radical scavenging activities of OPS at a concentration of 1 mg/mL were recorded as 39.3 % and 13%, respectively. Likewise, OPS recorded significantly greater DPPH and ABTS radical scavenging activities than OPP fraction ($P<0.05$). The total antioxidant radical scavenging activity and ABTS scavenging effects of polyphenol fraction OPP (as determined by

DPPH radical scavenging activity) at 1mg/mL were 45.7% and 16.2% respectively.Likewise, the methanolic extract of mollusk *Pleuroploca trapezium* was found to exhibit a good scavenger of DPPH radical with an IC_{50} value of 4021 µg/mL (Premanand *et al*., 2010).

Table 4.3. *In vitro* antioxidative activity of aqueous and solvent fractions of *O. dolffusi*

	Concentration (mg/mL)	FDOP	OCM	OEM	OPS	OPP
DPPH radical scavenging activity (% TARSA)	0.5	5.72±0.02^{a}	21.26±0.16^{b}	40.34±0.34^{c}	20.74±0.19^{b}	34.27±0.12d
	0.75	8.93±0.04^{a}	31.84±0.23^{b}	47.27±0.26^{c}	32.62±0.24^{b}	41.72±0.24d
	1	11.91±0.09^{a}	35.16±0.27^{b}	56.93±0.15^{c}	39.25±0.28^{d}	45.65±0.28e
ABTS radical scavenging activity (% TEAC)	0.1	0.11±0.01^{a}	8.35±0.01^{b}	11.77±0.03^{c}	7.24±0.05^{b}	8.51±0.01b
	0.3	0.15±0.01^{a}	12.44±0.11^{b}	16.36±0.13^{c}	9.76±0.03^{d}	13.83±0.05b
	0.5	0.19±0.^{01a}	13.76±0.09^{b}	20.75±0.16^{c}	13.01±0.06^{b}	16.24±0.09d

DPPH and ABTS radical scavenging activities at 0.1 mg/mL were expressed in percent;; FDOP– Freeze dried *O. dolffusi* extract; OCM- *O. dolffusi* chloform fraction; OEM- *O. dolffusi* ethyl acetate fraction; OPS- *O. dolffusi* polysaccharide; OPP- *O. dolffusi* polyphenol fraction.

4C.2.2 *In vitro* anti-inflammatory activity of aqueous and solvent fractions of *O. dolffusi*

The OEM fraction registered significantly greater COX-1 and 5-LOX inhibition (54.8% and 54.6%) than OCM fraction (51.6% and 51.8%, 5 mg/mL, $P<0.05$). No significant difference in anti-COX-2 activity was apparent between OEM fraction and OCM fraction in higher concentration whereas significant difference was observed in lower concentrations (**Fig 4.5**). Also no significant differences in the 5-LOX activity were apparent between OEM (54.6%) and indomethacin (54.8%) in higher concentration (0.5 mg/mL). The OEM fraction registered significantly higher 5-LOX inhibition than aspirin at lower concentrations (0.5 and 1 mg/mL), whereas no significant difference was observed at higher concentration between aspirin (54.8%) and OEM fraction (54.6%). OPS registered significantly greater COX-2 inhibition (70.7%) than aspirin and indomethacin (58.6% and 59%, respectively, at 5

mg/mL). Also no significant differences were apparent between OPS (53.1%) and aspirin (52.2%) at lower concentration (1 mg/mL). OPS registered significantly higher 5-LOX inhibition (66.8%, 5 mg/mL) than OPP fraction at higher concentration, and no significant differences were observed at lower concentrations. OPS fraction recorded significantly higher 5-LOX inhibition (66.8%) than aspirin and indomethacin (54.9 and 66.3%, respectively, 5 mg/mL). However the OPP fraction recorded significantly lower COX-2 inhibition (25.9%) and 5-LOX inhibition (28.4%, 5 mg/mL) than aspirin and indomethacin.

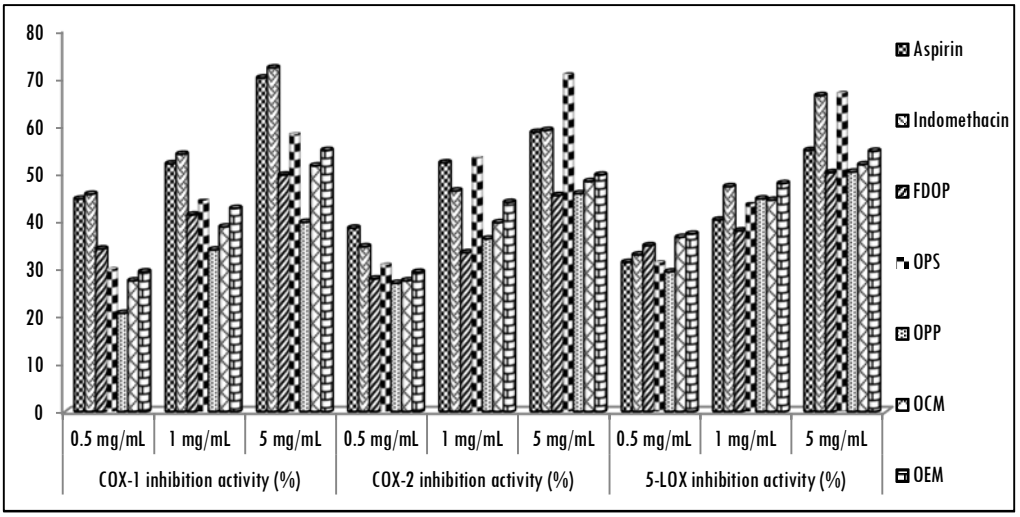


Fig 4.5 *In vitro* anti-inflammatory activity of aqueous and solvent fractions of *O. dolffusi*

4C.2.3 *In vivo* anti-inflammatory activity of aqueous and solvent fractions of *O. dolffusi*

The carrageenanan induced paw edema method has been used to evaluate the effect of NSAIDs and anti-inflammatory extracts (Phadke and Anderson, 1988). The *in-vivo* anti-inflammatory activity of carrageenan induced paw edema recorded greater activity of OPP fraction (62.1%) in 3rd hour, and the activity was found to be decreased to 43.4% during 6th h (**Fig**

4.6). OEM and aspirin showed highest activity in 4th h (65.65% and 79.84%, respectively) and started losing activity at 5th h (67.4% and 59.1%, respectively. Maximum inhibition of edema by OCH fraction was achieved in 6th h (60.07%). OPS fraction recorded significantly greater anti-inflammatory activity ($P<0.05$) than OPP fraction. Maximum inhibition of paw edema for OPS fraction was observed in 4th hour and decreased to 49.9 during 6th h. Kumar (Kumar, 2003) reported anti-inflammatory effect of the methanolic extracts of *Cypraea errones* and *Cypraea Arabica,* against carrageenan-induced inflammation. Novel anti-inflammatory drugs were isolated from corals and sponges. It was observed that the increase in the paw thickness was inhibited to about 73.6% by 100% acetone column-purified fraction of *T. tentorium* at a concentration of 50 mg/kg, whereas standard anti-inflammatory drug, diclofenac sodium (50 mg/kg) inhibited the paw thickness to 65% (Chellaram *et al*., 2012).

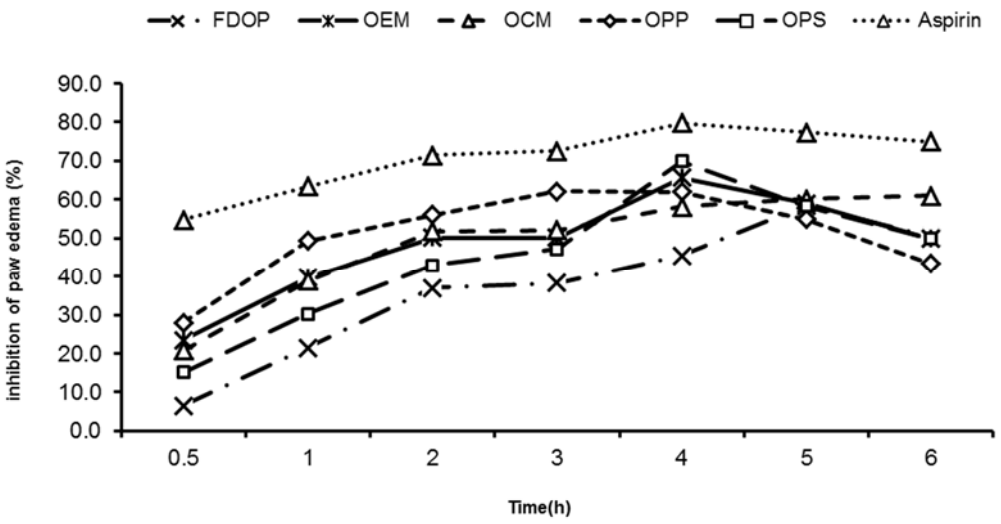


Fig 4.6 Inhibition of rat paw edema (%) after the administration of solvent and aqueous fractions of *O. dolffusi* (%)

4.2 Conclusions

The present study demonstrated the importance of *P. viridis*, *C. madrasensis* and *O. dolffusi*, in terms of their potential radical scavenging and anti-inflammatory properties. The antioxidant and anti-inflammatory assay measured the biological resistance of the organisms to the reactive oxygen species and oxyradical-medicated adverse disorders. The present findings implied that the methanolic extracts derived from these candidate bivalve species could be used to isolate potentially valuable antioxidative and anti-inflammatory compounds. This forms a first comprehensive report on the antioxidative and anti-inflammatory properties of these seafood species as a novel source of antioxidants and anti-inflammatory leads. The following chapter focussed towards bioactivity-guided isolation and characterization of bioactive lead molecules with anti-oxidative and anti-inflammatory properties.

Chapter 5

ISOLATION AND CHARACTERIZATION OF SECONDARY METABOLITES FROM *PERNA VIRIDIS, CRASSOSTREA MADRASENSIS* AND *OCTOPUS DOLFFUSI*

Contents

Background of the study

Marine environment encompasses of complex ecosystems and many of its inhabitants are living under constant stress from free radicals, reactive oxygen species, and prooxidants generated both exogenously (heat and light) and endogenously (H_2O_2 and transition metals).These inhabitants developed bioactive compounds as part of highly toxic defense mechanisms which is a reflection of the highly competitive environment in which the organisms reside (Grabley and Thierjcke, 1999). These antioxidant compounds include flavonoids, phenolic acids, carotenoids, and tocopherols that can inhibit Fe^{3+}/arachidonate induced oxidation, scavenge free radicals, and act as reductants. Many bioactive compounds have been extracted, characterized and purified from various marine animals,such as, bacteria, algae, dino-flagellates, tunicates, sponges, soft corals, bryozoans, cephalopods, and echinoderms.

Mollusks in the oceans are a common sight and are virtually untapped resources for the discovery of novel bioactive compounds. Chemical compounds with antioxidant and anti-inflammatory properties are present in mollusks as a protective mechanism against various stress factors in oceanic ecosystem. Even though many bioactive compounds has been extracted, characterized and purified from various marine animals, much of the potential compounds from mollusks still remain unharnessed especially the organisms belonging to class bivalves and cephalopods. The absence of oxidative damage in the stress-induced biochemical parameters of mollusks from coastal ecosystem suggested that their cells are equipped with powerful anti-stress secondary metabolites with antioxidative and anti-inflammatory activities, which provide competitive advantages against various oxidative stress factors leading to the development of harmful reactive oxygen species. These sessile species are the richest natural sources of bioactive compounds, many of which belong to novel chemical classes not found in terrestrial sources.

Mollusks are considered as one of the important sources to obtain bioactive compounds that exhibit anti-inflammatory and antioxidant activities (Sreejamole, 2011). Bioactive components, such as, peptides, enzyme and enzyme inhibitors have been identified from *Meretrixmeretrix* having useful effects including antihypertensive, hypolipidemic and antioxidant properties (Blunt *et al*., 2012). Mollusks were reported to contain heparin and heparin-like polysaccharide, such as,glycosaminoglycans. Anti-inflammatory compounds, such as,manoalide, pseudopterosins, topsentins, and debromohymenialdisine were isolated from mollusks (Blunt *et al*., 2013). Cyclotides represent the small molecular weight circular peptides with an extremely stable structural framework due to the knotted arrangement of disulfide bonds, and were reported to be endowed with antioxidative activities in mollusks. Antiproliferative and

antioxidant properties of mollusks, *Tambjaceutae*, and *Bugula dentate* were reported to possess tambjamines (Blunt *et al*., 2012). Dolastatins are a group of cyclic and linear peptides with free radical scavenging and anticarcinogenic activities isolated from the marine shell-less mollusk*Dolabellaauricularia*. Mytilin and molluskdefensins were isolated from the mollusk*Mytilisedulis* (Gestal*et al*., 2007). Polysaccharides and polysaccharide–protein complexes have been found as common polymers in shellfish and received considerable attention in recent years because of their anti-inflammatory, antioxidant, antitumor and immunoregulatory activities (Blunt *et al*., 2013). Polysaccharides have been used to stimulate the immune system and to fight against cancer, and researches shown polysaccharides to possess antioxidant activity. A glycogen complex extracted from *Pernacanaliculus*, had an anti-inflammatory activity (Blunt *et al*., 2014). Bioactive lipids, and specifically phospholipids are important part of the intestinal mucus, and an important regulator of epithelial function during inflammation by inhibiting cytokine tumor necrosis factor (TNF-α). Green Lipped Mussel from New Zealand has been shown to contain unique *n*-3 fatty acids, which appear to act as dual inhibitor of arachidonic acid oxygenation by both COX and LOX pathways (Tiffany *et al*., 2002), thus decreasing prostaglandin and leukotriene syntheses, and downregulating the inflammatory sequence.

This chapter envisages the isolation and characterization of secondary metabolites with antioxidant and anti-inflammatory activities from *Pernaviridis*, *Crassostreamadrasensis*and *Octopus dolffusi*. Hence, the purification of these crude extracts was carried out by repeated purifications with the aid of various chromatographic techniques. A systematic search for the development of new sources of bioactive pharmacophores from these molluskswill be helpful for the development of bioactive supplements.

5.1. Materials and methods

5.1.1. Chemicals and instrumentation

All compounds, reagents and solvents were of analytical, spectroscopic or chromatographic reagent grade and were obtained from Merck (Darmstadt, Germany).

5.1.1.1. Chromatographic analyses

5.1.1.1A. Preparative thin layer chromatography (TLC)

TLC was performed on pre-coated TLC plates with silica gel 60 F_{254} (layer thickness 0.2 mm; 20 x 20 cm; Merck KGaA, Darmstadt, Germany) using different solvent systems as needed. The band separation on TLC was detected under UV lamp at the wavelength of 254 and 366 nm

5.1.1.1B. Vacuum column chromatography (CC)

Normally, the columns were dry silica gel GF_{254} pre-packed, of 18 cm height and inner diameter of 12 cm, vertically clamped. The column was filled and saturated with the desired non-polar solvent in the mobile phase (*eg.*,*n*-hexane) just prior to sample loading. Samples were dissolved in a small volume of the same solvent used, and the resulting mixture was then packed onto the top of the column using special syringe. Using step gradient elution with non-polar solvent (*n*-hexane or $CHCl_3$) and increasing amounts of polar solvents (e.g. EtOAc or MeOH) the successive fractions were collected. The mobile phase (gradient elution) was pumped through the column with the help of air pressure resulting in sample separation.

5.1.1.2. Spectroscopic analyses

5.1.1.2A.Fourier transform infra-red spectrometer (FTIR)

Fourier transform infra-red spectrometer (FTIR) spectra of the compounds under KBr pellets were recorded in a Thermo Nicolet, Avatar 370. The scanning was conducted in to mid IR range, i.e., between 4000-400cm^{-1}. UV spectra were obtained on a Varian Cary 50 UV-VIS spectrophotometer (Varian Cary, USA).

5.1.1.2B. Mass spectrometry (MS) and elemental analysis

Elemental analysis of the compounds was carried out using a Euro Vector elemental analyzer (model no. EA3011). Liquid chromatography–mass spectrometry experiments were performed on an Applied BiosystemsQTrap 2000 (Applied Biosystems, Darmstadt, Germany) coupled to an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) using a Luna 5 μ C18 column (100 A, 100 × 4.6 mm, Phenomenex, Aschaffenburg, Germany) or a Luna 3 μ C18 column (100 A, 50 × 1.0 mm, Phenomenex, Aschaffenburg, Germany) and a gradient of solvents A (0.1% HCOOH) and B (CH_3CN + 0.1% HCOOH; gradient 0% B to 100% B in 10 min) with a flow rate of 1.5 mL/min or 60 μL/min, respectively. ESI-MS spectra were acquired in the positive and negative modes with a turboion spray voltage, curtain gas, turbo temperature, and nebulizer gas of -4500 V, 30 psi, 500 °C, and 50 psi (positive mode, flow rate at 1.5 mL/min. The exact molecular ion weights of the pure compounds have been acquired by direct injection in a high resolution mass spectrometer, and were compared with the MarinLit database (Royal Society of Chemistry, London, Burlington House, London W1J 0BA), dedicated to marine natural products.

5.1.1.2C. Nuclear magnetic resonance spectroscopy (NMR)

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE III 500 MHz (AV 500) DRX 500 NMR spectrometer (Bruker, Karlsruhe, Germany) in $CDCl_3$ as aprotic solvent at ambient temperature with TMS as the internal standard (δ 0 ppm) equipped with 5 mm probes. The number of attached protons for the ^{13}C NMR signals was determined from DEPT experiments. Standard pulse sequences were used for Distortionless Enhancement by Polarization Transfer (DEPT), ^{1}H–^{1}H Correlation spectroscopy (^{1}H–^{1}H COSY for determining basic connectivity via J couplings throughbond), Nuclear Overhauser Effect Spectroscopy (NOESY for see through-space and conformation and for determining proximity of adjacent spin systems), Heteronuclear Single Quantum Correlation (HSQC for determining the narrower resonances for ^{1}H- ^{13}C correlations), and Heteronuclear Multiple Bond Correlation (HMBC to correlate Xnucleus shifts that are typically 2-4 bonds away from a proton) experiments

5.1.2. Isolation and purification of secondary metabolites of *Pernaviridis, Crassostreamadrasensis and Octopus dolffusi*

5.1.2.1. Isolation of secondary metabolites from *Pernaviridis*

The dried mussel powder (1 kg) was extracted three times with MeOH (3 h) and filtered through theWhatman No. 1 filter paper. The filtrate was concentrated (50 °C) *in vacuo*, and successively partitioned with $CHCl_3$ and EtOAc before being concentrated to furnish the solvent fractions designated as MCH (9.8 g) and MEA (8.5 g), respectively. The $CHCl_3$ andEtOAc fractions were pooled together to afford the composite solvent fraction designated as MCT. The latter was subjected to further chromatographic fractionation. The schematic diagram showing the purification of *P. viridis*extract has been shown in **Fig 5.1**.

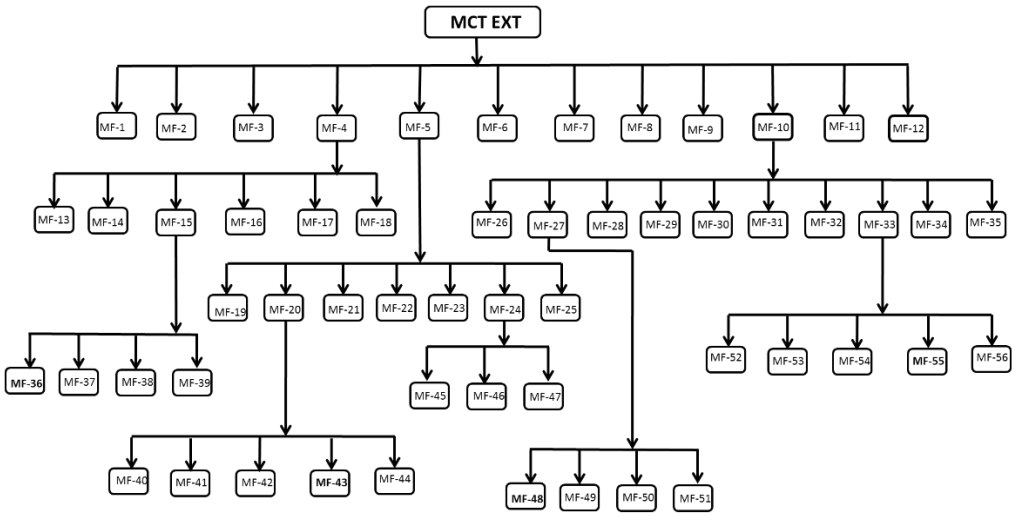


Fig 5.1 Schematic diagram showing the purification of the $CHCl_3$ fraction derived from *P. viridis*

5.1.2.1A Chromatographic purification of secondary metabolites from *Pernaviridis*

The compositefraction(MCT, 8.5 g) of *P. viridis*was subjected to vaccum liquid chromatography and eluted using a stepwise gradient system from *n*-hexane, EtOAc and MeOH to obtain a total of 12 column fractions (F_1 - F_{12}). The fraction F_4 obtained by eluting with *n*-hexane: EtOAc (4:1, v/v), on further purification using EtOAc/*n*-hexane(0-5% EtOAc) afforded 6 fractions (F_{13} – F_{18}).The fraction F_{15} on subsequent preparative TLC purification using 10% EtOAc:*n*-hexane afforded compound **1** (243 mg). The fraction F_5 on vaccum chromatographic purification yielded seven subfractions (F_{19} – F_{25}). The subfractionF_{20} on further purificationwith preparative TLC using 30 % EtOAc: *n*-hexane afforded compound **2** (46 mg). The subfraction F_{24} onsubsequent column chromatographic purification using EtOAc:*n*-hexane(0-70% EtOAc) afforded two compounds,**3**, (34 mg) and **4** (31 mg). The fraction F_{10} was flash chromatographed using a stepwise gradient using *n*-hexane, EtOAc and methanol to afford a total of 12 fractions (F_{26} – F_{35}). The column subfractionF_{26} on purification with preparative TLC (10%

MeOH/$CHCl_3$)afforded compound **5** (61 mg). The compound **6** (73 mg) was obtained after purification by preparative TLC of the column subfraction F_{27} by using 15% MeOH/$CHCl_3$.

5.1.2.1B Physicochemical data of compound (1): 3-Hydroxy-13-vinyl-dodecahydro-11-phenanthrenone

Light yellow crystalline solid; m.p. 135^oC; UV (MeOH) λ_{max} (log ε): 242 nm (1.74); TLC (Si gel GF_{254} 15 mm; $CHCl_3$/MeOH 40:60, v/v) R_f: 0.55; R_t: 7.51; IR (KBr,cm^{-1}) 1740.12 (C=O ν), 3250.90 br (O-H ν), 2942.02 (C-H ν), 1536 (C=C ν), 724.28 (C-H ρ), 1377.22 (C-H δ); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.91 – 5.68 (m, 1H), 5.36 – 5.30 (m, 1H), 5.06 – 4.84 (m, 2H), 3.62 – 3.45 (m, 1H), 2.41 – 2.24 (m, 3H), 2.02 (dt, 2H), 1.90 – 1.80 (m, 3H), 1.50-1.01 (m, 6H), 0.94 – 0.76 (m, 3H).HRESIMS *m/z* found 247.1698 (M+H), calcd 247.1709.

5.1.2.1CPhysicochemical data ofcompound (2): 4,4,9-Trimethyl-13-vinyl-dodecahydro-2-phenanthrenone

White crystalline solid; m.p. 122^oC; UV (MeOH) λ_{max} (log ε): 258 nm (3.41); TLC (Si gel GF_{254} 15 mm; $CHCl_3$/MeOH 80:20, v/v) R_f: 0.45; R_t: 4.70; IR (KBr, cm^{-1}) ν_{max}1736.96 (C=O ν), 2924.18 (C-Hν),1526.00 (C=C ν), 724.28 (C-H ρ), 1377.22 (C-H δ); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.60 (s, 1H), 6.03 – 5.72 (m, 1H), 5.02 – 4.90 (m, 2H), 4.33 – 4.04 (m, 2H), 2.95 – 2.62 (m, 1H), 2.41 – 2.25 (m, 4H), 2.18 (s, 3H), 2.10 – 1.99 (m, 3H), 1.86 – 1.74 (m, 1H), 1.42 (s, 1H), 1.38 – 1.29 (m, 4H), 1.02 (m, 3H), 0.98 (s, 3H). HRESIMS *m/z* ($C_{19}H_{28}O$) found 273.2219(M+H) calcd 273.2401.

5.1.2.1D Physicochemical data of compound (3): 11,20-Dihydroxy-6,6-dimethyl-decahydro-5H-benzo[h]naphtho[1,2-c]chromene-16-carbaldehyde

Yellowish crystalline solid; m.p. 165^oC; UV (MeOH) λ_{max} (log ε): 252 nm (1.61); TLC (Si gel GF_{254} 15 mm; $CHCl_3$/MeOH 40:60, v/ v) R_f: 0.35; R_t:

3.20; IR (KBr, cm^{-1}) ν_{max} 1738.56(C-O ν), 3219.90 br(O-H ν), 2924.18 (C-H ν), 1680 (aromatic C=C ν), 1545 (C=C ν), 724.28 (C-H ρ); 1377.22 (C-H); ^1H NMR (500 MHz, $CDCl_3$) δ 9.69(s,1H), 7.65 (dd, *J* = 5.7Hz, 2H), 7.46 (dt, *J* = 5.7 Hz, 2H), 6.44 (s, 1H), 5.33 – 5.25 (dd,1H), 4.31 – 4.19 (m, 2H), 4.10 (p, 1H), 3.60 (s, 1H), 2.37 – 2.17 (m, 4H), 1.94 (t, 2H), 1.62 – 1.47 (m, 9H), 0.91 (dd, 1H), 0.89 – 0.64 (m, 2H); HRESIMS *m/z*: ($C_{24}H_{28}O_4$) found 381.2067($M+H)^+$calcd 381.2188.

5.1.2.1E Physicochemical data ofcompound (4): 16-Acetyl-20-hydroxy-6,6-dimethyl-dodecahydro-5H-benzo[h]naphtho[1,2-c]chromen-12-one

Pale yellow powder; m.p. 129°C; UV (MeOH) λ_{max} (log ε): 252 nm (2.02); TLC (Si gel GF_{254} 15 mm; $CHCl_3$/MeOH 40:60, v/ v) R_f: 0.50; R_t: 5.53; IR (KBr, cm^{-1}) ν_{max} 1731.12 (C=O ν), 3219.05br (O-H ν), 2921.46 (C-H ν), 1556(C=C ν), 1377.22 (C-H δ), 724.28 (C-H ρ). ^1H NMR (500 MHz, $CDCl_3$) δ 7.72 (dd, 2H), 7.53 (dd, 2H), 5.58 – 5.33 (m, 1H), 5.27 (s, 1H), 4.28 (m, 3H), 4.14 – 4.08 (m, 1H), 3.81 – 3.57 (m, 1H), 2.35-2.31 (d, 5H), 2.02 (s, 2H), 1.79 (s, 6H), 1.29 – 0.94 (m, 6H); HRESIMS *m/z* $C_{25}H_{30}O_4$ found 395.2223 (M+H) calcd 394.2422 .

5.1.2.1F Physicochemical data of compound (5): Cholest-5-en-3β-3-yl-(30-hydroxy-3-methyl-36-methyleneundeca-30E,34E-dienoate)

Light yellow crystalline solid; m.p. 186°C; UV (MeOH) λ_{max} (log ε): 266 nm (2.61); TLC (Si gel GF_{254} 15 mm; MeOH/$CHCl_3$ 40:60, v/ v) R_f: 0.80; R_t: 13.40; IR (KBr, cm^{-1}) ν_{max}1736.96 (C=O ν), 3419.90 br (O-H ν), 2924.18 (C-H ν), 1545(C=C ν), 724.28 (C-H ρ), 1377.22 (C-H δ); ^1H NMR (500 MHz, $CDCl_3$) δ 5.81 (ddt, *J* = 16.9 Hz, 1H), 5.37 – 5.34 (m, 1H), 5.04 – 4.87 (m, 2H), 3.52 (tt, *J* = 11.0, 4.5 Hz, 1H), 2.34 – 2.20 (m, 4H), 2.17 (s, 3H), 2.07 – 1.92 (m, 6H), 1.83 (tq, *J* = 15.6, 6.2, 4.5 Hz, 10H), 1.57 (s, 1H), 1.55 – 1.43 (m, 4H), 1.33 (s, 3H), 1.28 (s, 2H), 1.18 – 1.04 (m, 10H), 1.01 (s, 3H), 0.91 (d,

J = 6.5 Hz, 2H), 0.88 – 0.85 (m, 9H), 0.68 (s, 3H). HRESIMS *m/z:* $C_{40}H_{64}O_3$ found 593.4935 (M+H) calcd 592.5146.

5.1.2.1G Physicochemical data ofcompound (6) Cholest-5-en-3β-3-yl-((E)-33-oxooct-31-enoate)

Yellow solid; m.p. 164°C; UV (MeOH) λ_{max} (log ε): 248nm (3.21); TLC (Si gel GF_{254} 15 mm; $CHCl_3$/MeOH 40:60, v/ v) R_f: 0.85; R_t: 18.20; IR - 1736.96 (C=O ν), 2924.18 (C-H ν), 1542 (C=C ν) , 1369.25 (C-H δ), 724.28 (C-H ρ);^{1}H NMR (500 MHz, $CDCl_3$) δ 5.90 – 5.74 (m, 1H), 5.35 (d, 1H), 5.04 – 4.88 (m, 1H), 3.58 – 3.47 (m, 1H), 2.38 – 2.19 (m, 6H), 2.07 – 1.92 (m, 4H), 1.99 – 1.77 (m, 7H), 1.60 – 1.40 (m, 5H), 1.42 – 1.31 (m, 5H), 1.21 – 0.89 (m, 8H), 0.86 – 0.75 (m, 6H), 0.73 – 0.59 (m, 6H); HRESIMS *m/z*: $C_{35}H_{56}O_3$ found 525.4309(M+H) calcd 524.4542.

5.1.2.2. Isolation of secondary metabolites from *Crassostreamadrasensis*

The freeze dried sample of *Crassostreamadrasensis* (100 g) was extracted with EtOAc: MeOH (500 mL X 2, 1:1, v/v) at an elevated temperature (50 ºC) under reflux for 2 h. The extracts were then filtered and concentrated (50 ºC) *in vacuo* to obtain the crude extract of *C. madrasensis* (CRCT, 12.56 g). The schematic diagram showing the purification of *C. madrasensis*has been illustrated under **Fig 5.2**.

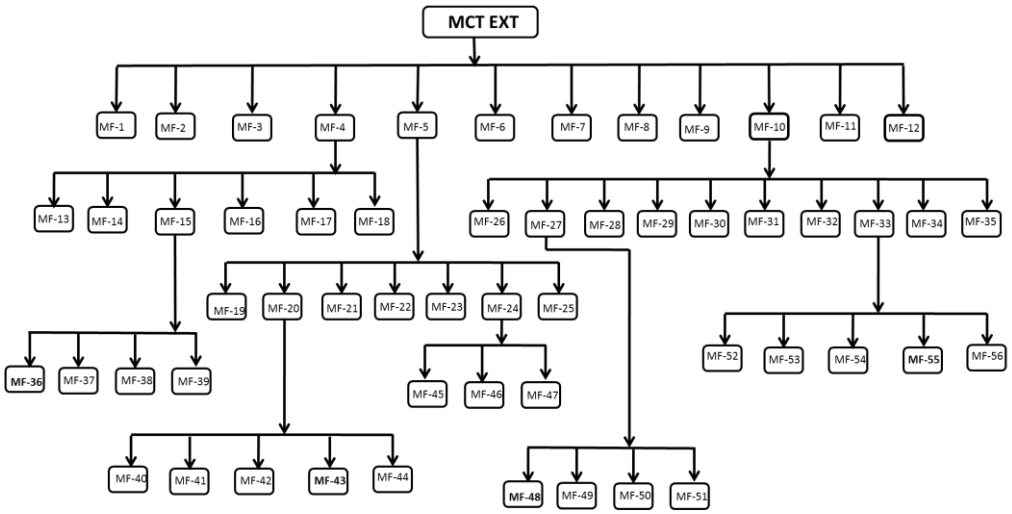


Fig 5.2 Schematic diagram showing the purification of EtOAc: MeOH fraction of *C. madrasensis*

5.1.2.2.AChromatographic purification of secondary metabolites from *C. madrasensis*

An aliquot of the crude *C. madrasensis* extract (CRCT,9.2 g) was slurried in silica gel (5 g, 60–120 mesh), and loaded into a glass column (90 cm x 4 cm) packed with silica gel (60–120 mesh, 90 g) as adsorbent before being subjected to vacuum liquid chromatography. The column was initially eluted with *n*-hexane and the eluent polarity was gradually increased by addition of EtOAc (*n*-hexane: EtOAc, 99:1 to 30:70, v/v) to furnish a total of 28 fractions of 35 mL each, which were reduced to 16 groups (CF_1 - CF_{16}) after TLC analysis (*n*-hexane: EtOAc, 9:1, v/v).The fraction CF_6 obtained by eluting with *n*-hexane: EtOAc (6:1, v/v) was found to be a mixture, which was flash chromatographed (Biotage AB SP1-B1A, 230–400 mesh, 12 g; Biotage AB, Uppsala, Sweden) on a silica gel column (Biotage, 230–400 mesh, 12 g; Sweden, Biotage No. 25+M 0489-1) at a collection UV wavelength of 236 nm using a step gradient of EtOAc/*n*-hexane (0-5% EtOAc) to afford 140 fractions (9 mL each). Based on analytical TLC, the fractions with similar patterns were pooled together to afford four pooled sub-fractions (CF_{17} – CF_{20}). The sub-

fraction CF_{18} on subsequent preparative TLC purification using 10% EtOAc/*n*-hexane yielded 12-hydroxy-2,9,9-trimethyl-10-[(2-phenylacetyl)oxy]-1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,12b,14a,14b-icosahydro-2-picenecarboxylate (**7**, 88 mg, **Table 5.9**). The fraction CF_9 on flash chromatographic purification on a silica gel column (Biotage, 230–400 mesh, 12 g; Sweden, Biotage No. 25+M 0489-1)at a collection UV wavelength of 256 nm using a step gradient of EtOAc/*n*-hexane (0-10% EtOAc) afforded 120 fractions (9 mL each). Based on analytical TLC, the fractions with similar patterns were pooled together to obtain six pooled fractions ($CF_{21} – CF_{26}$). The fraction CF_{22} on further purificationwith preparative TLC using 40 % EtOAc/*n*-hexane yielded methyl 1,12-dihydroxy-2,9,9-trimethyl-10-[(2-phenylacetyl)oxy]-1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,12b,14a,14b-icosahydro-2-picenecarboxylate(**8**, 52 mg, **Table 5.9**).

5.1.2.2.B Physicochemical data of methyl-3-(26-phenylacetyloxy)-icosahydro-19-hydroxy-4,4,20-trimethylpicene-23-carboxylate (7)

Yellow solid; m.p. 82 ^{o}C; UV (MeOH) λ_{max} (log ε): 256 nm (2.71); TLC (Si gel GF_{254} 15 mm; $CHCl_3$/MeOH 50:50, v/ v) R_f: 0.70; R_t: 12.20; IR (KBr, cm^{-1}) ν_{max} 1734.96 (C=O ν), 3419.90 br peak (O-H ν), 2924.18 (C-H ν), 1545 (C=C ν), 725.28 (C-H ρ), 1580(C=C aromatic ν) 1715 (6-membered C-C ring ν); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.75 – 7.66 (m, 2H), 7.53 (dd, 3H), 5.86 – 5.78 (m, 2H), 5.03 – 4.89 (m, 2H), 4.34 – 4.08 (m, 2H), 3.69 – 3.61 (m, 3H), 2.30 (dq, 5H), 2.17 (s, 2H), 2.03 (dd, 8H), 1.72 (s, 2H), 1.43 (d, 5H), 1.33 (d, 9H), 1.08 – 0.93 (m, 13H), 0.87 (d, 11H), 0.87 – 0.79 (m, 8H), 0.68 (s, 2H); HRESIMS *m/z*: $C_{35}H_{64}O_5$ found 549.3581(M+H) calcd 549.3685.

5.1.2.2.C Physicochemical data of methyl-3-(26-phenylacetyloxy)-icosahydro-1,19-dihydroxy-4,4,20-trimethylpicene-23-carboxylate (8)

White powder; m.p. 87 ^{o}C; UV (MeOH) λ_{max} (log ε): 267nm (2.89); TLC (Si gel GF_{254} 15 mm; $CHCl_3$/MeOH 40:60, v/ v) R_f: 0.20; R_t: 13.8; IR (KBr, cm^{-1}) ν_{max}724.28(C-H ρ), 1377.22(C-H δ), 1715 (6-membered cyclic ring ν), 1736.96(C=O ν), 2924.18(C-H ν),1642(C=C ν), 3010(C-H ν of C=C);^{1}H NMR (500 MHz, $CDCl_3$) δ 7.65 – 7.75 (m, 1H), 7.56 – 7.51 (m, 1H), 7.56 – 7.51 (m, 1H), 5.80(m,1H),(OH-5.32), 4.96(m,1H), 4.29(t,1H), 4.12(m,1H),4.08(dd,1H), 3.65(s,3H) 2.17(s,3H), 2.04 (m,1H), 2.02(m,2H), 1.96(m,1H), 1.85(m,2H) 1.71(m,2H), 1.62(q,1H), 1.62(m,2H), 1.61(m,1H), 1.31(m,2H), 1.15(m,2H), 1.28(t,1H), 1.36 (m,1H), , 1.12(m,1H),1.33(m,2H), 1.06(m,1H), 0.97(s,3H), 0.87(s,3H), 0.84(s,3H); HRESIMS *m/z*: ($C_{35}H_{48}O_6$) found 565.3530 (M+H) calcd565.3732.

5.1.2.3 Isolation of secondary metabolites from *Octopus dolffusi*

The freeze dried sample of *Octopus dolffusi* (100 g) was extracted with EtOAc: MeOH (500 mL X 2, 1:1, v/v) at an elevated temperature (50 ºC) under reflux for 2 h on a water bath. The extracts were filtered and concentrated (50 ºC) *in vacuo* to obtain the crude extract of *O. dolffusii* (OCT, 16.98 g).The schematic diagram showing the purification of *O. dolffusi* extract has been shown in **Fig 5.3.**

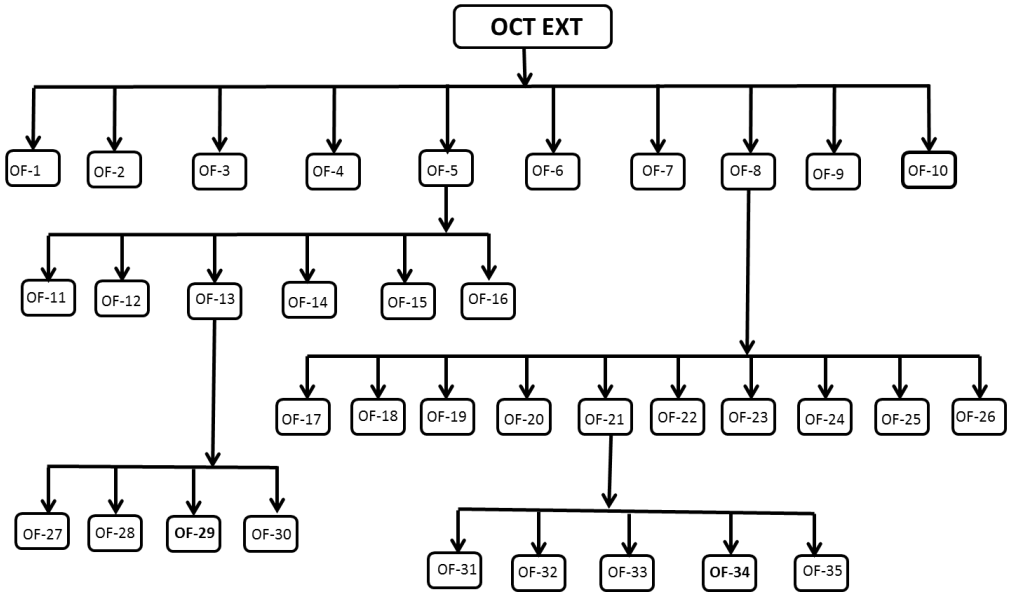


Fig 5.3 Schematic diagram showing the purification of *O. dolffusi* extract

5.1.2.3.AChromatographic purification of secondary metabolites from *Octopus dolffusi*

An aliquot of the *O.dolffusi*extract (9.0 g) was slurried in silica gel (5 g, 60–120 mesh), and loaded into a glass column (90 cm x 4 cm) packed with silica gel (60–120 mesh, 90 g) as adsorbent before being subjected to vacuum liquid chromatography. The column was initially eluted with *n*-hexane and the eluent polarity was gradually increased by addition of EtOAc (*n*-hexane: EtOAc 99:1 to 30:70, v/v) to furnish 14 fractions of 25 mL each, which were reduced to 10 groups (OF_1 - OF_{10}) after TLC analysis (*n*-hexane: EtOAc, 9:1, v/v). OF_5 obtained by eluting with *n*-hexane:EtOAc (4:1, v/v) was found to be a mixture, which was flash chromatographed (Biotage AB SP1-B1A, 230–400 mesh, 12 g; Biotage AB, Uppsala, Sweden) on a silica gel column (Biotage, 230–400 mesh, 12 g; Sweden, Biotage No. 25+M 0489-1) at a collection UV wavelength of 236 nm using a step gradient of EtOAc/*n*-hexane (0-5% EtOAc) to afford 110 fractions (9 mL each). Based on analytical TLC, the fractions

with similar patterns were pooled together to afford five pooled fractions (OF_{11} – OF_{16}). The fraction OF_{13} on subsequent preparative TLC purification using 10% EtOAc/*n*-hexane afforded 1-[17-(1,5-dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-3-yl] 5-methyl 3-[(*E*)-1-amino-3-ethyl-6-oxo-3-heptenyl]pentanedioate(**7**, 55 mg, **Table 5.12**). The fraction OF_8 was flash chromatographed on a silica gel column (Biotage, 230–400 mesh, 12 g; Sweden, Biotage No. 25+M 0489-1) at a collection UV wavelength of 280 nm using a step gradient of EtOAc/*n*-hexane (0-70% EtOAc) to afford 180 fractions (9 mL each). Based on analytical TLC, the fractions with similar patterns were pooled together to afford ten pooled fractions (OF_{17} – OF_{26}). The sub-fraction OF_{21} on purification by preparative TLC in 10% MeOH/$CHCl_3$ afforded 4-ethyl-6-vinyl-2,3,6,7,7a,8,9,9a-octahydroazuleno[1,8-*bc*]pyran-2,9-dione(**8**, 35 mg, **Table 5.12**).

5.1.2.3.B Physicochemical data of cholesta-5-en-3β-yl-(32-methyl-(30-((E)-34-amino-36-ethyl-39-oxohept-36-enyl)-pentanedioate (9)

Light yellow solid; m.p. 118 ^{o}C; UV (MeOH) λ_{max} (log ε): 252nm (3.61); TLC (Si gel GF_{254} 15 mm; $CHCl_3$/MeOH 40:60, v/ v) R_f: 0.70; R_t: 4.20; IR (KBr, cm^{-1}) ν_{max} 724.28(C-H ρ), 1068.21(C-O-C ν) 1377.22(C-H δ), 1715 (6-membered cyclic ν), 1736.96(C=O ν), 2924.18(C-H νof alkanes), 1642(C=C ν), 3010(C-H ν of C=C); ^{1}H NMR (500 MHz, Chloroform-d) δ 5.34 – 5.24 (m, 2H), 4.61 – 4.48 (m, 1H), 4.29 – 3.90 (m, 1H), 3.59 (s, 3H), 2.80 – 2.66 (m, 2H), 2.29 – 2.22 (m, 4H), 2.22 – 2.15 (m, 2H), 2.01 – 1.85 (m, 4H), 1.76 (tdd, 3H), 1.64 – 1.46 (m, 1H), 1.38 – 1.14 (m, 15H), 1.06 (ddd, 5H), 1.01 – 0.90 (m, 5H), 0.88 – 0.73 (m, 18H), 0.61 (s, 3H); HRESIMS *m/z*: $C_{42}H_{69}NO_5$ found 668.5255 (M+H) calcd668.5285

5.1.2.3.C Physicochemical data of 7-ethyl-9-vinyl-octahydroazuleno[1,8-bc]pyran-3,12-dione (10)

Yellow powder; m.p. 185 ^{o}C; UV (MeOH) λ_{max} (log ε): 227 nm (3.28); TLC (Si gel GF_{254} 15 mm; $CHCl_3$/MeOH 10:90, v/ v) R_f: 0.35; R_t: 6.20 min; IR (KBr, cm^{-1}) ν_{max} 721.29(C-H ρ), 1070(C-O-C ν), 1541(C=C ν), 1690.21 (C-CO-C ν) 1736.96(C=O ν), 2854.74(C-H ν of alkanes); ^{1}H NMR (500 MHz, Chloroform-d) δ 5.82 (ddt, 1H), 5.37 (d,1H), 5.04 – 4.89 (m, 2H), 3.69(s,1H),2.46 – 2.11 (m, 1H), 2.07 – 1.99 (m, 3H), 1.62 (d, 2H), 1.44 (d, 1H), 1.40 – 1.32 (m, 3H), 0.96 – 0.75 (m, 3H); ^{13}C-NMR, ^{1}H-^{1}H-COSY and HMBC data; HRESIMS *m/z*:$C_{16}H_{18}O_3$ found 259.1335(M+H) calcd258.1384).

5.1.3 Antioxidant and anti-inflammatory activities of the purified compounds

5.1.3.1Determination of antioxidant activities

1, 1-Diphenyl-2-picryl- hydrazil (DPPH•) radical scavenging activity of the samples was measured using the DPPH stable radical as described in a previous section 4.1.2.1.2 2,2′-Azino-bis-3 ethylbenzothiozoline-6-sulfonic acid diammonium salt (ABTS) radical scavenging activity was determined by ABTS radical cationdecolorization assay as described earlier (4.1.2.1.1).

5.1.3.2Determination of *in vitro* anti-inflammatory activities

The *in vitro* anti-inflammatory activities have been carried out in this study by cyclooxygenase (COX-2) inhibition assays as described by 2,7-dichloroluorescein method (Larsen *et al*., 1996) and 5-lipoxygenase (5-LOX)inhibition assay (Baylac and Racine, 2003).

5.1.4Statistical analysis

Statistical evaluation was carried out with the Statistical Program for Social Sciences 13.0 (SPSS Inc, Chicago, USA, ver. 13.0). Analyses were carried out in triplicate, and the means of all parameters were examined for significance by analysis of variance (ANOVA). The level of significance for all analyses was $P \leq 0.05$.

5.2 Results and discussion

5.2.1. Secondary metabolites from *Pernaviridis*

The $CHCl_3$ fraction (9.2 g) of *P. viridis*was subjected to vaccum liquid chromatography, and eluted using a stepwise gradient system from *n*-hexane, EtOAc and MeOH to obtain total of 12 column fractions (MF_1 - MF_{12}). The fraction MF_4 obtained by eluting with *n*-hexane: EtOAc (4:1, v/v) was further purified using EtOAc /*n*-hexane(0-10% EtOActo afford 6 sub-fractions (MF_{13} – MF_{18}).The fraction MF_{15} on subsequent preparative TLC purification using 10% EtOAc/*n*-hexane afforded compound **1** (243 mg). The fraction MF_5 on vaccum chromatographic purification yielded seven sub-fractions (MF_{19} – MF_{25}). The sub-fraction MF_{20} on further purificationwith preparative TLC using 30 % EtOAc/*n*-hexane afforded compound **2** (46 mg). The sub-fractionMF_{24} oncolumn chromatographic purification using EtOAc/*n*-hexane(0-70% EA) yielded two compounds**3**, (34 mg) and **4** (31 mg). The fraction MF_{10} was flash chromatographed using a stepwise gradient using *n*-hexane, ethyl acetate and methanol to afford 12 fractions (MF_{26} – MF_{37}). The fraction MF_{26} on subsequent purification by preparative TLC in 10% MeOH/$CHCl_3$ yielded compound **5** (61 mg). The fraction MF_{27} on preparative TLC purification using 15% MeOH/$CHCl_3$ resulted compound **6** (73 mg). The yield, R_f, antioxidant and anti-inflammatory activities of each column/P-TLC fractions and compoundswere given in **Table 5.1.**

Table 5.1. Yield, R_f, antioxidant and anti-inflammatory activities of each column/P-TLC fractions of *P. viridis*

	Yield (mg)	R_f	DPPH radical scavenging activity (%)	ABTS radical scavenging activity (%)	COX-2 inhibitory activity (%)	LOX-5 inhibitory activity (%)
MCT CC (EtOAc/HX)						
MF-1 (2% EtOAc/HX)	54	0.12	48.95±0.00	31.81±0.15	35.16±0.03	44.49±0.08
MF-2 (5% EtOAc/HX)	31	0.23	52.51±0.02	30.67±0.29	33.46±0.02	19.11±0.02
MF-3 (7% EtOAc/HX)	42	0.32	49.56±0.01	29.17±0.32	26.12±0.61	17.64±0.03
MF-4 (10% EtOAc/HX)	657	0.37	65.32±0.01	20.04±0.27	42.53±0.01	33.21±0.01
MF-5 (20% EtOAc/HX)	987	0.45	69.25±0.01	18.79±0.25	43.15±0.24	36.38±0.01
MF-6 (30% EtOAc/HX)	121	0.62	45.41±0.00	17.11±0.23	21.23±0.09	28.26±0.01
MF-7 (50% EtOAc/HX)	78	0.69	42.75±0.00	15.81±0.21	26.47±0.01	25.61±0.05
MF-8 (100% EtOAc)	44	0.72	32.49±0.00	14.81±0.20	23.58±0.01	18.42±0.06
MF-9 (2% MeOH/EtOAc)	65	0.79	49.16±0.00	9.841±0.13	19.76±1.02	13.73±0.03
MF-10 (5% MeOH/EtOAc)	1087	0.83	55.66±0.01	20.57±0.11	41.61±0.23	35.43±0.07
MF-11 (15% MeOH/EtOAc)	50	0.94	43.29±0.02	14.11±0.19	32.51±0.08	11.84±1.12
MF-12 (100% MeOH)	46	0.99	48.15±0.01	7.445±0.10	36.12±0.02	19.46±0.42
MF-4 (CC 0-10% HX/EtOAc)						
MF-13 (1% EtOAc/HX)	121	0.25	61.41±0.01	28.40±0.76	NA	NA
MF-14 (2% EtOAc/HX)	107	0.39	45.39±0.00	22.55±0.57	NA	NA
MF-15 (4% EtOAc/HX)	169	0.48	56.29±0.01	17.30±0.23	43.33±0.06	44.28±0.29
MF-16 (6% EtOAc/HX)	107	0.56	58.14±0.01	24.21±0.46	NA	NA
MF-17 (8% EtOAc/HX)	55	0.67	35.42±0.00	11.58±0.15	NA	NA
MF-18 (10% EtOAc/HX)	61	0.88	39.42±0.00	11.04±0.14	NA	NA
MF-5 (CC 0-15% HX/EtOAc)						
MF-19 (1% EtOAc/HX)	21	0.21	35.29±0.05	20.03±0.25	NA	NA
MF-20 (2% EtOAc/HX)	217	0.29	70.48±0.00	32.20±0.43	44.57±0.02	48.21±0.01
MF-21 (5% EtOAc/HX)	49	0.35	55.81±0.03	21.93±0.08	NA	NA
MF-22 (7% EtOAc/HX)	389	0.54	58.29±0.01	29.05±0.39	NA	NA
MF-23 (10% EtOAc/HX)	35	0.69	60.18±0.00	22.17±0.65	NA	NA
MF-24 (12% EtOAc/HX)	164	0.74	68.46±0.00	32.92±0.44	41.53±0.05	47.82±0.01
MF-25 (15% EtOAc/HX)	51	0.84	48.29±0.01	27.05±0.39	NA	NA
MF-10 (CC HX/MeOH/EtOAc)						
MF-26 (2% HX/EtOAc)	225	0.15	69.24±0.00	30.20±0.84	44.25±0.03	48.67±0.02
MF-27 (10% HX/EtOAc)	256	0.23	66.65±0.00	31.40±0.35	43.28±0.01	47.43±0.16
MF-28 (15% HX/EtOAc)	19	0.28	63.12±0.00	20.09±0.27	NA	NA
MF-29 (25% HX/EtOAc)	44	0.34	65.58±0.00	24.67±0.47	NA	NA
MF-30 (50% HX/EtOAc)	49	0.48	58.41±0.00	28.37±0.76	NA	NA
MF-31 (100% EtOAc)	55	0.51	60.66±0.00	27.57±0.73	NA	NA
MF-32 (2% MeOH/EtOAc)	66	0.67	35.42±0.00	11.58±0.15	NA	NA
MF-33 (5% MeOH/EtOAc)	84	0.78	39.42±0.00	11.04±0.14	NA	NA
MF-34 (10% MeOH/EtOAc)	28	0.84	35.42±0.00	11.58±0.15	NA	NA
MF-35 (25% MeOH)	66	0.88	39.42±0.00	11.04±0.14	NA	NA

MF-15 (PTLC 10% EA/HX)						
MF-36	31	0.32				
MF-37	24	0.59	70.18±0.00	21.53±0.56	NA	NA
MF-38	39	0.65	75.24±0.36	29.48±0.23	47.26±0.87	50.64±0.23
MF-39	55	0.89	79.46±0.00	35.21±0.27	47.15±0.07	47.86±0.01
MF-20 (PTLC 20% EA/HX)						
MF-40	27	0.25	33.35±0.01	15.08±0.20	NA	NA
MF-41	35	0.38	38.16±0.00	6.977±0.09	NA	NA
MF-42	24	0.46	65.24±0.01	24.65±0.19	NA	NA
MF-43	46	0.58	72.56±0.14	21.75±0.56	49.76±0.33	50.34±0.23
MF-44	49	0.75	68.19±0.01	29.19±0.12	48.29±0.02	51.67±0.02
MF-24(PTLC 20% EA/HX)						
MF-45	94	0.24	55.19±0.01	22.36±0.30	NA	NA
MF-46	34	0.57	78.65±0.25	31.4±0.89	45.28±0.55	47.43±0.45
MF-47	31	0.69	78.46±0.16	32.92±0.49	46.53±0.14	47.82±0.56
MF-26(PTLC 5% MeOH/$CHCl_3$)						
MF-48	32	0.24	51.44±0.00	24.44±0.30	NA	NA
MF-49	47	0.37	59.18±0.00	21.25±0.29	NA	NA
MF-50	61	0.54	71.47±0.14	33.29±0.23	47.25±0.54	48.67±0.8
MF-51	54	0.72	61.32±0.00	14.69±0.19	NA	NA
MF-27(PTLC 10% MeOH/$CHCl_3$)						
MF-52	34	0.23	55.34±0.02	24.13±0.19	NA	NA
MF-53	27	0.46	59.26±0.01	24.05±0.10	NA	NA
MF-54	73	0.55	68.21±0.18	28.67±0.14	49.28±0.23	48.57±0.49
MF-55	46	0.64	56.64±0.00	22.85±0.30	NA	NA
MF-56	55	0.76	59.82±0.00	21.43±0.29	NA	NA

DPPH radical scavenging activity at 0.1 mg/mL was expressed in percent;ABTS radical scavenging activity at 0.1 mg/mL is expressed in percent; COX-2 inhibitory activity at 0.1 mg/mL was expressed in percent; LOX-5 inhibitory activity at 0.05 mg/mL was expressed in percent; NA – not assayed, ie. the fractions with lesser yield were evaluated only for DPPH and ABTS radical scavenging activity. CC – Column chromatography; PTLC -Preparative thin layer chromatography; MeOH - Methanol; EA - Ethyl acetate; HX - *n*-hexane; $CHCl_3$ - Chloroform; MCT - Compositefraction of *P. viridis*

5.2.1.1Antioxidant and anti-inflammatory activities of the purified compounds

The order of DPPH radical-scavenging abilities of the test compounds were as follows: **3**>**4**>**1**>**2**>**5**>**6** (**Table 5.2**). The $ABTS^{\cdot+}$radical scavenging activity was found to be in the order: **5**>**4**>**3**>**1**>**6**>**2**. The COX-2 inhibition activity was found to be in the order, **2** >**6**>**1**>**5 a**nd 5-LOX inhibition activity was in the order, **1** >**2**>**5**>**6.**The anti-inflammatory activities of compounds **3**

and **4** were found to be lesser than other compounds purified from the $CHCl_3$ fraction of *P. viridis.*

Table 5.2. Radical scavenging and *in vitro* anti-inflammatory activities of the compounds isolated from the $CHCl_3$ fraction of *Pernaviridis*

Compounds	DPPH radical scavenging activity	ABTS radical scavenging activity	COX-2 inhibition activity	5-LOX inhibition activity
Compound **1**	75.24±0.36^{pq}	29.48±0.23^{p}	47.26±0.87^{p}	50.64±0.23^{p}
Compound **2**	72.56±0.14^{pq}	21.75±0.56^{p}	49.76±0.33^{p}	50.34±0.23^{p}
Compound **3**	78.65±0.25^{p}	31.4±0.89^{p}	45.28±0.55^{p}	47.43±0.45^{p}
Compound **4**	78.46±0.16^{p}	32.92±0.49^{p}	46.53±0.14^{p}	47.82±0.56^{p}
Compound **5**	71.47±0.14^{q}	33.29±0.23^{p}	47.25±0.54^{p}	48.67±0.8^{p}
Compound **6**	68.21±0.18^{q}	28.67±0.14^{p}	49.28±0.23^{p}	48.57±0.49^{p}

$DPPH^{\cdot}$ and $ABTS^{\cdot+}$ radical scavenging activities (at 0.1 mg/mL) were expressed as percent. COX-2 and 5-LOX inhibition activities(at 0.05 mg/mL) were expressed as percent. p, q - Column wise values with different superscripts indicates significant difference (P<0.05). Results were expressed as mean ± SD (n = 3).

5.2.1.2 Chromatographic purification and spectral analyses of secondary metabolites from *Pernaviridis*

Chromatographic separation led to the isolation of six new secondary metabolites. The novelty of these compounds has been verified with the MarinLit and SciFinder databases. The molecular structures of the purified compounds were proposed on the basis of comprehensive analysis of the ^{1}H NMR, ^{13}C NMR, including 2D-NMR experiments (^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY) and mass spectra.

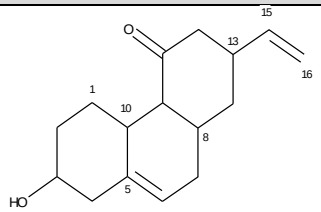
5.2.1.2A Chromatographic purification and spectral analyses of 3-hydroxy-13-vinyl-dodecahydro-11-phenanthrenone (1)

The compound 3-hydroxy-13-vinyl-dodecahydro-11-phenanthrenone (1**)**, a new phenanthrenone derivative was isolated as light yellow crystalline solid upon repeated chromatography over silica columns/TLC. The UV absorbance at λ_{max} (log ε) 242 nm (1.74) has been assigned to be a chromophore with olefinic system. Its mass spectrum exhibited a molecular

ion peak at *m/z*247 (HRESIMS *m/z* 247.1698amu, $[M+1]^+$), which in combination with its ^1H and ^{13}C NMR data (**Table 5.3**) indicated the elemental composition as 3-hydroxy-13-vinyl-dodecahydro-11-phenanthrenone (1) with six degrees of unsaturation. Three degrees of unsaturation from the double bond equivalent and the other three degrees of unsaturation were due to a cyclic ring system, which was not of aromatic type. This was based on the absence of the aromatic proton signal in the ^1H NMR spectrum.

Table 5.3. NMR spectroscopic data of 3-hydroxy-13-vinyl-dodecahydro-11-phenanthrenone (1) isolated from *Pernaviridis*

Compound 1



Position No.	^{13}C NMR	δ^1H NMR (int., mult., J in Hz)[b]	^1H-^1H COSY	HMBC(^1H-^{13}C)
1	22.69	1.28(m,2 H)	2-H	C-2,3
2	29.1	1.47(m,2H)	-	-
3	76.75	3.51 (m,1H)	4-H, 2-H	C-4,5
4	32.74	2.31(m,2H)	3-H	C-3
5	100	-	-	-
6	140.45	5.35 (m,1H)	7-H	C-7,4
7	29.15	1.99(m,2H)	8-H	C-6,8
8	31.92	1.30(m,1H)	-	C-7
9	29.69	1.08(t,1H)	10-H	C-11,10
10	29.15	1.85(m,1H)	-	-
11	187.35	-	-	-
12	29.1	2.02(m,2H)	13-H	C-13
13	50.36	2.21(m,1H)		C-12,15,16
14	14.11	0.88 (m,2H)	8-H	C-13
15	132.18	5.81 (m,1H)	16-H	C-12
16	116.46	4.97 (dd,2H)	15-H	C-15

[a] NMR spectra recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometers.
[b] Values in ppm, multiplicity and coupling constants (J¼ Hz) are indicated in parentheses. The assignments were made with the aid of the 1H-1H COSY, HSQC, HMBC and NOESY experiments.

The olefinic (C=C) and carbonyl (C=O) groups have been symbolized by the absorption bands at 1536 and 1740 cm^{-1},respectively. The characteristic IR absorption spectra at 3250 cm^{-1} (O-H stretching vibration), 2942.02 cm^{-1} (C-H stretching), 724.28 cm^{-1} (C-H rocking) and 1377.22 cm^{-1} (C-H bending) also support the structure of tricyclic moiety.

The ^{1}H, ^{13}C along with ^{135}DEPT NMR experiments confirmed the occurrences of seven each of methylenes and methines along with two quaternary carbons. The olefinic peaks were apparent at δ 132.18(C-15) and 116.46(C-16) attributing the presence of double bonds, which were correlated to the protons by HSQC with δ 5.81 and 4.97, respectively thereby ascribing the occurrence of vinyl (>CH=CH_2) moiety attached to the tricylic system. Its position was further corroborated by the HMBCs from δ 2.21(H-13) to δ 132.18(C-15) and 116.46(C-16). The deshielded peak at δ 3.51(H-3) exhibited HSQC correlation with the carbon at δ 76.75, which appropriately indicated the presence of possible oxygenation {could be a hydroxyl group directly linked to methine proton (>CH-OH)} in its vicinity. The relatively greater chemical shift of quaternary carbon at δ 187.35(C-11) referred to carbonyl directly bonded to ring system. The HMBCs from δ 1.08(H-9) to δ 187.35(C-11) validated the above assignments. The signals at δ 5.35(H-6) with δC 140.45 attributed the presence of an olefinic framework, which was bonded to the quaternary alkenic signal at δ 100.00(C-5). The correlations, such as δ 2.31(H-4)/3.51(H-3)/1.47(H-2)/1.28(H-1)/1.85(H-10)/1.08(H-9)/1.30(H-8)/1.99(H-7)/5.35(H-6) and δ 1.30(H-8)/0.88(H-14)/2.21(H-13)/2.02(H-12) supported the tricyclic framework along with δ 2.21(H-13)/5.81(H-15)/4.97(H-16), which established the exocyclic vinylic moiety in the

compound. Other long-range relations from δ 1.28(H-1) to δ 76.75(C-3); δ 3.51(H-3) to δ 100.00(C-5) and δ 5.81(H-15) to δ 29.12(C-12) further supported the presence of 3-hydroxydodecahydro-11-phenanthrenone framework. Hence, the double bond equivalence of six was accounted for three double bonds and three ring systems. The geometric isomerism of olefinic protons has been established by greater coupling constants (*J*) of 9.2(H-15) and 7.9(H-16) Hz, which appropriately validated the *trans*(*E*) configuration of the vinyl group. The NOE correlations were recorded between δ 3.51(H-3)/5.81(H-15)/1.08(H-9), which depicted that these were in identical plane of symmetry and α-orientated. The hydroxyl at C-3 was β-oriented being opposite to the α-disposed proton at δ 3.51(C-3). The NOESY cross-peaks between δ 1.85(H-10)/2.21(H-13)/1.30(H-8) indicated that these were disposed in identical plane of reference, and β-oriented.

The LCMS spectrum of 3-hydroxy-13-vinyl-dodecahydro-11-phenanthrenone (1) showed an adduct (2M+1) peak at *m/z* 492.3 ($C_{32}H_{44}O_4$ $^{\bullet}$+), which appeared to undergo elimination of hydroxyl groups (-OH) to form the daughter ion peaks at *m/z* 476.3 ($C_{32}H_{44}O_3$) and *m/z* 460.3 ($C_{32}H_{44}O_2$). The molecular ion was found to be at *m/z* 246.1 ($C_{16}H_{22}O_2$).

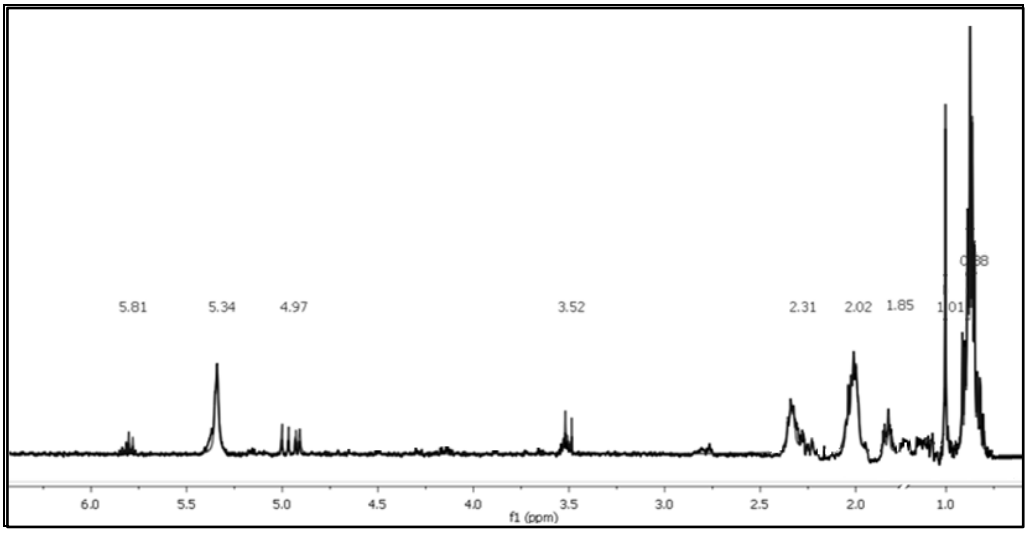


Fig 5.4.A. ^{1}H NMR spectrum of 3-hydroxy-13-vinyl-dodecahydro-11-phenanthrenone (1) isolated from *Pernaviridis*.

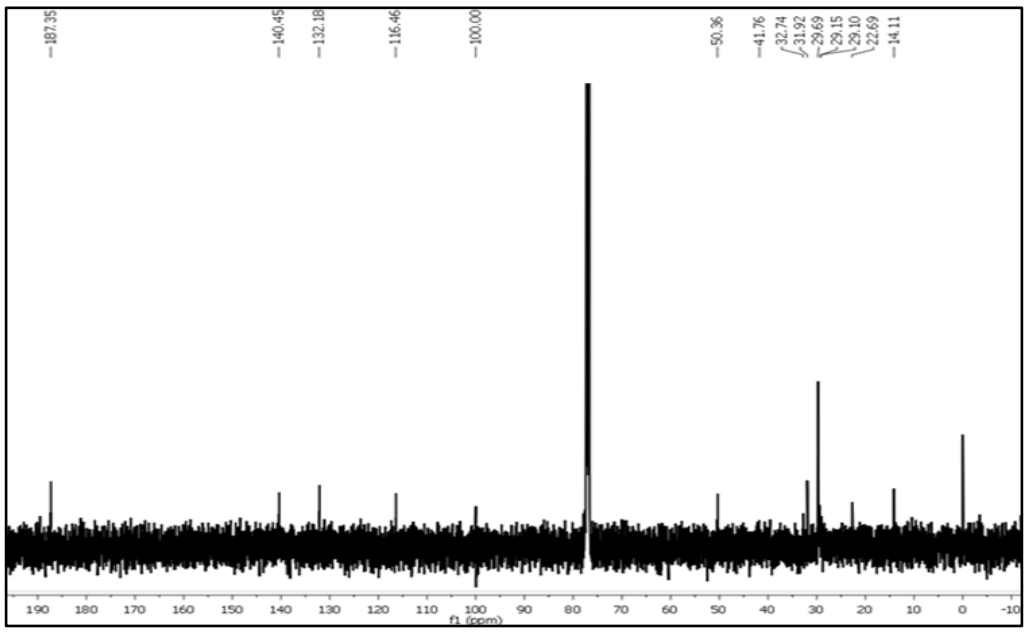


Fig 5.4. B.^{13}C NMR spectrum of 3-hydroxy-13-vinyl-dodecahydro-11-phenanthrenone (1)isolated from *Pernaviridis*.

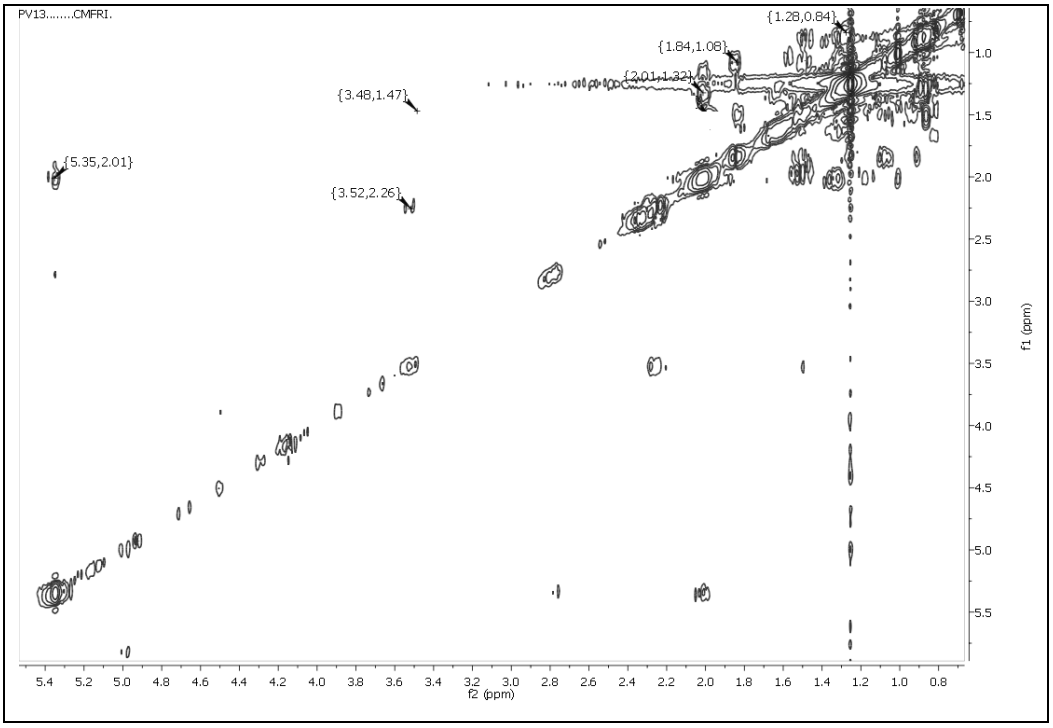


Fig 5.4.C. ^{1}H–^{1}H COSY spectrum of 3-hydroxy-13-vinyl-dodecahydro-11-phenanthrenone (1)isolated from *Pernaviridis*.

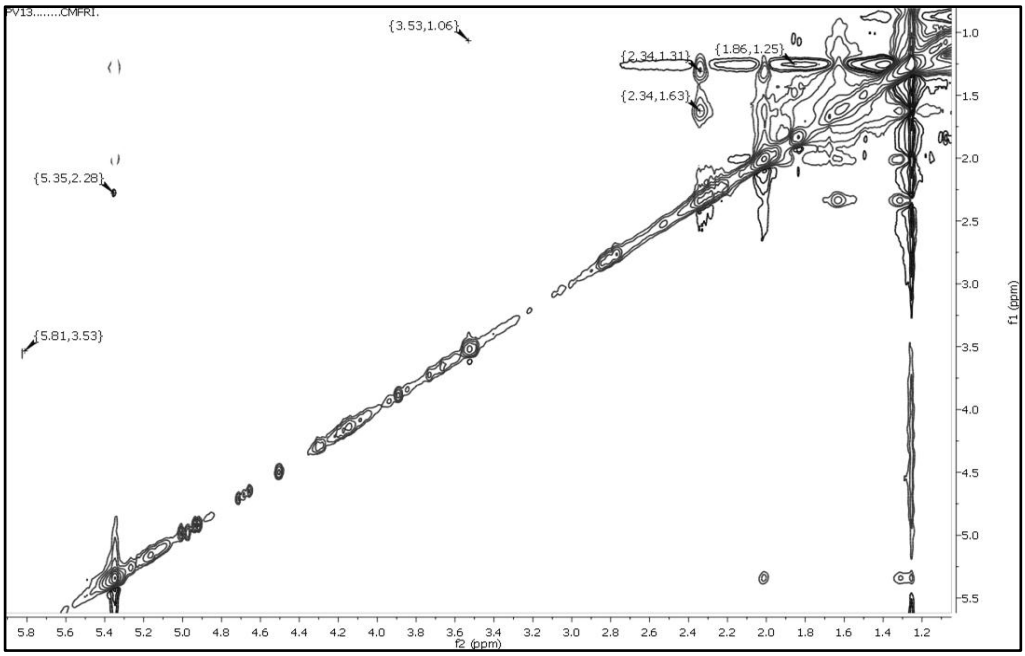


Fig 5.4.D. NOE spectrum of 3-Hydroxy-13-vinyl-dodecahydro-11-phenanthrenone (1) isolated from *Pernaviridis*

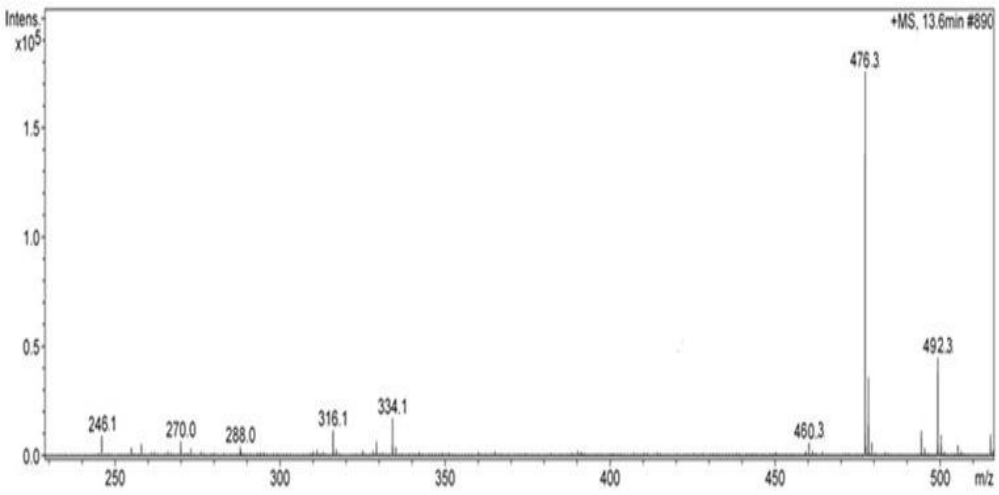


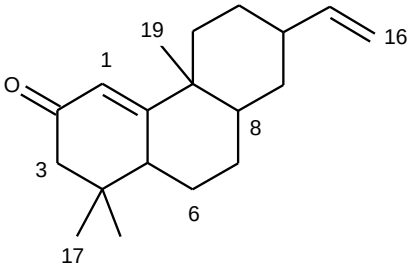
Fig 5.4.E Mass spectrum of 3-hydroxy-13-vinyl-dodecahydro-11-phenanthrenone (1)isolated from *Pernaviridis*

5.2.1.2B Chromatographic purification and spectral analyses of 4,4,9-trimethyl-13-vinyl-dodecahydro-2-phenanthrenone (2)

The compound 4,4,9-trimethyl-13-vinyl-dodecahydro-2-phenanthrenone **(2)** isolated as white crystalline solid which showed characteristic peaks as compound (**1**) although some differences were observed in the first ring. The peak position at H-3(δ3.51) was absent in compound (**2**) and extra methyl peaks at H-17 (δ 1.02), H-18(δ 0.98) and α, β unsaturatedolefinic proton at highly downfield shift δ6.59 were apparent. The UV absorbance at λ_{max} (log ε) 258nm (3.41) was assigned to be positioned at a chromophore with α, β unsaturated olefinic system. Its mass spectrum exhibited a molecular ion peak at *m/z*273 $(M+1)^+$(HRESIMS *m/z*273.2219amu, which in combination with its ^1H and ^{13}C NMR data (**Table 5.4**) indicated the elemental composition as 4,4,9-trimethyl-13-vinyl-dodecahydro-2-phenanthrenone (2) with six degree of unsaturation.

Table 5.4. NMR spectroscopic data of 4,4,9-trimethyl-13-vinyl-dodecahydro-2-phenanthrenone (2) isolated from the $CHCL_3$ extract of *Pernaviridis*

Compound 2



Position No.	^{13}CNMR	δ^{1}H NMR (int., mult., J in Hz)[b]	^{1}H-^{1}H COSY	HMBC(^{1}H-^{13}C)
1	129.55	6.59(S)	-	C-2,10
2	184.16	-	-	-
3	50.1	4.25(S)	-	C-2,1,10
4	33.86	-	-	-
5	46.87	2.79(dd)	6-H	C-4,10,6
6	33.55	2.33(m)	7-H	C-8,9
7	29.15	1.99(m)		C-8,5
8	31.92	1.88(t)	7-H	C-13,11,14
9	31.98	-	-	-
10	169	-	-	-
11	29.5	1.28(m)	12-H	C-19,12
12	28.96	1.35(m)	13-H, 15-H	C-13
13	33.51	2.01(m)	14-H	C-12,15
14	29.15	1.30(m)	8-H	-
15	139.5	5.82(m)	13-H	-
16	113.97	4.99(dd)	15-H	-
17	16.81	1.02(s)	-	C-4,5,2
18	14.1	0.98(s)		C-5
19	19.01	2.21(s)		C-10,11

[a] NMR spectra recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometers.

[b] Values in ppm, multiplicity and coupling constants (J¼ Hz) were indicated in parentheses. The assignments were made with the aid of the 1H-1H COSY, HSQC, HMBC and NOESY experiments.

The structure of **2** was similar to that of **1**, except for three additional methyl groups at C-4 and C-9 in **2**. Also, the carbonyl carbon (C=O) at C-11 in **1** was shifted to the C-2 position in **2**. The ^{1}H, ^{13}C along with ^{135}DEPT data established the occurrences of seven methylenes, four quaternary carbons, five methines and three methyl singlets. Olefinic signals were evident at δ 139.50(C-15) and 113.97(C-16), which indicated the presence of methine and methylene groups that were correlated to the protons at δ 5.82 and 4.99, respectively by HSQC. Thus, the presence of terminal vinyl (>$\underline{CH}$=$\underline{CH_2}$) moiety attached to the main tricylic system was appropriately validated, and its position was identified by HMBCs from δ 2.01(H-13) to δ 139.50(C-15). Higher chemical shift signals at δ 6.59(H-1) was found to be a singlet methine attached to δ 129.55(C-1), which apparently indicated the presence of olefinic framework in the ring. It was positioned between the highly deshielded quaternary alkenic carbon at δ 169.00(C-10) and carbonyl carbon (C=O) at δ 184.16(C-2). Therefore, the methine at C-1 was found to be singlet proton. The HMBCs from δ 6.59(H-1) to δ 184.16(C-2), 169.00(C-10) and δ 4.25(H-3) to δ 184.16(C-2), 169.00(C-10), 129.55(C-1) corroborated the dispositions of the protons at C-10, C-3, C-1 and C-2. The COSY correlations, such as δ 2.79(H-5)/2.33(H-6)/1.99(H-7)/1.88(H-8)/1.30(H-14)/2.01(H-13)/1.35(H-12)/1.28(H-11) supported the tricyclic framework along with δ 2.01(H-13)/5.82(H-15)/4.99(H-16) to assign that the exocyclic vinyl moiety was attached at C-13. Long range HMBCs from δ 2.79(H-5) to δ 169.00(C-10); δ 1.88(H-8) to δ 29.50(C-11), 29.16(C-14); δ 1.28(H-11) to δ 19.01(C-19); δ 1.02(H-17) to δ 46.87(C-5), 184.16(C-2); δ 0.98(H-18) to δ 46.87(C-5) and δ 2.21(H-19) to δ 169.00(C-10) supported the occurrence of 4,4,9-trimethyl-13-vinyl-dodecahydro-2-phenanthrenone part. Therefore, the double bond equivalences of six were deduced for three double bonds and three ring systems. The geometric isomerism of olefinic protons was established by the greater coupling constants (*J*) of 9.4(H-15) and 11.6(H-16) Hz that validated

the *trans*(*E*) configuration of vinyl group at C-13. The NOE correlations were apparent between δ 6.59(H-1)/2.21(H-19)/2.79(H-5), which depicted that these were in the identical plane and *α*-orientated. Other NOESY cross-peaks among δ 2.01(H-13)/5.82(H-15)/1.88(H-8)/1.02(H-17) indicated that these groups were located in an identical plane of reference and *β*-oriented.

The LCMS spectrum of the compound (**2**) formed a sodium adduct (M+Na) peak at *m/z* 295.2 ($C_{19}H_{28}ONa^{\bullet+}$) The molecular ion peak appeared to be at *m/z*272.2($C_{19}H_{28}O^{\bullet+}$) that undergoes elimination of methyl group and doublebond to form octahydro trimethyl phenanthrenone which appeared at *m/z*246.2 ($C_{17}H_{26}O$).

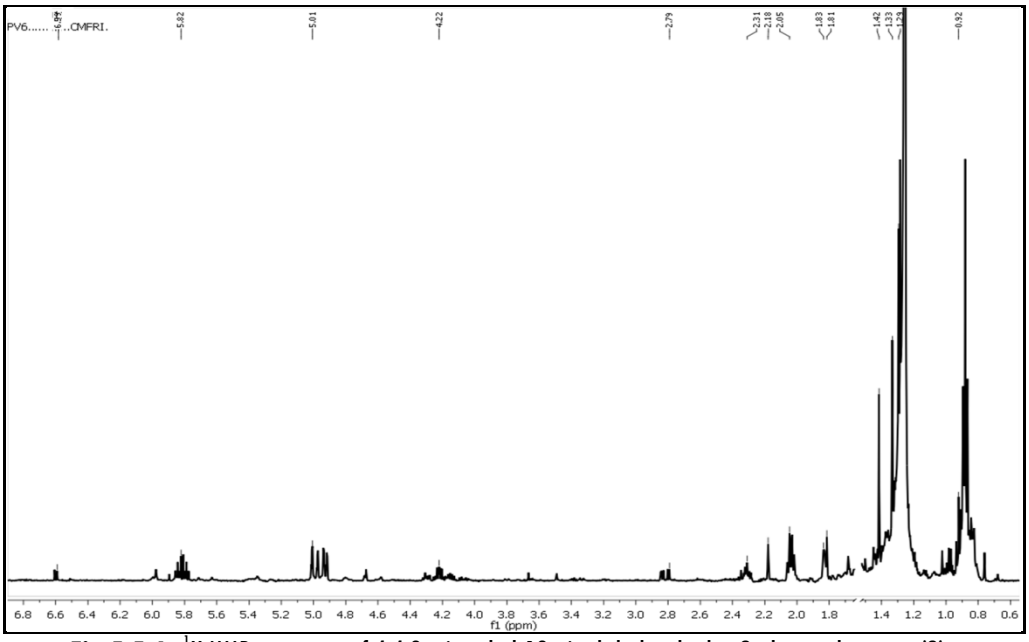


Fig 5.5.A. ^{1}H NMR spectrum of 4,4,9-trimethyl-13-vinyl-dodecahydro-2-phenanthrenone (2).

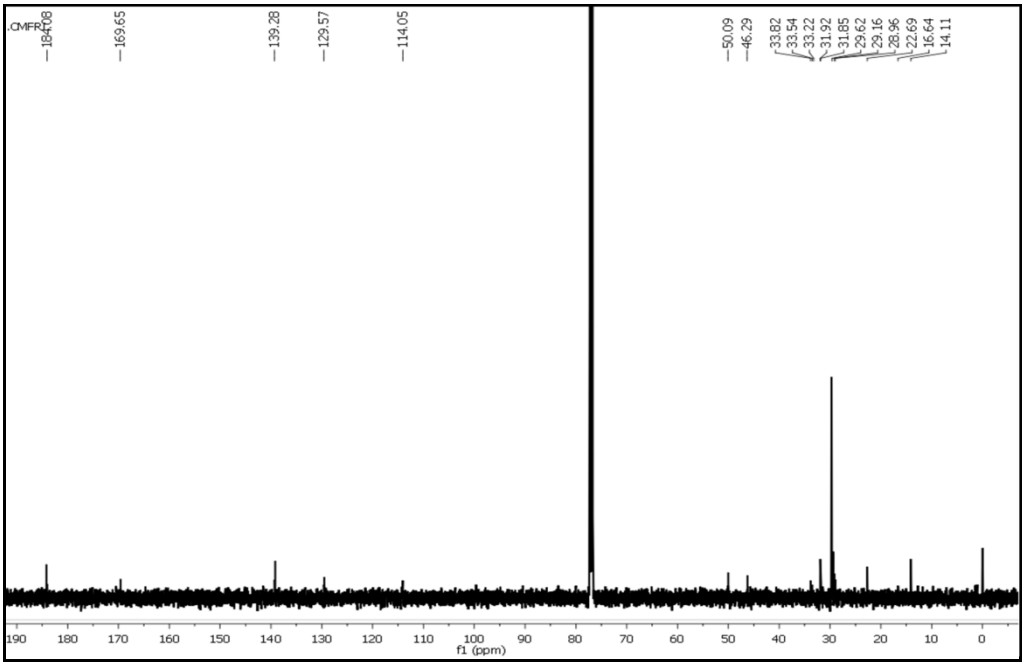


Fig 5.5.B. ^{13}C NMR spectrum of 4,4,9-trimethyl-13-vinyl-dodecahydro-2-phenanthrenone (2).

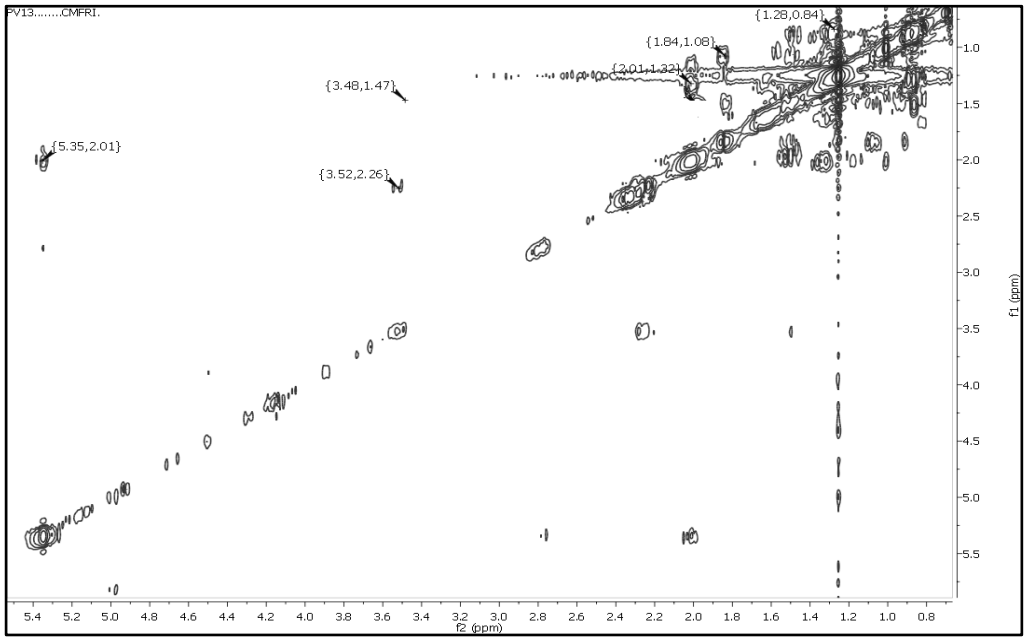


Fig 5.5.C. $^{1}H-^{1}H$ COSY NMR spectrum of 4,4,9-trimethyl-13-vinyl-dodecahydro-2-phenanthrenone (2).

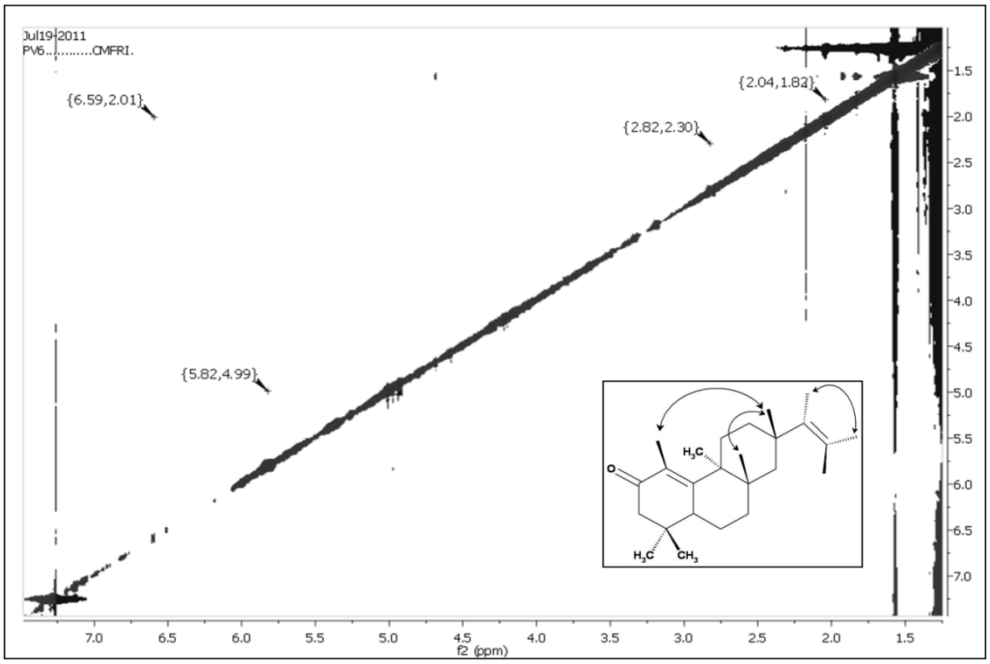


Fig 5.5.D. NOE spectrum of 4,4,9-trimethyl-13-vinyl-dodecahydro-2-phenanthrenone (2).

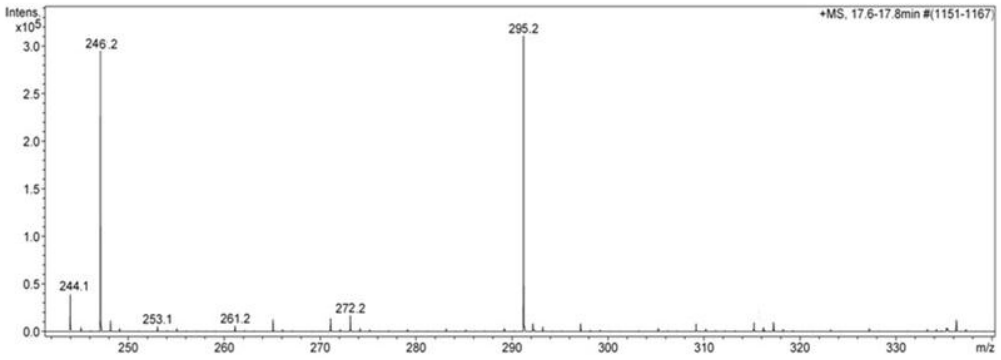


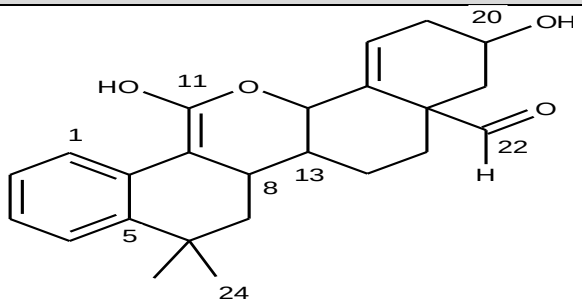
Fig 5.5.E. Mass spectrum of 4,4,9-trimethyl-13-vinyl-dodecahydro-2-phenanthrenone (2).

5.2.1.2C Chromatographic purification and spectral analyses of 11,20-dihydroxy-6,6-dimethyl-decahydro-5*H*-benzo[*h*]naphtho[1,2-c]chromene-16-carbaldehyde (3)

The compound 11,20-dihydroxy-6,6-dimethyl-decahydro-5H-benzo[h] naphtho[1,2-c]chromene-16-carbaldehyde (**3**) was isolated as yellowish crystalline solid upon repeated chromatography over silica columns/TLC. The ultraviolet absorbance at λ_{max} (log ε) 252 nm (1.61) and its mass spectrum exhibited a molecular ion peak at 380 (HRESIMS *m/z* 381.4684amu [M+1]), which in combination with its ^{1}H and ^{13}C NMR data (**Table 5.5**) indicated the molecular formula as $C_{24}H_{28}O_4$ (11,20-dihydroxy-6,6-dimethyl-decahydro-5H-benzo[h]naphtho[1,2-c]chromene-16-carbaldehyde) with 11 degrees of unsaturation. Four degrees of unsaturation were due to the aromatic cyclic ring system and three degrees of unsaturation were from double bonds, and the remaining four degrees of unsaturation were due to the cyclic ring systems.

Table 5.5 NMR spectroscopic data of 11,20-dihydroxy-6,6-dimethyl-decahydro-5H-benzo[h]naphtho[1,2-c]chromene-16-carbaldehyde (**3**) isolated from *Perna viridis*

Compound 3



Position No	^{13}C NMR	δ^{1}H NMR (int., mult., J in Hz)[b]	^{1}H ^{1}H COSY	HMBC(^{1}H-^{13}C)
1	130.9	7.46 (dt, J = 6.1,1H),	2-H	C-2,3
2	130.02	7.46 (dt, J = 6.1,1H),	-	-
3	129.94	7.65 (dd, J = 5.7,1H)	4-H	C-1,4
4	128.84	7.65 (dd, J = 5.7,1H)	-	-
5	153.13	-	-	-
6	33.94	-	-	-
7	31.93	2.29(m,2H)	8-H	C-6,8
8	34.12	4.10(m,1H)	13-H, 9-H	C-10,13
9	99.99	-	-	-
10	144.42	-	-	-
11	160.88	-	-	-
12	69.9	4.24(dd,1H)	13-H	C-13
13	29.7	3.66(m,1H)	14-H	C-7,14
14	14.11	0.88(m,2H)	15-H	C-15
15	22.69	1.54(m,2H)	-	C-13,16,22
16	59.68	-	-	-
17	107.09	-	-	-
18	129.73	5.26(dd,1H)	19-Ha	C-19
19	24.7	1.94(t,1H)2.01(m,1H)	-	C-18,22
20	65.04	4.08(m,1H)	20-H	C-19
21	27.23	2.24(m,2H)	-	C-22,19
22	179.25	-	20-H	-
23	29.36	1.23(m,3H)	-	C-7,8
24	29.07	1.25(m,3H)	-	C-4

[a] NMR spectra recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometers.

[b] Values in ppm, multiplicity and coupling constants (J¼ Hz) were indicated in parentheses. Assignments were made with the aid of the ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY experiments.

Aromatic proton signals were recorded at δ 7.46 and 7.65 with total proton integral of four, which validated the presence of di-substituted aromatic ring. Aromatic carbon signals were registered at δ 130.90(C-1), 130.02(C-2), 129.94(C-3) and 128.84(C-4), which were corresponded to methine carbons, whereas the resonances at δ 153.13(C-5) and 144.42(C-10) were attributed to the aromatic quaternary carbons as deduced from the combined ^{13}C and ^{135}DEPT spectral data. The combined ^{1}H, ^{13}C and DEPT NMR experiments recorded the occurrences of twenty-four carbon atoms with five each of methylenes and methines, along with two methanes (singlets) and seven quaternary carbons. The olefinic signals were apparent at δ 99.99(C-9), 160.88(C-11), 107.09(C-17) and δC 129.73(C-18)/δH 5.26. The highly deshielded carbon at δ 160.88(C-11) represented a directly linked electronegative group in its vicinity. Hence, the four valences of carbon at C-11 were satisfied with the highly electronegative oxygen, hydroxyl and alkenic bonds {>C-9=C-11(-O-)-OH}. The hydroxymethyl at C-20 was attributed to δ 4.08, which displayed HSQC correlation with δC 65.04, and this greater chemical shift was because of the presence of adjacent alkene at C-18. The methine at δ 4.10(H-8) and 3.66(H-13) were characteristic to the junction point of a cyclic moiety. An aldehyde group was apparent at the ring junction, C-16 with δ 59.68 and carbonyl carbon of aldehyde part {-C(=O)H} was recorded at δ 179.25(C-22), which was supported by HMBCs from δ 1.54(H-15) to δ 179.25(C-22); δ 1.94(H-19) to δ 179.25(C-22) and δ 2.24(H-21) to δ 179.25(C-22). The two singlet methyl groups were recorded at δH 1.23/δC 29.36 and δH 1.25/δC 29.07, which were linked at δ 33.94(C-6). It exhibited four significant spin systems, such as δ 7.46(H-1)/7.65(H-2) and δ 7.65(H-

3)/7.46(H-4) for aromatic ring, δ 2.29(H-7)/4.10(H-8)/3.66(H-13)/4.24(H-12) along with δ 3.66(H-13)/0.88(H-14)/1.54(H-15) and δ 5.26(H-18)/1.94(H-19)/4.08(H-20)/2.24(H-21), appropriately supported the presence of tetracyclic ring system. The HMBC resonances from δ 4.10(H-8) to δ 144.42(C-10), 29.70(C-13); δ 3.66(H-13) to δ 31.93(C-7); δ 1.54(H-15) to δ 29.70(C-13); δ 2.24(H-21) to δ 24.70(C-19); δ 1.23(H-23) to δ 31.93(C-7) and δ 1.25(H-24) to δ 29.07(C-4) further supported the structural. Hence, the eleven degrees of hydrogen deficiencies were deduced for one aromatic ring, threedouble bonds and four cyclic rings. The NOE couplings among δ 0.88(H-14)/4.08(H-20) depicted that these were in indistinguishable plane of orientation, and therefore, were designated to be *α*-orientated. The hydroxyl at C-20 was located at *β*-position being opposite to *α*-proton at C-20. Other NOESY correlations among δ 4.10(H-8)/5.26(H-18)/4.24(H-12) indicated that these were aligned in same plane of reference, and therefore, these protons were *β*-oriented.

The LCMS spectrum of the compound 11,20-dihydroxy-6,6-dimethyl-decahydro-5H-benzo[h]naphtho[1,2-c]chromene-16-carbaldehyde (**3)** formed an sodium adduct (M+Na) peak at *m/z* 403.8 ($C_{24}H_{28}O_4Na$). The molecular ion peak appeared to be at *m/z*380.5($C_{24}H_{28}O_4$ $^{\bullet+}$), undergo elimination of hydroxyl groups and aldehyde to form daughter ion at *m/z*364.5 ($C_{24}H_{28}O_3$) and *m/z*320.5 ($C_{23}H_{28}O$).The base peak was found to be at *m/z* 292.4 ($C_{21}H_{24}O$) indicates the presence of be as benzo naphtho chromene fragment.

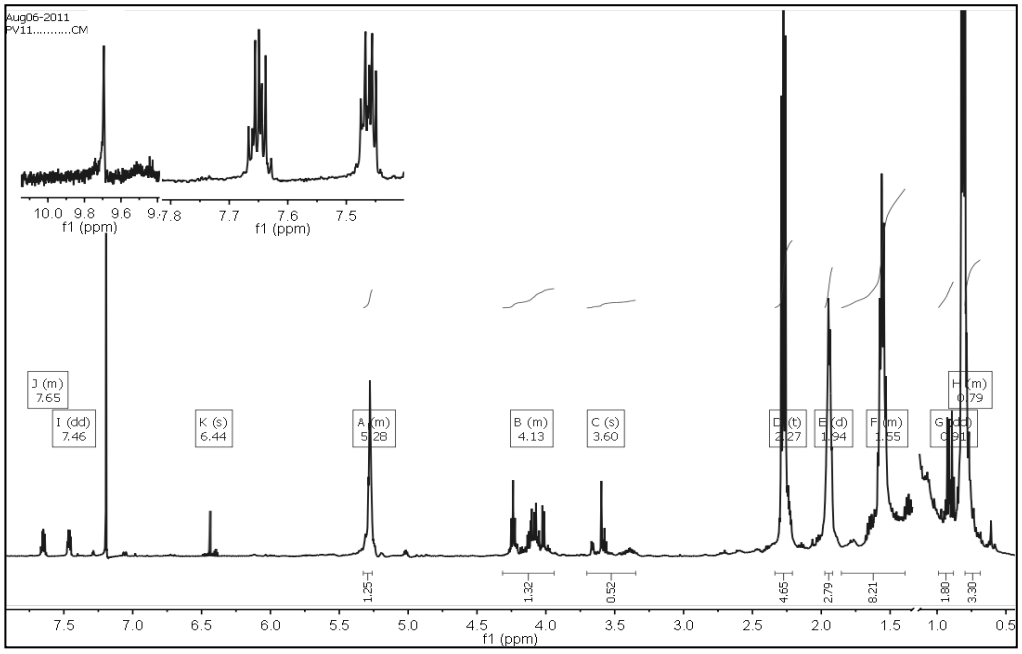


Fig 5.6.A. ^{1}H NMR spectrum of 11,20-dihydroxy-6,6-dimethyl-decahydro-5H-benzo[h] naphtho[1,2-c]chromene-16-carbaldehyde (**3**)

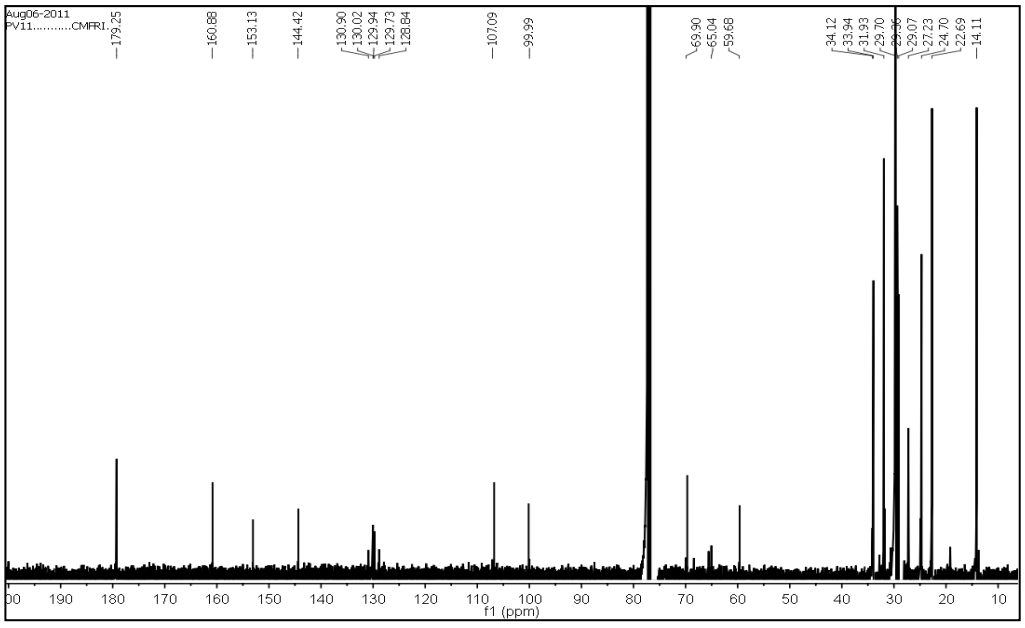


Fig 5.6.B. ^{13}C NMR spectrum of 11,20-dihydroxy-6,6-dimethyl-decahydro-5H-benzo[h]naphtha [1,2-c]chromene-16-carbaldehyde (**3**).

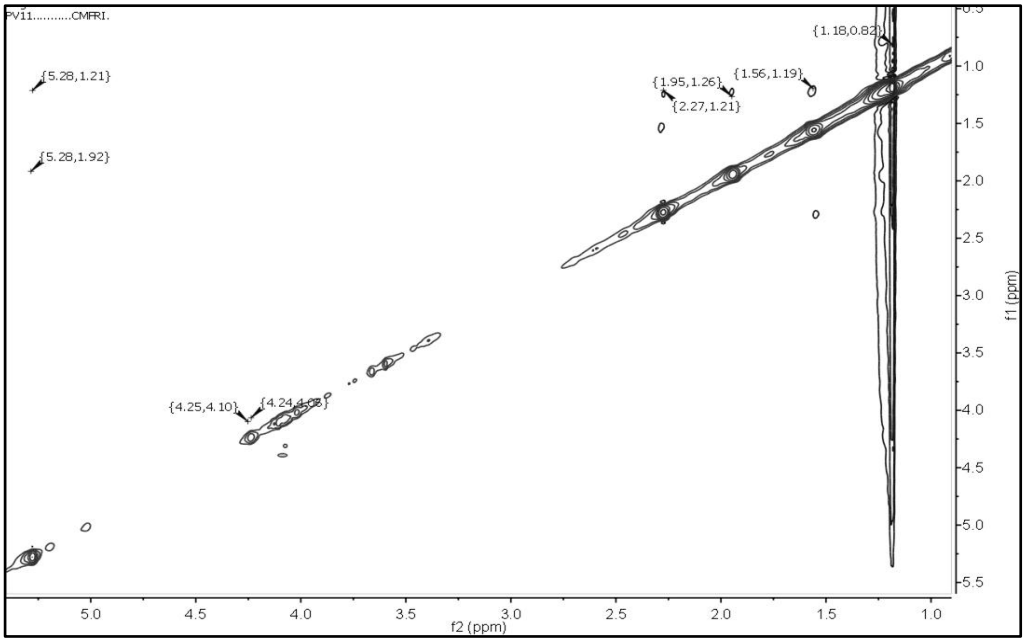


Fig 5.6.C. NOE spectrum of 11,20-dihydroxy-6,6-dimethyl-decahydro-5H-benzo[h]naphtho[1,2-c] chromene-16-carbaldehyde (**3**).

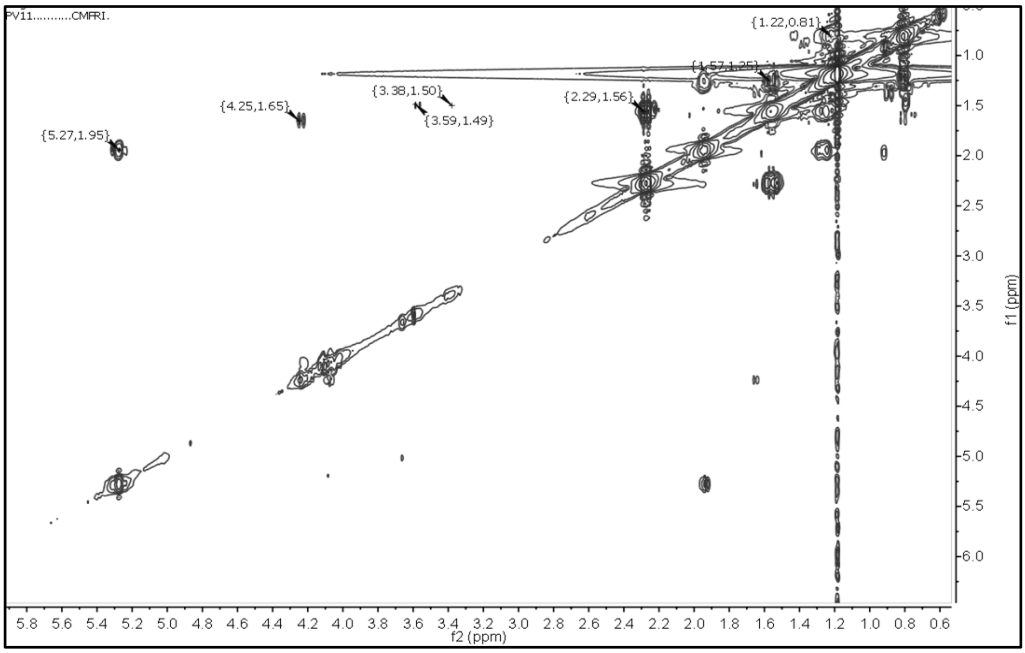


Fig 5.6.D. $^{1}H—^{1}H$ COSY spectrum of 11,20-dihydroxy-6,6-dimethyl-decahydro-5H-benzo [h]naphtho[1,2-c]chromene-16-carbaldehyde (**3**).

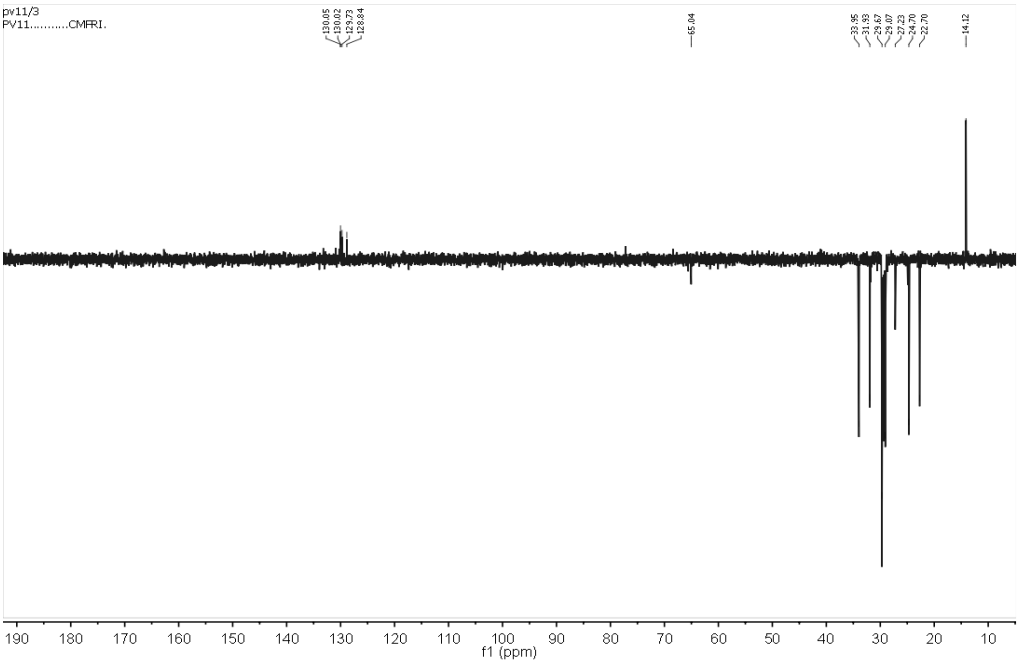


Fig 5.6.E. DEPT NMR spectrum of 11,20-dihydroxy-6,6-dimethyl-decahydro-5H-benzo[h] naphtho[1,2-c]chromene-16-carbaldehyde (**3**).

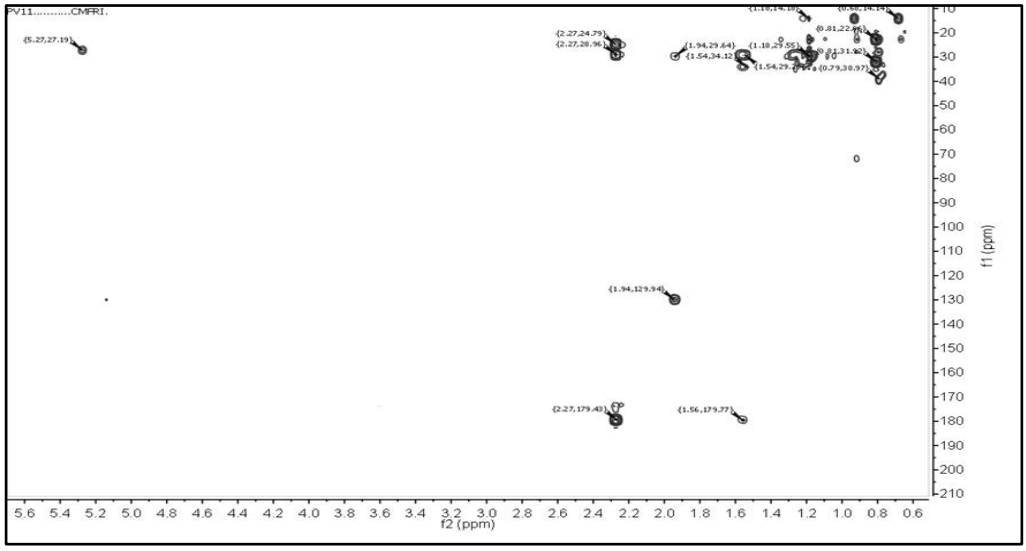


Fig 5.6.F. HMBC NMR spectrum of 11,20-dihydroxy-6,6-dimethyl-decahydro-5H-benzo[h] naphtho[1,2-c]chromene-16-carbaldehyde (**3**).

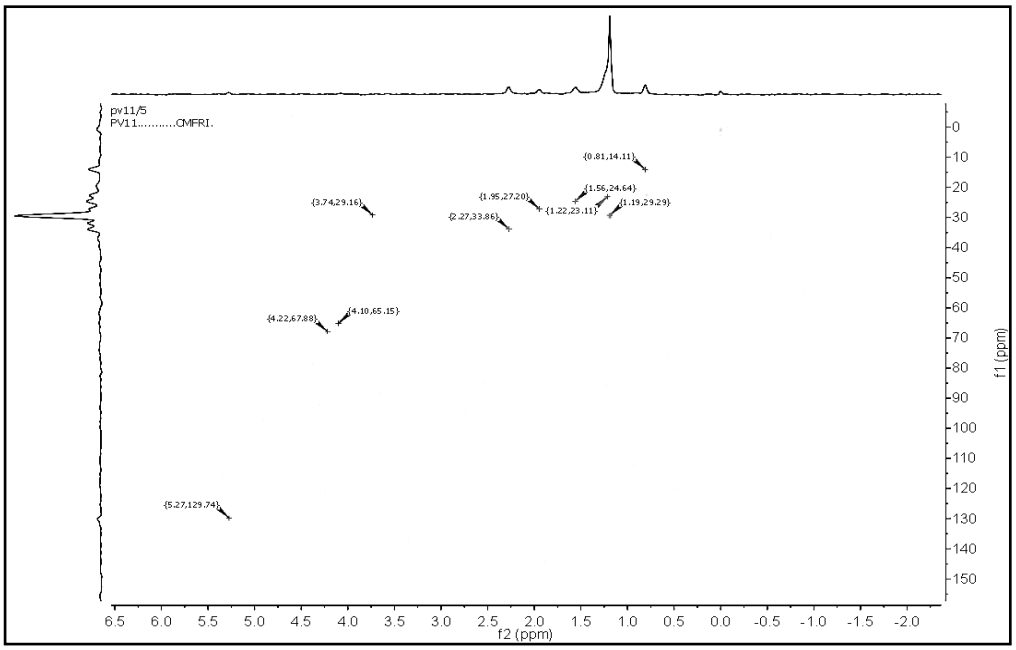


Fig 5.6.G. HSQC NMR spectrum of 11,20-dihydroxy-6,6-dimethyl-decahydro-5H-benzo[h] naphtho[1,2-c]chromene-16-carbaldehyde (**3**).

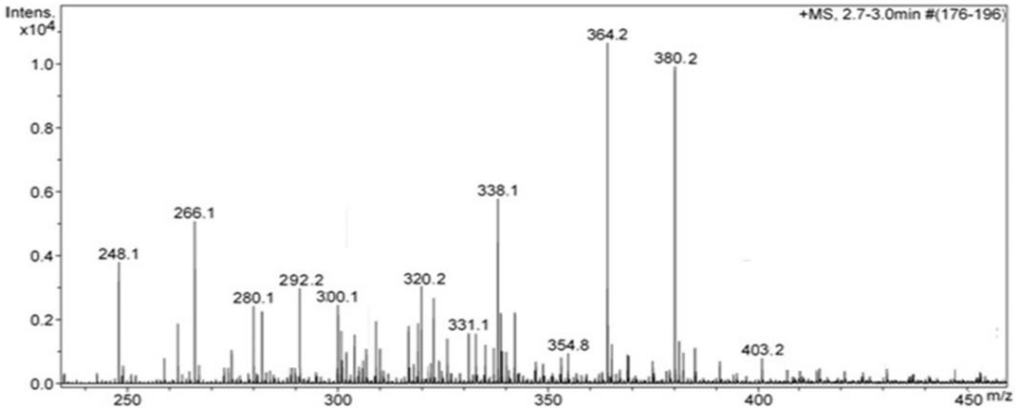


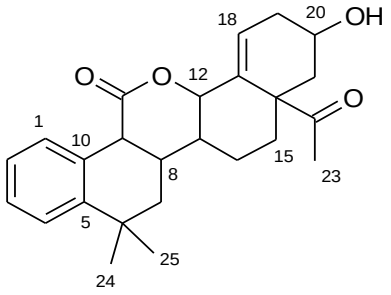
Fig 5.6.H. Mass spectrum of 11,20-dihydroxy-6,6-dimethyl-decahydro-5H-benzo[h]naphtho [1,2-c] chromene-16-carbaldehyde (3).

5.2.1.2D Chromatographic purification and spectral analyses of 16-acetyl-20-hydroxy-6,6-dimethyl-dodecahydro-5*H*-benzo[*h*]naphtho[1,2-c]chromen-12-one (4)

The compound 16-acetyl-20-hydroxy-6,6-dimethyl-dodecahydro-5H-benzo[h]naphtho[1,2-c]chromen-12-one (**4)** isolated aspale yellow powder and showed close structural similarity with 11,20-dihydroxy-6,6-dimethyl-decahydro-5H-benzo[h]naphtho[1,2-c]chromene-16-carbaldehyde (**3**) except the additional carbonyl group in third ring in compound **4** and the extra peak at H-23(2.35) signifying the methyl singlet attached to the carbonyl group. Mass spectrum displayed the molecular ion peak at 394.3 (HRESIMS *m/z* 394.5682 amuwhich in combination with its ^{1}H and ^{13}C NMR data (**Table 5.6**) indicated the molecular formula as $C_{25}H_{30}O_4$with 11 degree of unsaturation.

Table 5.6. NMR spectroscopic data of 16-acetyl-20-hydroxy-6,6-dimethyl-dodecahydro-5H-benzo[h]naphtho[1,2-c]chromen-12-one

Compound 4



Position No	^{13}CNMR	δ^{1}H NMR (int., mult., J in Hz)[b]	^{1}H-^{1}H COSY	HMBC(^{1}H-^{13}C)
1	128.84	7.71(m)	2-H	C-2,3
2	130.94	7.53(m)	-	-
3	130.9	7.53(m)	-	C-1,4
4	128.03	7.71(m)	4-H	-
5	120.98	-	-	-
6	48.78	-	-	-
7	33.15	2.32(dt)	8-H	C-6,8,10
8	62.13	4.14(m)	13-H, 9-H	C-11,9,12
9	68.79	5.26(s)	8-H	C-11,7,13
10	120	-	-	-
11	173.31	-	-	-
12	52.7	4.30(m)	13-H	C-11,18,14
13	62.12	3.66(d)	14-H	C-7,14
14	34.73	1.73(m)	15-H	C-13,12,17
15	31.9	1.32(t)	-	C-16,22
16	37.8	-	-	-
17	114	-	-	-
18	129.9	5.34(t)	19-Ha	C-19,20,16
19		2.01(dd)		
	27.21	1.92(m)	20-H	C-18,22,20
20	61.2	4.09(m)	-	C-19
21	24	1.56(s)	20-H	C-22
22	179.81	-	-	-
23	34.24	2.35(s)	-	-
24	22.71	1.23(s)	-	C-7,5,8
25	21.1	1.25(s)	-	C-7

[a] NMR spectra recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometers.

[b] Values in ppm, multiplicity and coupling constants (J¼ Hz) were indicated in parentheses. The assignments were made with the aid of the 1H-1H COSY, HSQC, HMBC and NOESY experiments.

The structure of the studied compound was found to be similar with **3** except the acetyl side chain at C-16 in **4**,whereas, the carbaldehyde moiety was positioned at C-16 in **3**. The aromatic proton signals were displayed at δ 7.71 and 7.53 with total proton integral of four, which appropriately validated the occurrence of di-substituted aromatic ring. Aromatic methines were registered at δ 128.84(C-1), 130.94(C-2), 130.90(C-3) and 128.03(C-4), whereas the aromatic quaternary carbons were recorded at δ 120.98(C-5) and 120.01(C-10). The ^{13}C and ^{135}DEPT experiments recorded 24 carbons with five methylenes, ten methines, three methanes (singlets) and seven quaternary carbons. The proton at δ 4.09 (HSQC with δC 61.20) was due to the presence ofhydroxylatedmethine (-CH-OH) at C-20 and this higher chemical shift was attributed to the adjacent alkene at C-18 (δH 5.34/δC 129.90). The acetyl {-C(=O)-$\underline{C}H_3$} group was recorded at C-16(δ 37.80) and carbonyl carbon of ketone was found at δ 179.81(C-22). This was supported by the HMBCs from δ 2.01(H-19) to δ 179.81(C-22); δ 1.56(H-21) to δ 179.81(C-22) and 1.32(H-15) to δ 179.81(C-22). The singlet methyl at δ 2.35 correlated to δ 34.24(C-23), was ascribed to be a part of acetyl moiety and its attachment at C-16 was corroborated by the HMBCs from δ 2.35(H-23) to δ 179.81(C-22) and δ 1.56(H-21) to δ 34.24(C-23). The singlet methyls at δH 1.23/δC 22.71 and δH 1.25/δC 21.10 were linked at the quaternary carbon of δ 48.78 at C-6. The assignments were made according to long-range coupling values at δ 1.23(H-24) to δ 33.15(C-7), 120.98(C-5), 62.13(C-8) and δ 1.25(H-25) to δ 33.15(C-7). Four significant spin systems were validated by COSYs at δ 7.71(H-1)/7.53(H-2) and δ 7.53(H-3)/7.71(H-4) for aromatic ring, δ 2.32(H-7)/4.14(H-8)/5.26(H-9), 3.66(H-13) along with δ 4.30(H-12)/3.66(H-13)/1.73(H-14)/1.32(H-15) and δ 5.34(H-18)/2.01, 1.92(H-19)/4.09(H-20)/1.56(H-21) for the tetracyclic moiety in **4** (Fig. 2C). The HMBCs from δ 2.32(H-7) to δ

48.78(C-6), 120.01(C-10); δ 4.14(H-8) to δ 173.31(C-11), 52.70(C-13); δ 5.26(H-9) to δ 33.15(C-7), 173.31(C-11), 62.12(C-12); δ 4.30(H-12) to δ 34.73(C-14), 129.90(C-18); δ 1.73(H-14) to δ 114.00(C-17); δ 5.34(H-18) to δ 61.20(C-20), 37.80(C-16) and δ 1.56(H-21) to δ 179.81(C-22) further supported the structural attributions. Hence, the double bond equivalences of eleven were deduced for one aromatic ring, threedouble bonds and four rings. The NOE couplings were displayed between the protons at δ 1.73(H-14)/4.09(H-20)/5.26(H-9), which represented that these protons were in same plane of orientation, and considered as *α*-orientated. The hydroxyl group at C-20 was *β*-disposedbeing opposite to *α*-proton at C-20. Other NOESYs among δ 4.14(H-8)/5.34(H-18)/4.30(H-12) indicated that these groups were located in the identical plane of reference, and opposite to *α*-orientated protons, thus, considered as *β*-aligned.

The LCMS spectrum of the compound 4, 16-acetyl-20-hydroxy-6,6-dimethyl-dodecahydro-5H-benzo[h]naphtho[1,2-c]chromen-12-one (**4) s**pectra are presented in **Fig 5.7**.**Its** molecular ion peak was appeared at *m/z*394.2($C_{25}H_{30}O_4$ $^{\bullet+}$).

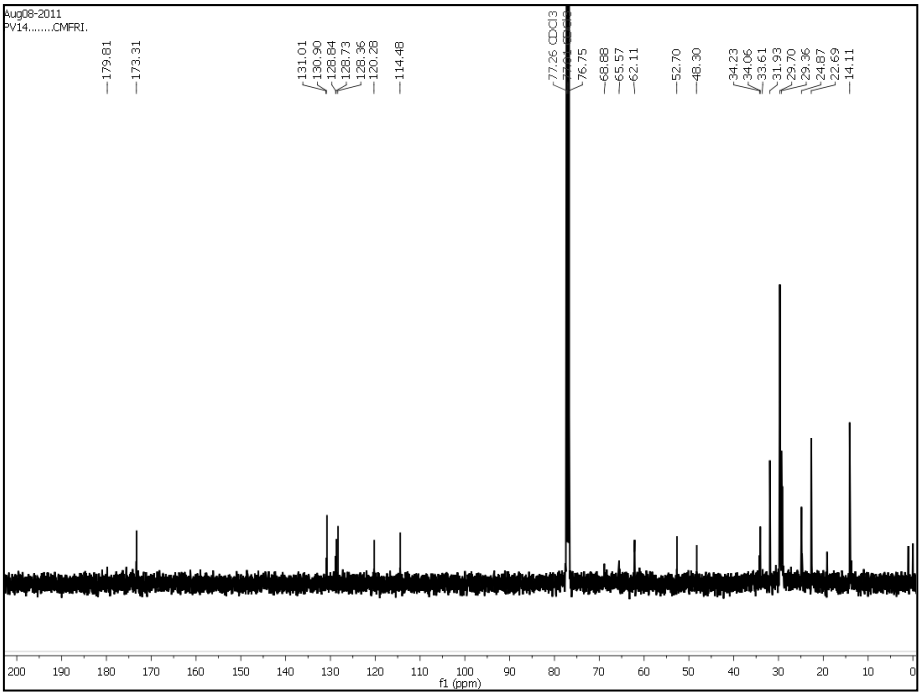


Fig 5.7. A. ^{13}C NMR spectrum of 16-acetyl-20-hydroxy-6,6-dimethyl-dodecahydro-5H-benzo[h] naphtho[1,2-c]chromen-12-one (**4**).

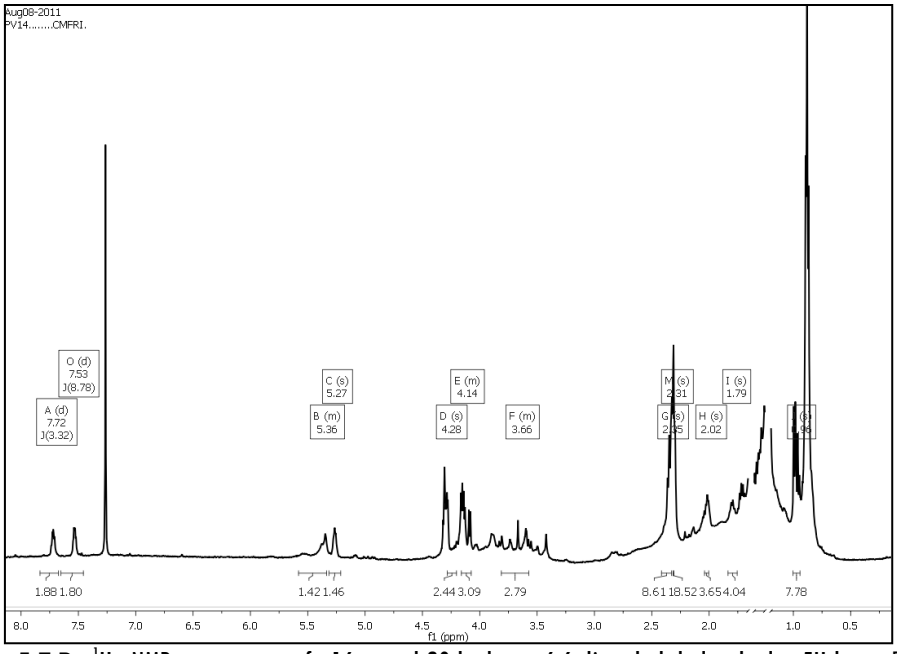


Fig 5.7.B. ^{1}H NMR spectrum of 16-acetyl-20-hydroxy-6,6-dimethyl-dodecahydro-5H-benzo[h] naphtho[1,2-c]chromen-12-one (**4**).

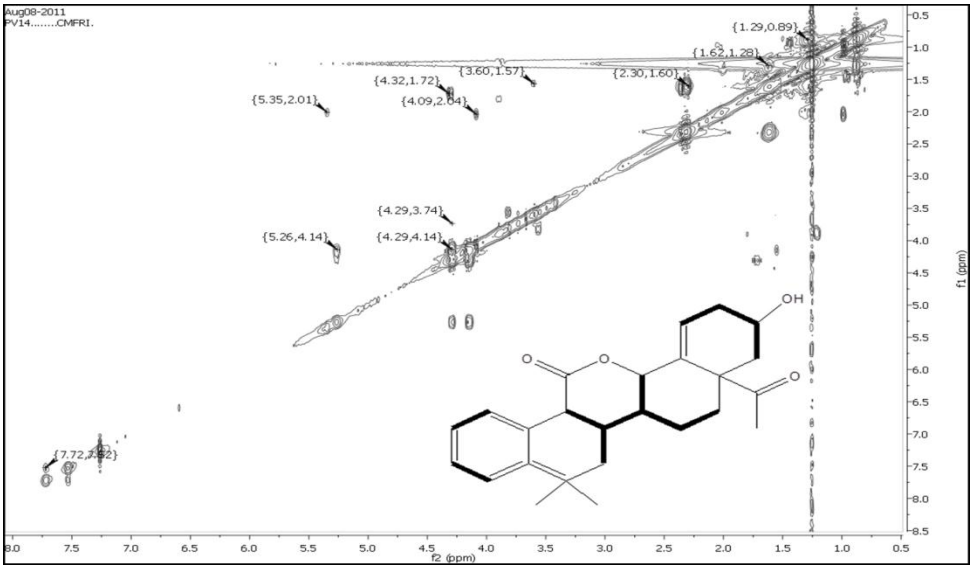


Fig 5.7.C. ^{1}H–^{1}H COSY spectrum of 16-acetyl-20-hydroxy-6,6-dimethyl-dodecahydro-5H-benzo[h]naphtho[1,2-c]chromen-12-one (**4**).The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds.

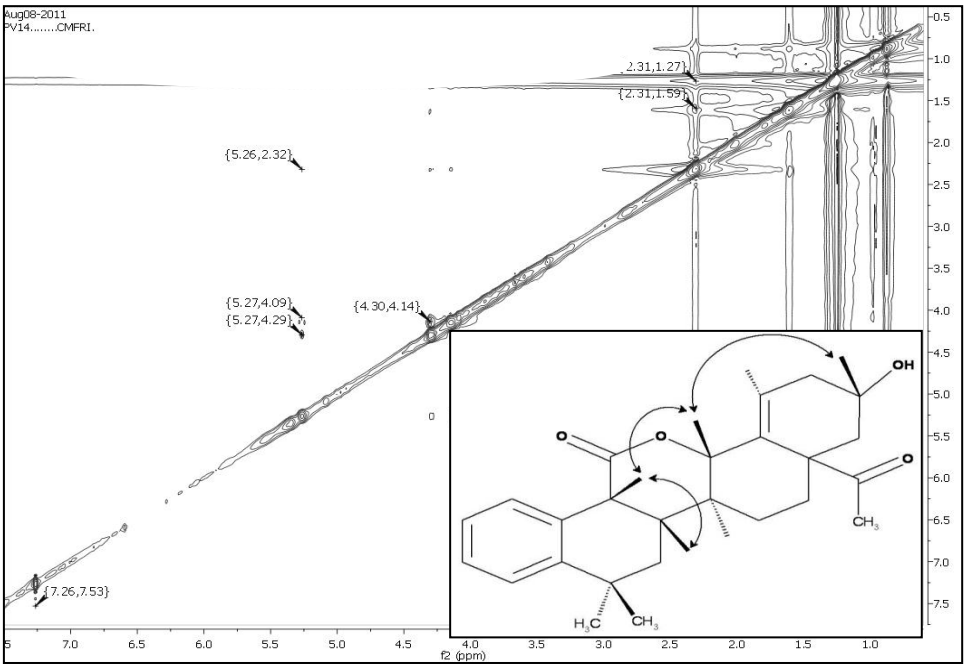


Fig 5.7.D. NOE NMR spectrum of 16-acetyl-20-hydroxy-6,6-dimethyl-dodecahydro-5H-benzo[h]naphtho[1,2-c]chromen-12-one (**4**).

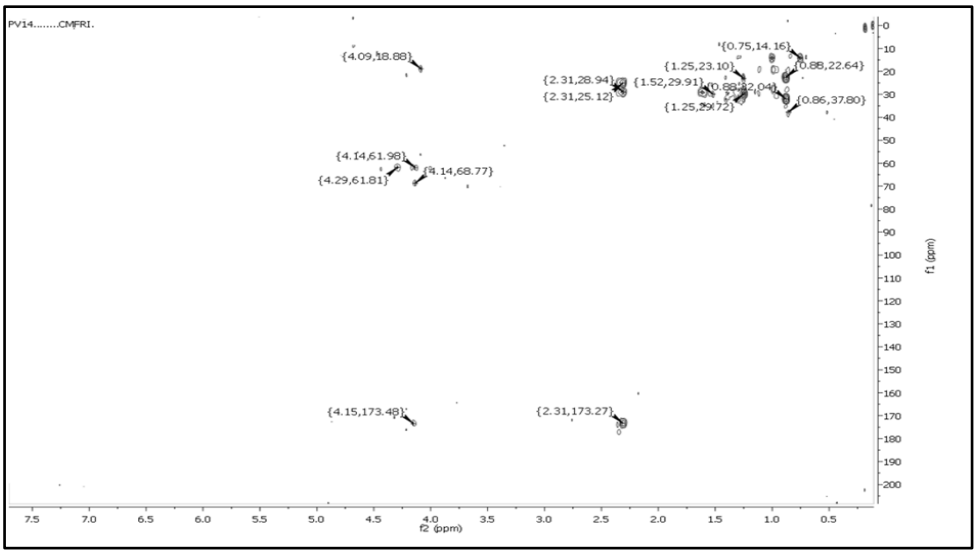


Fig 5.7.E. HMBC NMR spectrum of 16-acetyl-20-hydroxy-6,6-dimethyl-dodecahydro-5H-benzo[h] naphtho[1,2-c]chromen-12-one (**4**).

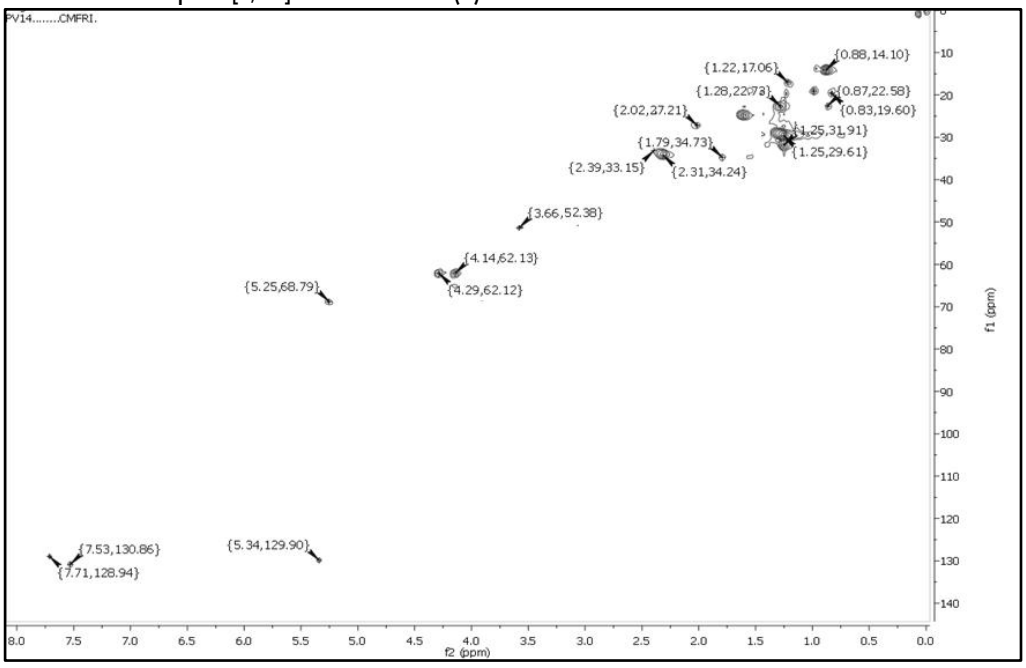


Fig 5.7.F HSQC NMR spectrum of 16-acetyl-20-hydroxy-6,6-dimethyl-dodecahydro-5H-benzo[h] naphtho[1,2-c]chromen-12-one (**4**).

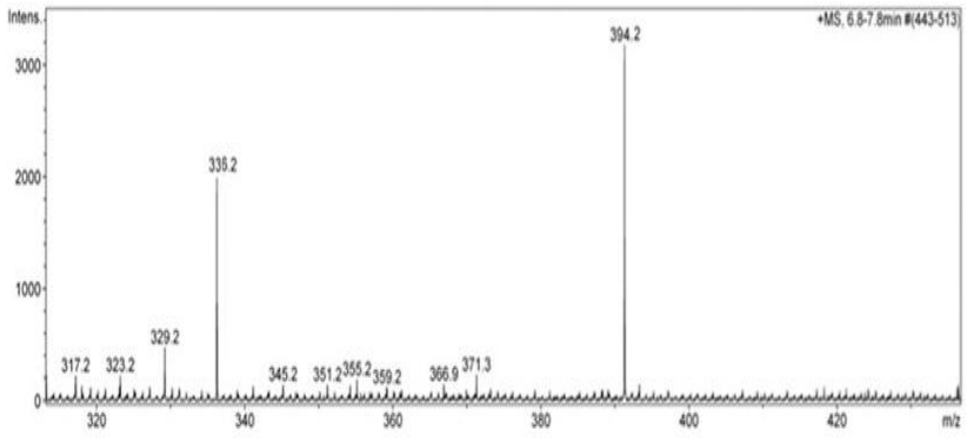


Fig 5.7.G. Mass spectrum of 16-acetyl-20-hydroxy-6,6-dimethyl-dodecahydro-5H-benzo[h] naphtho[1,2-c]chromen-12-one.

5.2.1.2E Chromatographic purification and spectral analyses of cholest-5-en-3-3-yl-(30-hydroxy-3-methyl-36-methyleneundeca-30*E*,34*E*-dienoate) (5)

The compound cholest-5-en-3β-3-yl-(30-hydroxy-3-methyl-36-methyleneundeca-30E,34E-dienoate) (**5)**was isolated as a light yellow crystalline solid upon repeated chromatography over silica columns. The mass spectrum of the compound exhibited the molecular ion peak at *m/z* 592 (HRESIMS at *m/z* 592.9562 $[M]^+$) that alongwith the detailed NMR spectroscopic experiments established the molecular formula as $C_{40}H_{64}O_3$.

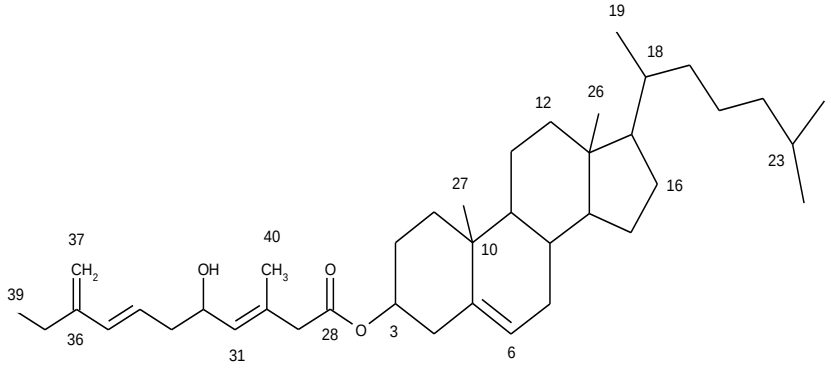
The number of carbon atoms was established as 40 through the combined ^1H, ^{13}C and ^{135}DEPT analyses, which included seven methyls, twelve methines, fifteen methylenes and six quaternary carbons. The alkenic resonances appeared at δ 140.75(C-5) and 121.70(C-6) in the carbon spectrum attributed the presence of a double bond. Significantly higher downfield shift at δC 140.75 suggested that it belonged to a quaternary carbon subsequent to the alkenic bond in a ring system. One highly deshielded signal was found at δ 5.35(H-6) (HSQC with δ 121.70), which was deduced to be a triplet due to the

nearby proton at δ 2.28(C-7) (HSQC with δC 31.92; >C=C<u>H</u>-C<u>H</u>$_2$-). The deshielded proton at δ 3.52(δC 71.80) was due to the presence of methine, which was directly linked to an oxygenated side chain. Two spin systems were recorded in the studied cholestane, wherein two spin systems were accounted for the side chain, which were tabulated by the COSYs, δ 1.28(H-1)/1.97(H-2)/3.52(H-3)/2.30(H-4); δ 5.35(H-6)/2.28(H-7)/1.98(H-8)/0.95(H-9)/1.49(H-11)/1.46(H-12); δ 1.98(H-8)/1.11(H-14)/1.58(H-15)/1.86(H-16)/1.13(H-17)/1.33(H-20)/1.82(H-22), 0.98(H-21) and δ 1.85(H-24)/1.99(H-25)/0.89(H-26), 0.87(H-27) to support the presence of cholestene framework. The three characteristic shielded methyl protons of the sterol framework were appeared at δ 0.68 (s), 1.01 (s) and 0.98 (d), which were due to the carbons at C-18, C-19 and C-21 positions, respectively. The HMBCs from δ 2.30(H-4) to δ 31.66(C-2), 140.75(C-5), 121.70(C-6); δ 5.35(H-6) to δ 42.30(C-4), 31.00(C-8); δ 1.98(H-8) to δ 24.30(C-15); δ 0.95(H-9) to δ 37.27(C-1), 21.10(C-11) and δ 1.01(H-19) to δ 50.15(C-9), 21.10(C-11), 36.51(C-10), 71.80(C-3) further supported the occurrence of C-27 sterol moiety. The 30-hydroxy-3-methyl-36-methyleneundeca-30,34-dienoate chain was attached at C-3 position of the sterol skeleton, and was confirmed by HMBCs from δ 2.38(H-29) to δ 71.80(C-3). The side chain enclosing three spin systems were validated by COSYs from H-31 to H-35 {δ 5.80(H-31)/4.16(H-32)/1.57, 1.03(H-33)/5.17(H-34)/4.32(H-35)} and H-37 to H-38 {δ 1.84(H-37)/0.80(H-38)}. A carbonyl carbon was positioned at δ 173.92(C-28) and hydroxyl attached methine was recorded at δ 65.09, which exhibited C-H correlation with δ 4.16(H-32). The long-range C-H couplings from δ 5.17(H-34) to δ 37.15(C-37); δ 4.32(H-35) to δ 114.06(C-39); δ 4.94(H-39) to δ 37.15(C-37), 14.11(C-38) and δ 2.17(H-40) to δ 138.10(C-30) deduced the presence of side chain. The greater coupling constants at H-34(J=8.3 Hz) and H-35(10.9 Hz)

supported the *trans* (*E*) orientation of double bond at -CH(34)=CH(35)- position. Hence, the double bond equivalences of nine were deduced for five double bonds and four ring skeletons. The NOESY correlations, δ 3.50(H-3)/5.35(H-6)/1.11(H-14)/4.16(H-32) suggested that they were located in same plane, and were considered as α protons. This, in turn, indicated the β-position of hydroxyl at C-3 and C-32. The methyls at H-18 and H-19 of the cholestane were correlated with δ 1.98(H-8) and 1.13(H-17) that apparently attributed their β-orientation.

Table 5.7. NMR spectroscopic data of cholest-5-en-3β-3-yl-(30-hydroxy-3-methyl-36-methyleneundeca-30E,34E-dienoate) (**5**) isolated from *Pernaviridis*

Compound 5



Position No	^{13}C NMR	δ^{1}H NMR (int., mult., J in Hz)^{b}	^{1}H-^{1}H COSY	HMBC(^{1}H-^{13}C)
1	37.27	1.28(m)	2-H	-
2	31.66	1.98(m)	-	C-1,C-3
3	71.8	3.52 (p, J = 11.0, 4.5 Hz, 1H)	1-H,2-H,4-H	-
4	42.3	2.30(m)	-	C-2,1,3,5,6
5	140.75	-	-	-
6	121.7	5.35 (td, J = 5.2, 2.3 Hz)	7-H	C-4,7,8
7	31.92	2.28(m)	8-H	C-5,C-6
8	31	1.98(m)	14-H	C-9, C-15
9	50.15	0.95(m)	11-H	C-1,C-11
10	36.51	-	-	-

11	21.1	1.49(m)	12-H	C-5,6,9,12
12	39.8	1. 46(m)	11-H	C-9,C-13
13	42.33	-	-	-
14	56.78	1.11	8-H	C-26
15	24.3	1.58(m)	16-H	C-13
16	28.24	1.86(m)	17-H,15-H	C-15,C-18,C-20
17	56.16	1.13	18-H	C-13
18	35.79	1.33	19-H	C-22,C-16
19	18.73	0.98(d)	20-H	C-20
20	36.2	1.82(tq)	-	-
21	23.84	1.80 (tq)	22-H	C-24,20,23
22	39.53	1.85(tq)	23-H	C-24
23	28.02	1.99	25-H,24-H	C-25,24
24	22.56	0.89	23-H	C-25
25	22.69	0.87(m)	23-H	C-24
26	11.87	0.68(s)	-	C-17,13,12
27	19.4	1.01(s)	-	C-9,11,10,3
28	173.92	-	-	-
29	34.19	2.38(d) 2.27(m)	-	C-28
30	138.1	-	-	-
31	126.73	5.80(dd)	32-H	C-32
32	65.09	4.16(m)	33-H	
33	24.2	1.57(m) , 1.03(m)	34-H	C-34
34	131.82	5.17(q)	35-H	C-38
35	106	4.32(d)	-	C-37
36	156	-	-	-
37	114.06	4.94(ddd)	-	C-39,38
38	37.15	1.84(m)	39-H	
39	14.11	0.80(t)	38-H	C-40
40	31.07	2.17(s)	-	C-30

[a] NMR spectra were recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometers. [b]Values in ppm, multiplicity and coupling constants (J¼ Hz) were indicated in parentheses. Assignments were made with the aid of the ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY experiments.

The ultraviolet absorbance at λmax (log ε) 266 nm (2.61) has been assigned to be a chromophore with multiple olefinic system. The H–H and C–H connectivities apparent in the ^{1}H–^{1}H COSY and HMBC spectra indicated nine degrees of unsaturation in **5**. Five degrees of unsaturation from the double bond equivalency and the other was due to a cyclic ring system. The olefinic (C=C), carbonyl (C=O) and ether (C-O-C) groups have been symbolized by the absorption bands at 1642, 1736.96 and 1068 cm^{-1},respectively. The characteristic IR absorption spectra atν_{max}1736.96 cm^{-1} (C=O stretching), 3419.90cm^{-1} (O-H stretching vibration), 2924.18 cm^{-1} (C-H stretching of alkanes), 1545cm^{-1} (C=C stretching), 724.28 cm^{-1} (C-H rocking), 1377.22 cm^{-1} (C-H bending) supported the structure of steroid framework. The ^{1}H, ^{13}C, ^{1}H–^{1}H COSY, HMBC, and NOE spectra were presented in **Fig 5.8**.

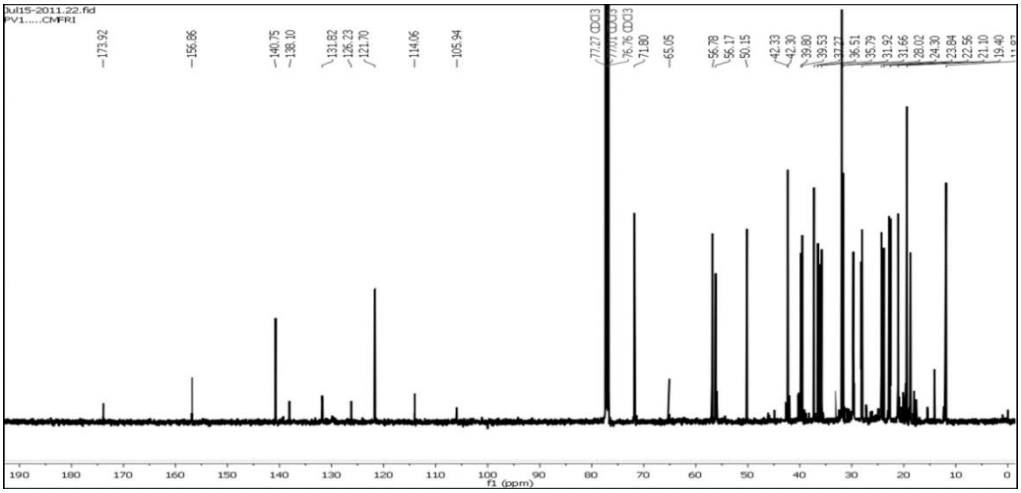


Fig 5.8.A. ^{13}C NMR spectrum of cholest-5-en-3β-3-yl-(30-hydroxy-3-methyl-36-methyleneundeca-30E,34E-dienoate (**5**).

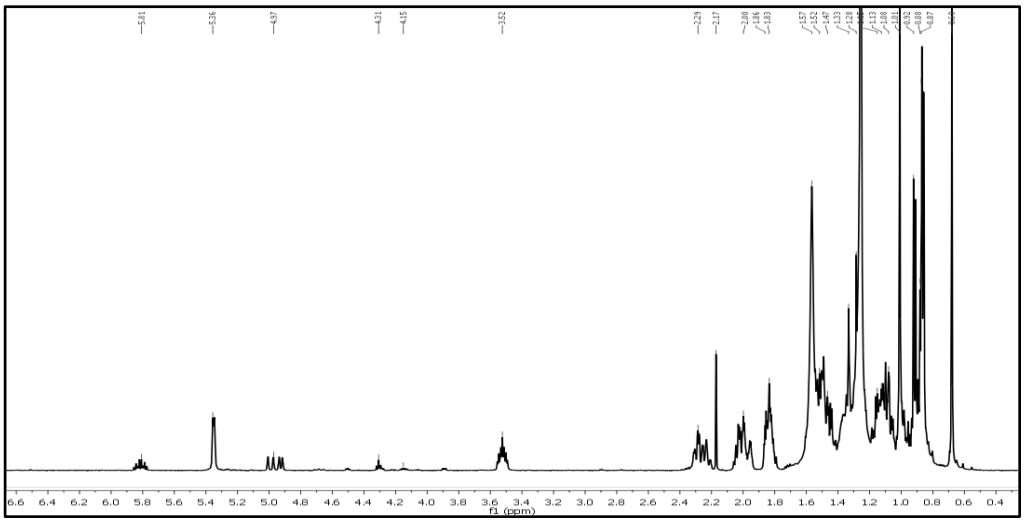


Fig 5.8.B. ^{1}H NMR spectrum of cholest-5-en-3β-3-yl-(30-hydroxy-3-methyl-36-methyleneundeca-30E,34E-dienoate (**5**).

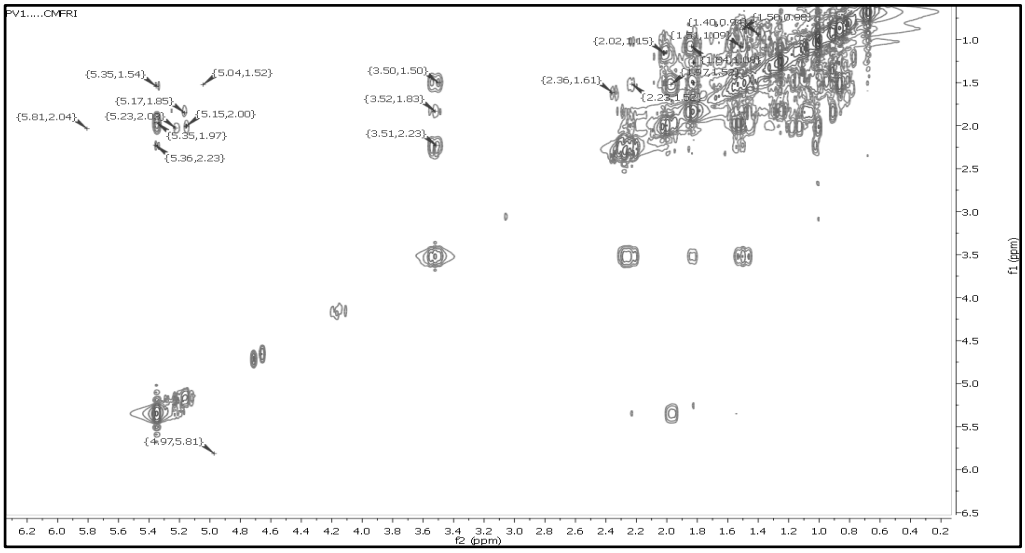


Fig 5.8.C. ^{1}H–^{1}H COSY spectrum of cholest-5-en-3β-3-yl-(30-hydroxy-3-methyl-36-methyleneundeca-30E,34E-dienoate (**5**).

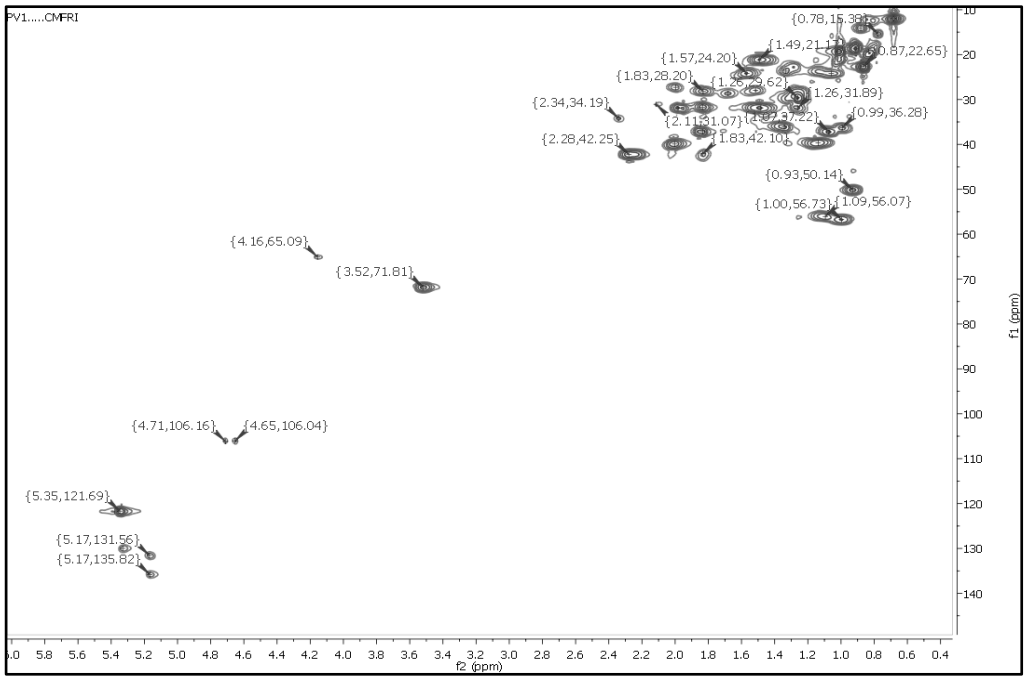


Fig 5.8.D. HSQC Spectrum of cholest-5-en-3β-3-yl-(30-hydroxy-3-methyl-36-methyleneundeca-30E,34E-dienoate (**5**).

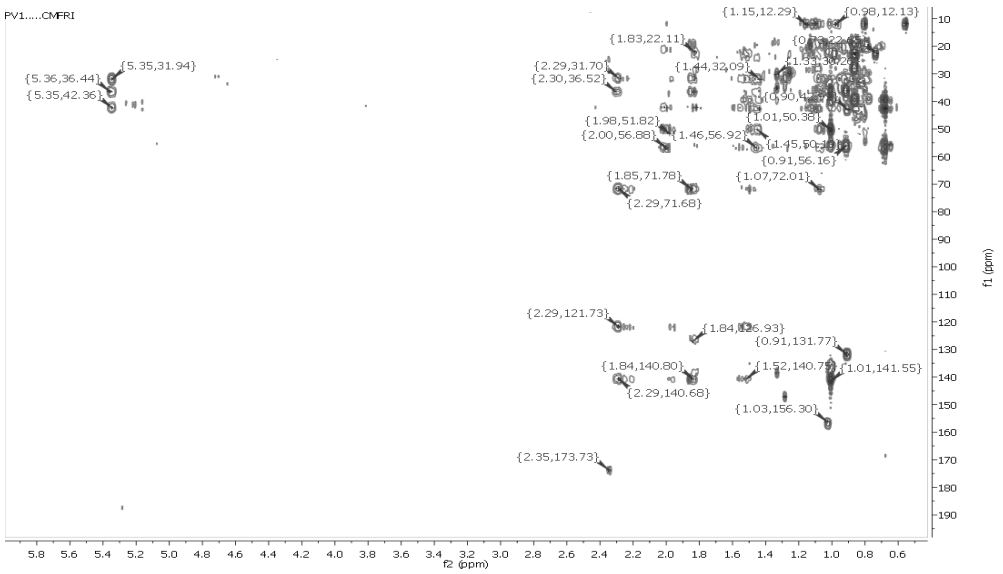


Fig 5.8.E. HMBC spectrum of cholest-5-en-3β-3-yl-(30-hydroxy-3-methyl-36-methyleneundeca-30E,34E-dienoate (**5**).

5.2.1.2F. Chromatographic purification and spectral analyses of cholest-5-en-3β-3-yl-((E)-33-oxooct-31-enoate (6)

The compound cholest-5-en-3β-3-yl-((E)-33-oxooct-31-enoate (**6**) isolated as yellow solid upon repeated chromatography over silica columns/TLC.Its mass spectrum exhibited a molecular ion peak at *m/z* 524(HRESIMS *m/z* 524.8364 which in combination with its ^{1}H and ^{13}C NMR data (**Table 5.8**) indicated the molecular formula as ($C_{35}H_{56}O_3$) as cholest-5-en-3β-3-yl-((E)-33-oxooct-31-enoate with eight degree of unsaturation. Four degree of unsaturation from double bonds and the remaining four degree of unsaturation were due to a cyclic ring system.

The number of carbons was established as 35 through combined ^{1}H, ^{13}C and ^{135}DEPT experiments, which included six methyls, fourteen methylenes, ten methines and five quaternary carbons. Considerably deshielded shift at δC 140.76 was belonged to the quaternary carbon adjacent to an alkenic bond in a cyclic ring. Highly deshielded signal at δ 5.35(H-6) exhibited HSQC correlation with δ 121.72, and was found to be a triplet due to the nearby proton at δ 2.29(C-7) bonded to δC 31.92 (>C=C<u>H</u>-C<u>H</u>$_2$-). The deshielded proton at δ 3.52 was belonged to the methine with δC 71.81, which confirmed that it was directly linked to an oxygenated end. The presence of two spin systems in sterol skeleton was established by COSY correlations, δ 1.32(H-1)/1.97(H-2)/3.52(H-3)/2.28(H-4); δ 5.35(H-6)/2.29(H-7)/1.98(H-8)/1.34(H-9)/1.49(H-11)/1.15(H-12); δ 1.98(H-8)/1.53(H-14)/1.58(H-15)/ 1.86(H-16)/0.99(H-17)/1.33(H-20)/0.91(H-21), 1.33(H-22) and δ 1.26(H-24)/1.92(H-25)/0.89(H-26)/0.91(H-27). The shielded protons of sterol framework were found to appear at δ 0.69 (s), 1.01 (s) and 0.91 (d), which exhibited HSQC correlations with the carbons at δ 18.69(C-18), δ 19.38(C-19) and δ 18.73(C-21), respectively. The HMBCs from δ 2.28(H-4) to δ 31.62(C-

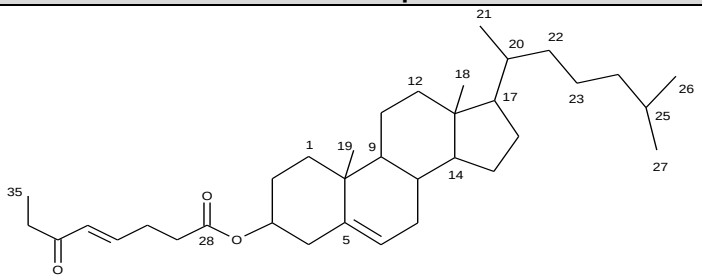
2), 140.76(C-5), 121.72(C-6); δ 2.29(H-7) to δ 140.76(C-5); δ 1.34(H-9) to δ 24.30(C-15); δ 1.15(H-12) to δ 42.30(C-13) and δ 1.01(H-19) to δ 37.26(C-1) supported the occurrence of C-27 sterol moiety. The 33-oxooct-31-enoate side chain placed at C-3 of sterol skeleton was confirmed from HMBC correlation from δ 2.33(H-29) to δ 71.81(C-3). The presence of side chain was corroborated by COSYs from δ 2.33(H-29)/2.04(H-30); δ 5.80(H-31)/4.88(H-32) and δ 2.00, 1.86(H-34)/0.88(H-35). The chemical shifts due to the carbons associated with ester and ketone groups (δ 174.00 and 194.01, respectively) were assigned to C-28 and C-33, respectively based upon the HMBC experiment. The long-range C-H correlations from δ 2.04(H-30) to δ 174.00(C-28); δ 5.80(H-31) to δ 29.70(C-29); δ 4.88(H-32) to δ 29.37(C-30), 194.01(C-33) and δ 0.88(H-35) to δ 136.00(C-31), 194.01(C-33) further validated the presence of side chain assignments. Therefore, the eight degrees of unsaturation were assigned to each of four double bonds and ring moieties. The greater coupling constants at H-31(J = 9.1 Hz) and H-32(10.4 Hz) attributed the *trans* (*E*) configuration of the olefinic bond at -CH-(34)=CH-(35)-The NOESYs at δ 3.52(H-3)/5.35(H-6)/1.53(H-14) appropriately suggested that they were aligned towards the identical plane of reference, and considered as α-protons. This, in turn, indicated the β-disposition of hydroxyl at C-3. The methyls at δ 0.69(H-18) and 1.01(H-19) of cholestane were related with the protons at δ 1.98(H-8) and 0.99(H-17), which did not exhibit any cross-peaks with the α-assigned protons, and considered as β-protons.

The LCMS spectrum of the compound 5, cholest-5-en-3β-3-yl-((E)-33-oxooct-31-enoate is given in **Fig 5.9.** The molecular ion peak appeared to be at *m/z*524.4 ($C_{35}H_{56}O_3$ $^{\bullet+}$), undergo elimination of methyl groups to form daughter ions at *m/z*496.4 ($C_{33}H_{52}O_3$).These daughter ion undergo further elimination of series

of methyl groups to form daughter ions at *m/z*482.4($C_{32}H_{50}O_3$), *m/z*468.4 ($C_{31}H_{48}O_3$) and *m/z*454.3 ($C_{30}H_{46}O_3$) respectively.

Table 5.8. NMR spectroscopic data of cholest-5-en-3β-3-yl-((E)-33-oxooct-31-enoate (**6**)

Compound 6



Position No	^{13}CNMR	δ^{1}H NMR (int., mult., J in Hz)[b]	^{1}H-^{1}H COSY	HMBC(^{1}H-^{13}C)
1	37.26	1.32(m)	2-H	-
2	31.62	1.98(m)	-	C-1,C-3
3	71.81	3.52(td)	1-H,2-H,4-H	-
4	42.31	2.28	3-H	C-3,5,6,2
5	140.76	-	-	-
6	121.72	5.35	7-H	C-7
7	31.92	2.28(m)	8-H	C-5,C-6
8	31.92	1.98(t)	14-H,9-H	C-9,
9	50.14	1.34(m)	8-H	C-15
10	36.52	-	-	C-11,19,12
11	21.1	1.49(m)	12-H	-
12	39.79	1.15(t)	11-H	C-13,C-12
13	42.3	-	-	C-18,C-13
14	56.78	1.53(m)	8-H	-
15	24.3	1.58(m)	16-H	C-13
16	28.24	1.86(m)	17-H,15-H	-
17	56.16	0.99(m)	20-H	C-15,C-22,C-20
18	18.69	0.69(s)	-	C-14,C-13
19	19.38	1.01(s)	-	C-13,C-17,C-11
20	35.79	1.33	21-H	-
21	18.73	0.91(d)	20-H	C-22,C-16
22	36.21	1.33(m)	23-H	C-20
23	23.83	1.15	22-H	C-20,C-16
24	39.53	1.26	25-H	C-25,C-24
25	28.02	1.92	26-H	C-22

26	22.82	0.89(d)	25-H	-
27	22.83	0.91(d)	-	C-25
28	174	-	-	-
29	29.7	2.33(m)	30-H	-
30	29.37	2.04(m)	-	C-28
31	136	5.80(m)	35-H	C-29
32	111	4.88(d),4.95(d)	37-H	C-30,C-32
33	194	-	-	-
34	29.67	2.00(m), 1.86(m)	38-H	-
35	14.12	0.88(t)	37-Ha	C-31,C-35

[a] NMR spectra were recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometers.[b]Values in ppm, multiplicity and coupling constants (J¼ Hz) are indicated in parentheses. Assignments were made with the aid of the 1H-1H COSY, HSQC, HMBC and NOESY experiments.

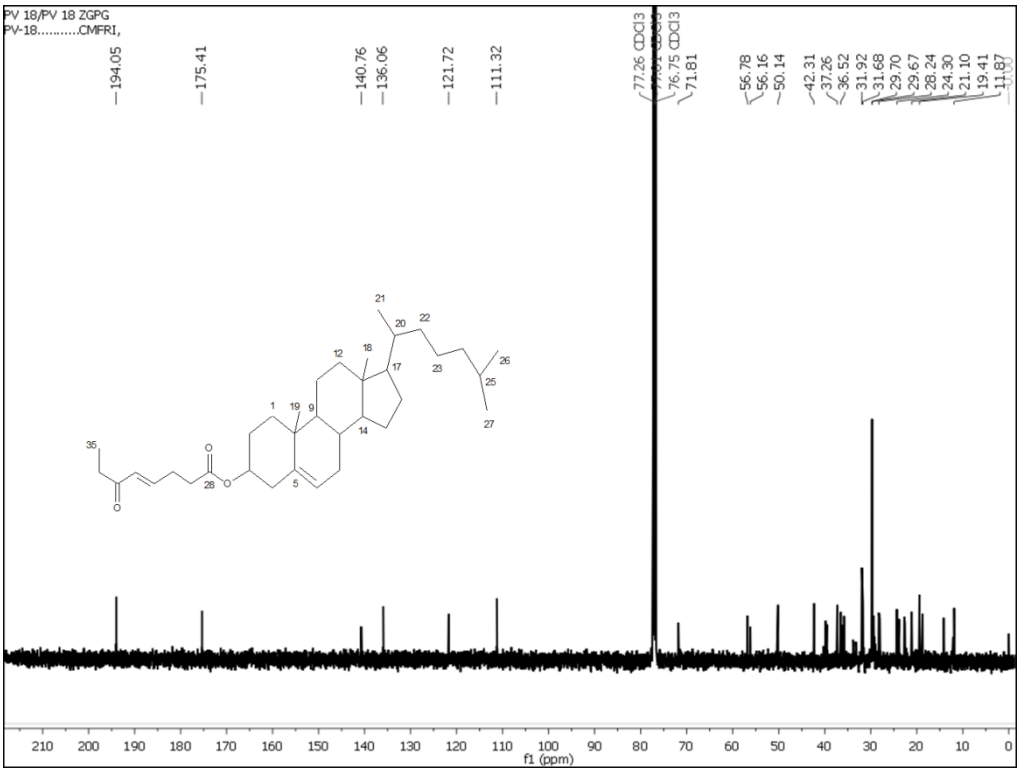


Fig 5.9.A. ^{13}C NMR spectrum of cholest-5-en-3β-3-yl-((E)-33-oxooct-31-enoate (**6**).

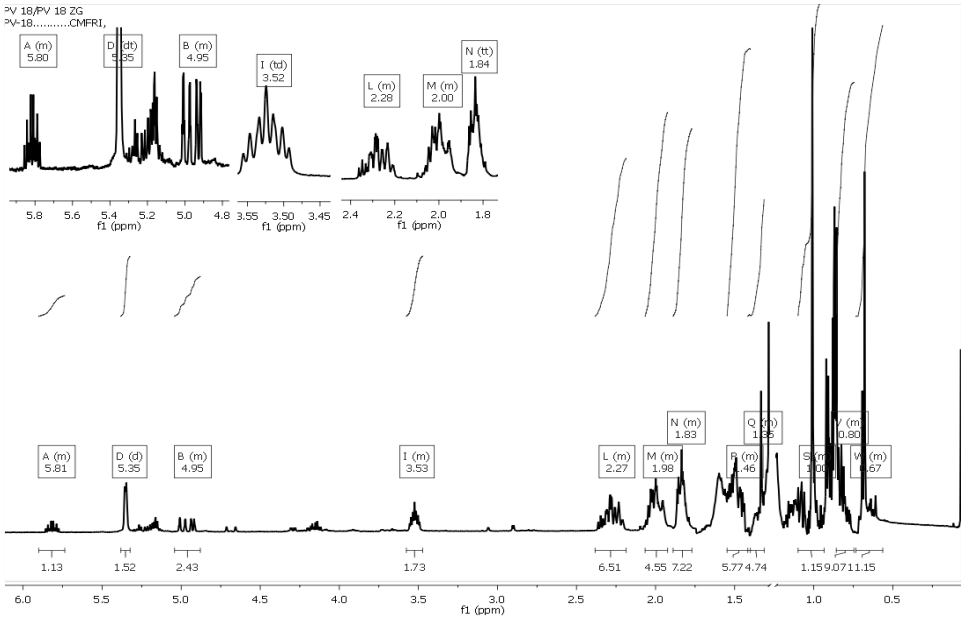


Fig 5.9. B. ^{1}H NMR spectrum cholest-5-en-3β-3-yl-((E)-33-oxooct-31-enoate (**6**).

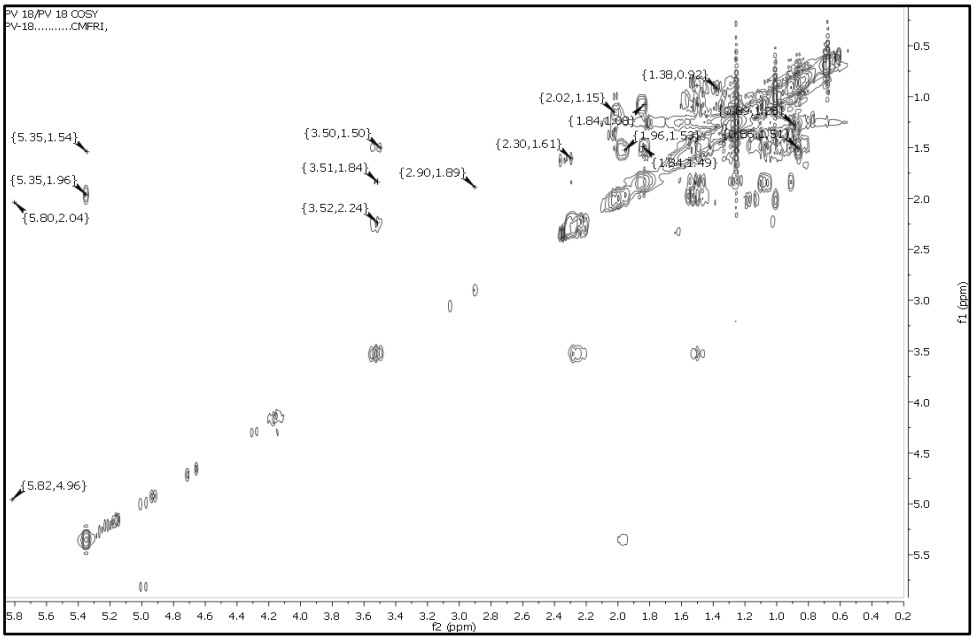


Fig 5.9.C. ^{1}H–^{1}H COSY spectrum of cholest-5-en-3β-3-yl-((E)-33-oxooct-31-enoate (**6**). The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds.

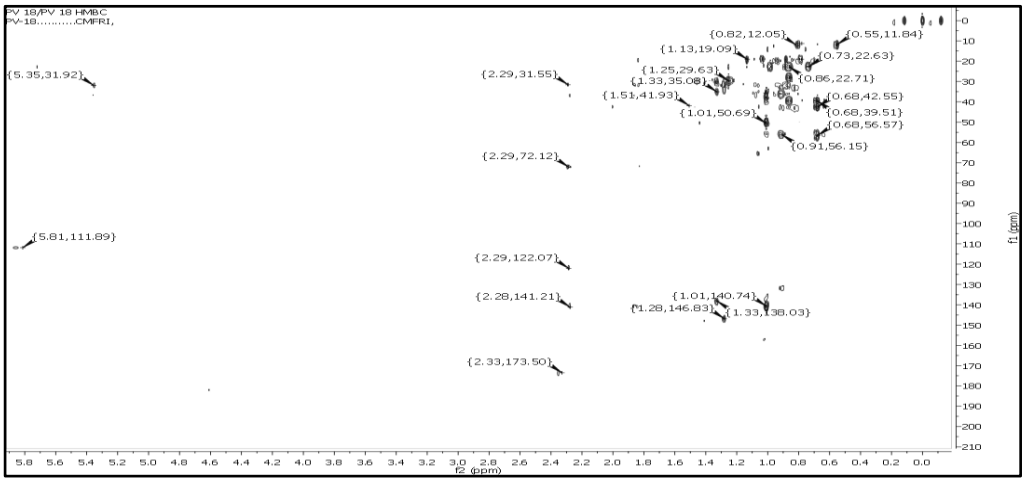


Fig 5.9.D. HMBC spectrum of cholest-5-en-3β-3-yl-((E)-33-oxooct-31-enoate (**6**).

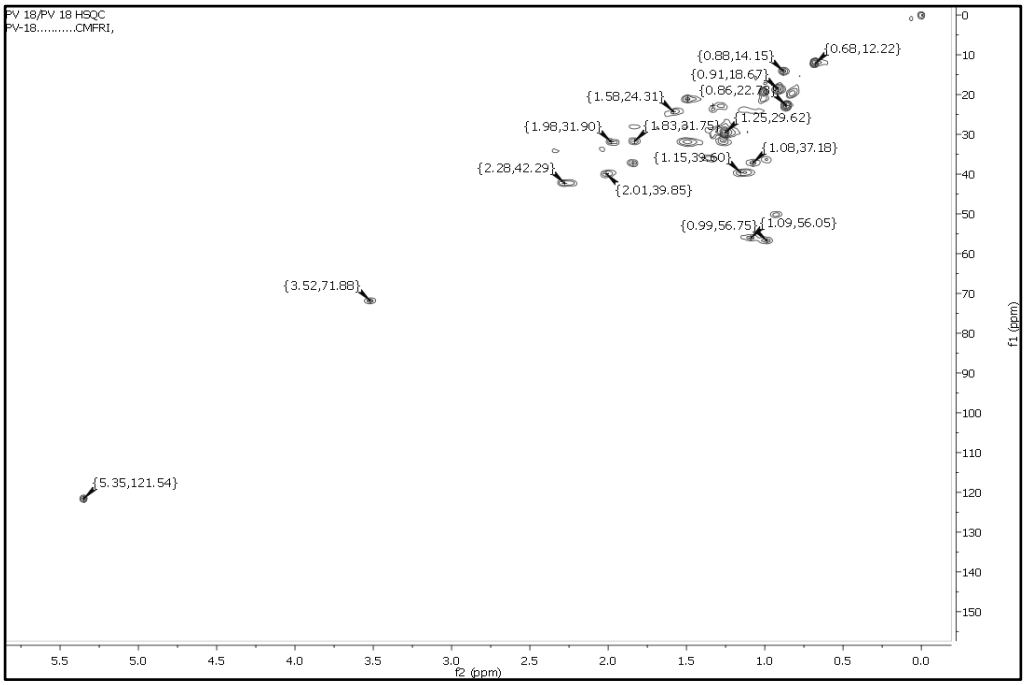


Fig 5.9.E. HSQC spectrum of cholest-5-en-3β-3-yl-((E)-33-oxooct-31-enoate (**6**).

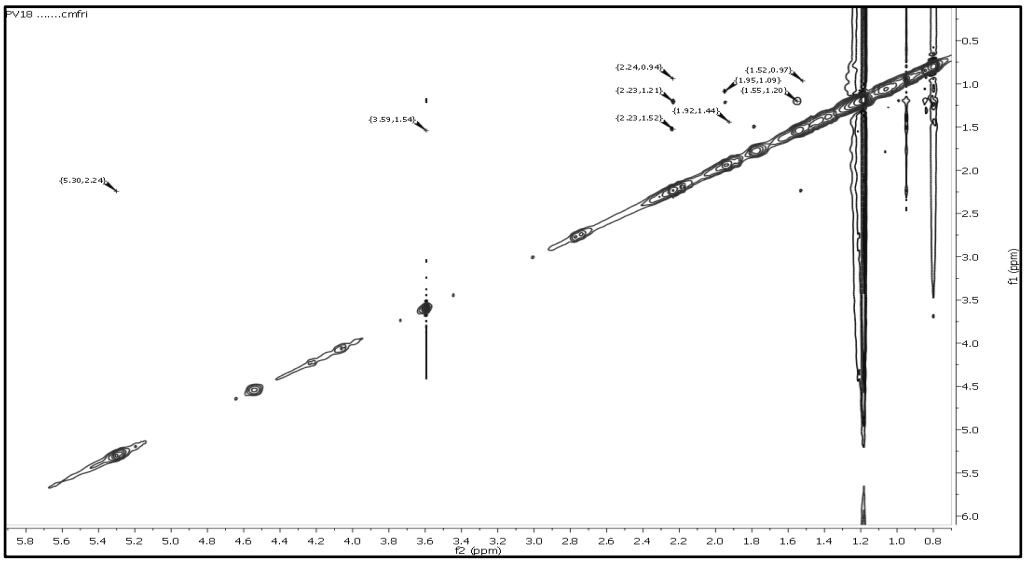


Fig 5.9.F. NOEspectrum of cholest-5-en-3β-3-yl-((E)-33-oxooct-31-enoate (**6**).

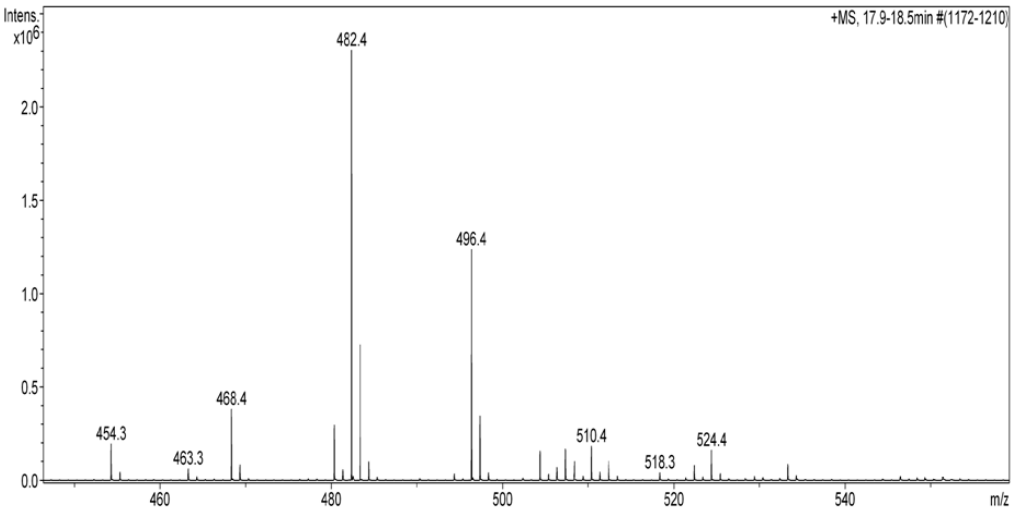


Fig 5.9.G. Mass spectrum of cholest-5-en-3β-3-yl-((E)-33-oxooct-31-enoate (**6**).

5.2.1.3Conclusion

Currently, the reactive oxygen species and lipid oxidation in food industry are being controlled or minimized by the addition of synthetic antioxidants. The chloroform partitioned methanolic extract of *Pernaviridis* was chromatographically fractionated over silica columns to yield 3-hydroxy-13-vinyl-dodecahydro-11-phenanthrenone (**1**), 4,4,9-trimethyl-13-vinyl-dodecahydro -2-phenanthrenone (**2**), 11,20-dihydroxy-6,6-dimethyl-decahydro-5H-benzo[h] naphtho[1,2-c]chromene-16-carbaldehyde (**3**), 16-acetyl-20-hydroxy-6,6-dimethyl-dodecahydro-5H-benzo[h]naphtho[1,2-c]chromen-12-one (**4**), cholest-5-en-3β-3-yl-(30-hydroxy-3-methyl-36-methyleneundeca-30E,34E-dienoate (**5**) and cholest-5-en-3β-3-yl-((E)-33-oxooct-31-enoate (**6**).The structures of the compounds, as well as their relative stereochemistries, were established by means of spectral data analyses, including 2D-NMR experiments. The scavenging effect of the purified compounds decreased with the order: **3**>**4**>**1**>**2**>**5**>**6**.These compounds could effectively inhibit COX-2 as well as LOX-5 enzymes.This study established the potential of marine derived compounds as potential natural lead antioxidative and anti-inflammatory molecules for use in pharmaceutical and food industries.The knowledge on the structural features responsible for antioxidative and anti-inflammatory activities will guide us to synthesize the molecules in commercial scale for use as new generation antioxidant and anti-inflammatory leads.

5.2.2. Secondary metabolites from *Crassostrea madrasensis*

An aliquot of the *C. madrasensis* extract (CRCT,9.2 g) was slurried in the silica gel (5 g, 60–120 mesh), and loaded into a glass column (90 cm X 4 cm) packed with silica gel (60–120 mesh, 90 g) as adsorbent before being subjected to vacuum liquid chromatography. The column was initially eluted with *n*-hexane and the eluent polarity was gradually increased by addition of EtOAc (*n*-hexane:EtOAc 99:1 to 30:70, v/v) to furnish a total of 28 fractions

of 35 mL each, which were reduced to 16 groups (CF_1 - CF_{16}) after TLC analysis (*n*-hexane: EtOAc, 9:1, v/v).CF_6 obtained by eluting with *n*-hexane:EtOAc (4:1, v/v) was found to be a mixture, which was flash chromatographed (Biotage AB SP1-B1A, 230–400 mesh, 12 g; Biotage AB, Uppsala, Sweden) on a silica gel column (Biotage, 230–400 mesh, 12 g; Sweden, Biotage No. 25+M 0489-1) at a collection UV wavelength of 236 nm using a step gradient of ethyl acetate/*n*-hexane (0-5% ethyl acetate) to afford 140 fractions (9 mL each). Based on analytical TLC, the fractions with similar patterns were pooled together to afford four pooled fractions (CF_{17} – CF_{20}). The fraction F_{14} on subsequent preparative TLC purification using 10% EtOAc /*n*-hexane afforded methyl-3-(26-phenylacetyloxy)-icosahydro-19-hydroxy-4,4,20-trimethylpicene-23-carboxylate0020(**7**, 88 mg). The fraction CF9 on flash chromatographic purification on a silica gel column (Biotage, 230–400 mesh, 12 g; Sweden, Biotage No. 25+M 0489-1)at a collection UV wavelength of 256 nm using a step gradient of EtOAc/*n*-hexane (0-10 % EtOAc) to afford 120 fractions (9 mL each). Based on analytical TLC, the fractions with similar patterns were pooled together to afford six pooled fractions (CF_{21} – CF_{26}). CF_{22} on further purificationwith P-TLC using 40% EtOAc/*n*-hexane afforded the compound, methyl-3-(26-phenylacetyloxy)-icosahydro-1,19-dihydroxy-4,4,20-trimethylpicene-23-carboxylate (**8**, 52 mg). The yield and antioxidant activities of each column/P-TLC fractions and anti-inflammatory activities of the selected fractions were presented in Table 5.8. The R_f of all the P-TLC fractions were also shown in **Table 5.9**.

Table 5.9. Yield, Rf, antioxidant and anti-inflammatory activities of each column/P-TLC fractions of *C. madrasensis.*

	Yield (mg)	R_f	DPPH radical scavenging activity (%)	ABTS radical scavenging activity (%)	COX-2 inhibitory activity (%)	LOX-5 inhibitory activity (%)
CRCT (HX/EA)						
CF-1 (1% EA/HX)	102	0.13	50.14±0.32	20.60±0.42	NA	NA
CF-2 (2% EA/HX)	245	0.18	49.67±0.11	19.27±0.39	NA	NA
CF-3 (3% EA/HX)	83	0.21	35.89±0.24	14.33±0.26	NA	NA
CF-4 (4% EA/HX)	158	0.28	40.02±0.16	15.85±0.30	NA	NA
CF-5 (5% EA/HX)	67	0.31	33.98±0.18	15.43±0.29	NA	NA
CF-6 (6% EA/HX)	530	0.34	48.34±0.12	19.250.29	29.49±0.14	30.46±0.08
CF-7 (7% EA/HX)	102	0.38	47.81±0.12	25.81±0.12	NA	NA
CF-8 (10% EA/HX)	42	0.42	32.22±0.15	12.79±0.25	NA	NA
CF-9 (12% EA/HX)	34	0.45	47.38±0.22	18.46±0.14	28.46±0.05	32.17±0.21
CF-10 (14% EA/HX)	51	0.49	48.59±0.01	22.79±0.44	NA	NA
CF-11 (16% EA/HX)	49	0.56	44.28±0.00	23.32±0.18	NA	NA
CF-12 (18% EA/HX)	65	0.63	59.38±0.00	25.31±0.09	NA	NA
CF-13 (20% EA/HX)	72	0.84	49.32±0.00	24.67±0.22	NA	NA
CF-14 (22% EA/HX)	805	0.89	53.59±0.00	21.90±0.16	NA	NA
CF-15 (25% EA/HX)	43	0.92	63.96±0.01	18.31±0.11	NA	NA
CF-16 (30% EA/HX)	22	0.95	56.32±0.09	20.89±0.09	NA	NA
CF-6 (P-TLC 10% EA/HX)						
CF-17	51	0.32	31.24±0.11	24.65±0.11	NA	NA
CF-18	356	0.56	56.57±0.25	20.64±0.25	40.56±0.33	38.29±0.02
CF-19	84	0.73	57.77±0.08	21.87±0.08	NA	NA
CF-20	39	0.85	50.65±0.07	181.34±0.07	NA	NA
CF-14 (P-TLC 10% EA/HX)						
CF-21	21	0.25	59.44±0.17	26.65±0.17	NA	NA
CF-22	304	0.32	60.73±0.01	27.98±0.01	46.73±0.11	44.28±0.25
CF-23	168	0.44	54.61±0.08	23.51±0.08	NA	NA
CF-24	89	0.67	49.64±0.14	24.84±0.14	NA	NA
CF-25	37	0.72	47.76±0.06	20.69±0.06	NA	NA
CF-26	66	0.92	53.13±0.07	23.56±0.07	NA	NA
CF-18 (P-TLC 5% EA/HX)						
CF-27	88	0.24	72.47±0.23	31.47±0.23	49.56±0.11	48.16±0.01
CF-28	52	0.35	61.64±0.25	28.64±0.25	NA	NA
CF-29	34	0.46	63.46±0.09	29.41±0.09	NA	NA
CF-30	18	0.57	70.85±0.16	29.96±0.16	NA	NA
CF-31	54	0.68	64.45±0.46	28.49±0.46	NA	NA
CF-22 (P-TLC 40% EA/HX)						
CF-32	68	0.27	57.1±0.04	20.1±0.04	NA	NA
CF-33	52	0.46	68.76±0.04	32.76±0.04	48.22±0.0	48.38±0.26
CF-34	52	0.59	55.8±0.33	22.42±0.33	NA	NA
CF-35	72	0.63	49.35±0.18	20.54±0.18	NA	NA
CF-36	43	0.75	48.47±0.08	18.65±0.08	NA	NA

DPPH radical scavenging activities at 0.1 mg/mL were expressed in percent; COX-2 inhibitory activity at 0.1 mg/mL was expressed in percent; LOX-5 inhibitory activity at 0.05 mg/mL was expressed in percent; NA -not assayed, *ie*, the fractions with low yield were evaluated only for DPPH and ABTS radical scavenging activity. CC -column chromatography; P-TLC -preparative thin layer chromatography; EA - ethyl acetate; HX- *n*-hexane; CRCT -EtOAc- methanol fraction of *C.madrasensis*

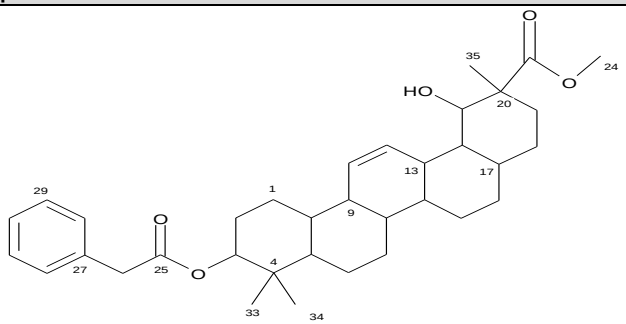
5.2.2.1 Chromatographic purification and spectral analyses of secondary metabolites from *Crassostreamadrasensis*

Chromatographic separation led to the isolation of two new derivatives. The molecular structures of the purified compounds was proposed on the basis of comprehensive analysis of the ^{1}H NMR, ^{13}C NMR, including 2D-NMR experiments (^{1}H–^{1}H–COSY, HMQC, HMBC, and NOESY), and mass spectra.

5.2.2.1.A Chromatographic purification and spectral analyses of methyl-3-(26-phenylacetyloxy)-icosahydro-19-hydroxy-4,4,20-trimethylpicene-23-carboxylate (7)

The compound methyl-3-(26-phenylacetyloxy)-icosahydro-19-hydroxy-4,4,20-trimethylpicene-23-carboxylate(7), a new picene derivative was isolated as yellow solid upon repeated chromatography over silica columns/TLC. The olefinic (C=C), carbonyl (C=O), alcoholic (O-H) and aromatic (C=C) groups were assigned by the absorption bands at 1545, 1734, 3419 and 1580 cm^{-1}respectively. The UV absorbances at λ_{max} (log ε) 256 nm (2.71) were assigned to be a chromophore with carbonyl groups and olefinic system.

Table 5.10. NMR spectroscopic data of methyl-3-(26-phenylacetyloxy)-icosahydro-19-hydroxy-4,4,20-trimethylpicene-23-carboxylate (**7**) isolated from *Crassostrea madrasensis*

Compound 7				
				
Position No.	**^{13}CNMR**	**δ^{1}H NMR (int., mult., J in Hz)^{b}**	**^{1}H-^{1}H COSY**	**HMBC (^{1}H-^{13}C)**
1	19.19	1-H	1.44(m,2H)	2-H
2	30.58	2-H	1.71(m,2H)	3-H

3	65.56	3-H	4.29(t,1H)	-
4	34.06	-	-	-
5	30.2	5-H	1.62(q,1H)	6-H
6	28.96	6-H	1.31(m,2H)	7-H
7	24.87	7-H	1.15(m,2H)	8-H
8	29.7	8-H	1.28(t,1H)	14-H,9-H
9	33.82	9-H	1.36 (m,1H)	11-H
10	32.71	10-H	2.04 (m,1H)	1-H
11	128.84	11-H	5.80(m,1H)	12-H
12	114.05	12-H	4.96(m,1H)	13-H
13	31.44	13-H	1.96(m,1H)	14-H
14	38.88	14-H	1.06(m,1H)	15-H
15	25.93	15-H	1.85(m,2H)	-
16	33.75	16-H	2.02(m,2H)	17-H,15-H
17	38.88	17-H	1.12(m,1H)	-
18	43.39	18-H	1.61(m,1H)	13-H
19	64.8	19-H	4.12(m,1H)	18-H
	-	-	(OH-5.32)	-
20	56.14	-	-	-
21	30.56	21-H	1.62(m,2H)	22-H
22	29.51	22-H	1.33(m,2H)	17-H
23	172.97	-	-	-
24	51.44	24-H	3.65(s,3H)	
25	167.7	-	-	-
26	49.57	26-H	2.17(s,3H)	-
27	139.27	-	-	-
28	132.52	28-H	7.56 – 7.51 (m, 1H)	29-H
29	130.89	29-H	7.65 – 7.75 (m, 1H)	-
30	124.05	30-H	7.65 – 7.75 (m, 1H)	-
31	130.29	31-H	7.65 – 7.75 (m, 1H)	32-H
32	132.53	32-H	7.56 – 7.51 (m, 1H)	31-H
33	14.11	33-H	0.97(s,3H)	-
34	13.72	34-H	0.87(s,3H)	-
35	22.69	35-H	0.84(s,3H)	-

[a] NMR spectra were recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometers.

[b] Values in ppm, multiplicity and coupling constants (J¼ Hz) were indicated as parentheses. The assignments were made with the aid of the 1H-1H COSY, HSQC, HMBC and NOESY experiments.

The mass spectrum exhibited a molecular ion peak at *m/z*548 (HRESIMS *m/z* 549.7785(M+H) amu), which in combination with ^{1}H and ^{13}C NMR data (**Table 5.10**) assigned the molecular formula as $C_{35}H_{64}O_5$with 12 degrees of unsaturation. Six degrees of unsaturation were from double bonds and the remaining six degrees of unsaturation were due to a cyclic ring system, from which one was of aromatic type.

The 1D (^{13}C and DEPT) NMR data demonstrated the presence of nine methylenes, seventeen methines and five quaternary carbons. Highly deshielded olefinic signals were apparent at δ 128.8 (C-11) and 114.0 (C-12), which were correlated to δ 5.80 (H-11) and 4.96 (H-12), respectively indicating the presence of isolated double bond in the compound. The position of the double bond was confirmed by the long-range C-H correlation from δ 5.80 (H-11) to δ 31.4 (C-13) and 114.0 (C-12) along with δ 4.96 (H-12) to δ 31.4 (C-13) and 43.3 (C-18). The relatively downfield shift of methine proton at δ 4.29 (H-3) exhibited HSQC correlations with δ 65.5 (C-3), which referred to a possible electronegative atom in its locality and was the characteristic junction point of the cyclic moiety with the ester linked side chain. The occurrence of three singlets at δ 0.97, 0.87 and 0.84 corresponded to the ^{13}C signals at δ 14.1, 13.7 and 22.6, respectively indicating that the methyl groups were positioned at C-33, C-34 and C-35, respectively. The presence of hydroxyl at C-19 {-CH(OH)} and the long range C-H correlation between δ_H 4.12/δ_C 64.8 was deduced from the HMBCs between δ 4.12 (H-19) to δ 172.9 (C-23). The ^{1}H-^{1}H COSY correlations established the occurrence of four spin systems, which were due to the signals at δ 1.44 (assigned to H-1)/1.71 (H-2)/4.29 (H-3) in ring A, δ 1.62 (H-5)/1.31 (H-6)/1.15 (H-7)/1.28 (H-8)/1.36 (H-9)/2.04 (H-10) along with δ 1.36 (H-9)/5.80 (H-11)/4.96 (H-

12)/1.96 (H-13)/1.06 (H-14)/1.85 (H-15)/2.02 (H-16)/1.12 (H-17)/1.33 (H-22)/1.62 (H-21) and δ 1.28 (H-8)/1.06 (H-14), which were found to correspond to the B/C/D/E rings (tetracyclic moiety), whereas that at δ 7.55 (H-28)/7.70 (H-29) were assigned to the aromatic ring system. The HMBC correlations deduced an acetate group {-C(O)O-(CH_3)} at C-20 from the cross-peaks between δ 3.65 (H-24) to δ 172.9 (C-23) and δ 1.62 (H-21) to δ 172.9 (C-23). The HMBC correlations from δ 4.29 (H-3) to δ 34.0 (C-4); δ 2.02 (H-16) to δ 29.5 (C-22); δ 1.36 (H-9) to δ 128.8 (C-11) and δ 1.28 (H-8) to δ 38.8 (C-14) further corroborated the presence of the picene-23-carboxylate framework in **7**. The aromatic signals in the region δ_H 7.56-7.75 with an integral value of five demonstrated the presence of mono-substituted aromatic ring and the deshielded quaternary carbon at δ 139.2 suggested the side chain remained attached to the picene framework. The ^{13}C NMR peaks at δ 139.2 (C-27), 132.5 (C-28), 130.8 (C-29), 124.0 (C-30), 130.2 (C-31), 132.5 (C-32) along with the HSQC and HMBC spectroscopic data deduced the 2-phenylacetate group attached to the C-3 carbon of icosahydropicenecarboxylate moiety. The attachment of 2-phenyl acetate with C-3 was confirmed by the HMBC correlation of δ 4.29 (H-3) with δ 167.7 (C-25). The relative stereochemistry of the chiral centres, particularly those of δ 4.29 (C-3) and δ 4.12 (C-19) was deduced by the NOESY correlation indicating that these two were oriented towards the same plane and was arbitrarily considered as *α*-disposed. The protons at H-5, H-9, H-14 and H-18 exhibited NOESY correlations with each other and considered to be aligned towards the *β*-side of the molecule, being opposite to the *α*-protons.

The compound **7** formed an sodium adduct peak at *m/z* 571.3 ($C_{35}H_{48}O_5Na^{\bullet+}$) which appeared to undergo elimination of $C_9H_{10}O_2$(*m/z*:150.07)

to yield a peak at *m/z* 414.3 ($C_{27}H_{42}O_3$•$^+$).The latter appeared to undego molecular fragmentatation to afford the fragments with *m/z* 74.04 ($C_3H_6O_2$•+)assigned to be as methylacetate, and icosahydro-trimethylpicenol with the *m/z*of 356.3.

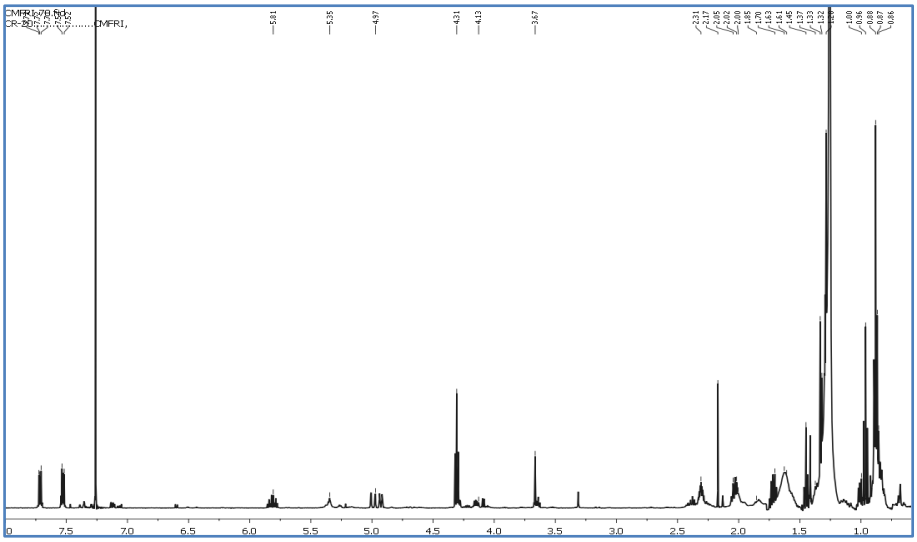


Fig 5.10.A. ^{1}HNMR spectrum of methyl-3-(26-phenylacetyloxy)-icosahydro-19-hydroxy-4,4,20-trimethylpicene-23-carboxylate (**7**)

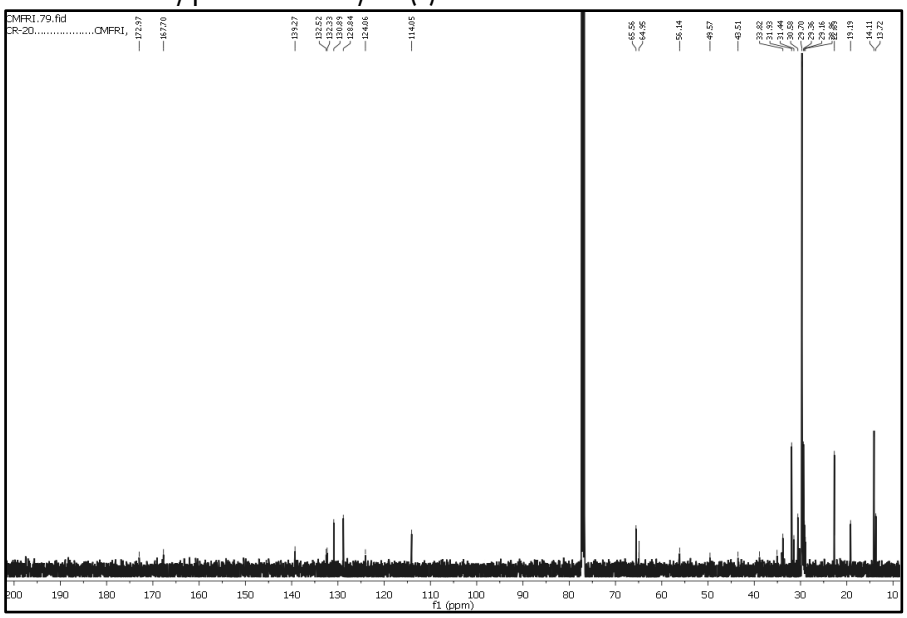


Fig 5.10.B. ^{13}C NMR spectrum of methyl-3-(26-phenylacetyloxy)-icosahydro-19-hydroxy-4,4,20-trimethylpicene-23-carboxylate (**7**)

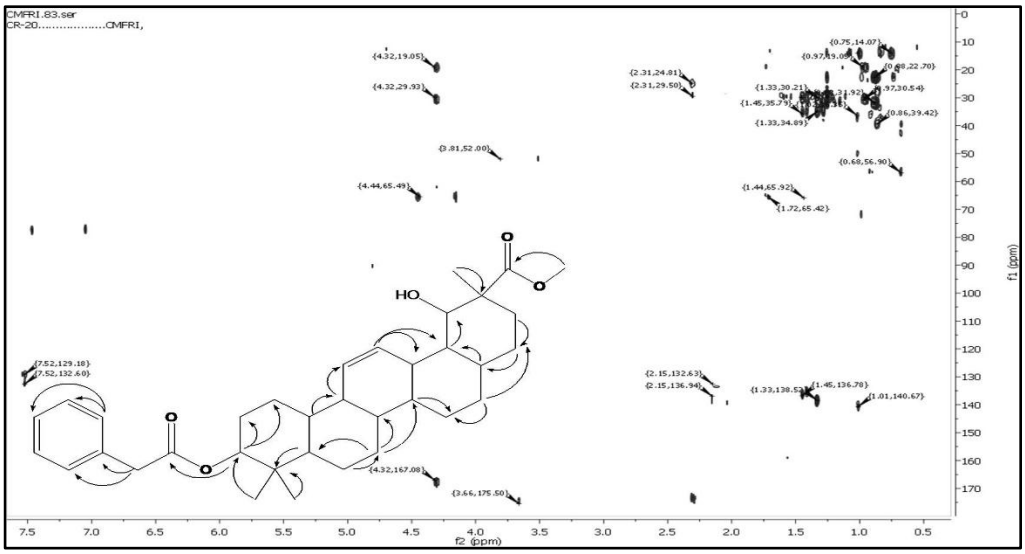


Fig 5.10.C. HMBC spectrum of methyl-3-(26-phenylacetyloxy)-icosahydro-19-hydroxy-4,4,20-trimethylpicene-23-carboxylate (**7**)

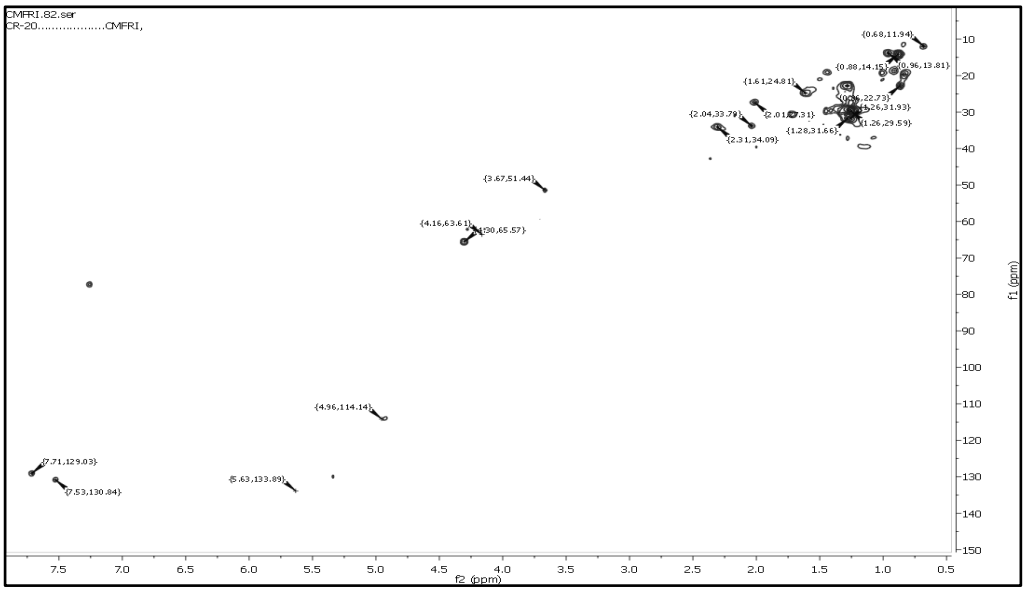


Fig 5.10.D. HSQC spectrum of methyl-3-(26-phenylacetyloxy)-icosahydro-19-hydroxy-4,4,20-trimethylpicene-23-carboxylate (**7**)

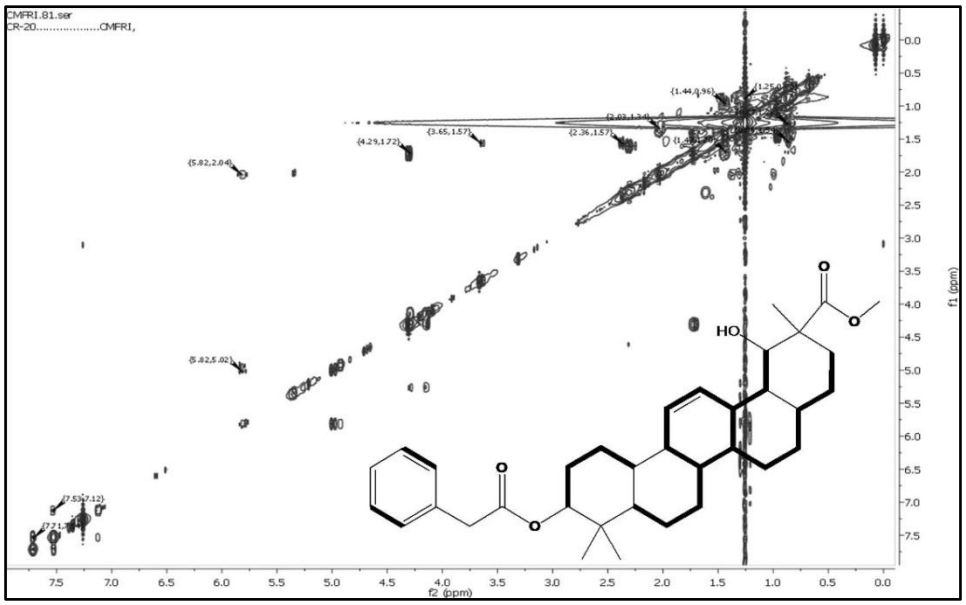


Fig 5.10.E. ^{1}H–^{1}H COSY spectrum of methyl-3-(26-phenylacetyloxy)-icosahydro-19-hydroxy-4,4,20-trimethylpicene-23-carboxylate (**7**)

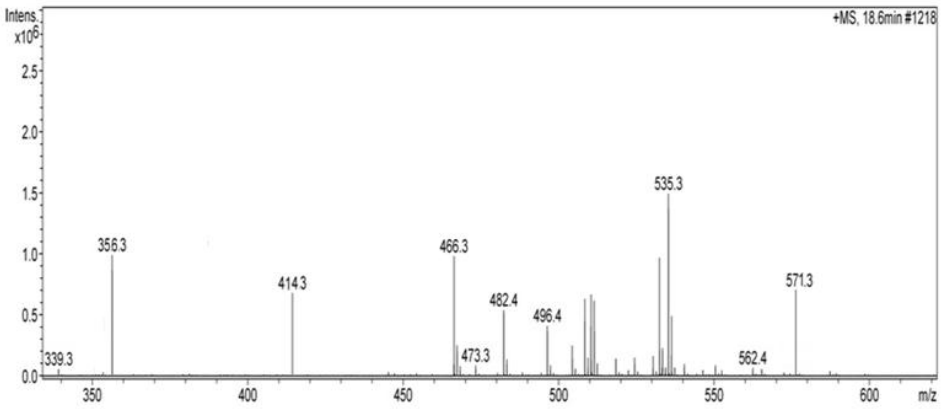


Fig 5.10.F. Mass spectra of methyl-3-(26-phenylacetyloxy)-icosahydro-19-hydroxy-4,4,20-trimethylpicene-23-carboxylate (**7**).

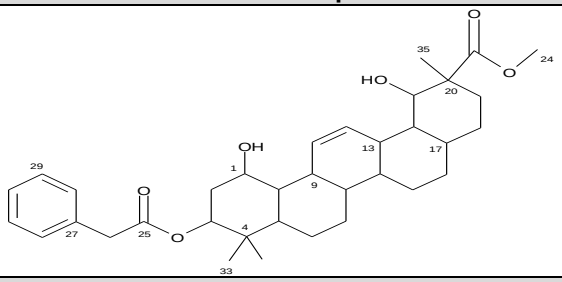
5.2.2.1.B Chromatographic purification and spectral analyses of methyl-3-(26-phenylacetyloxy)-icosahydro-1,19-dihydroxy-4,4,20-trimethylpicene-23-carboxylate(8)

Methyl-3-(26-phenylacetyloxy)-icosahydro-1,19-dihydroxy-4,4,20-trimethylpicene-23-carboxylate, a new picene derivative was isolated as white powder upon repeated chromatography over silica columns/TLC. The olefinic

(C=C) and (C=O stretch) groups were depicted by the absorption bands at 1642 and 1736.96 cm^{-1} respectively. The UV absorbances at λ_{max} (log ε) 252nm (3.61) was assigned to be a chromophore with carbonyl groups and olefinic system. Its mass spectrum exhibited a molecular ion peak at *m/z*580.7732(HRESIMS m/z 580.7732 [M+1] amu), which in combination with its ^{1}H and ^{13}C NMR data (**Table 5.11**) indicated the molecular formula as $C_{36}H_{52}O_6$ with twelve degrees of unsaturation. Three degree of unsaturation was from the double bonds, five degrees of unsaturation were due to a cyclic ring system, which was not of aromatic type and the remaining four degrees of unsaturation was due to aromatic ring system.

Table5.11. NMR spectroscopic data of methyl-3-(26-phenylacetyloxy)-icosahydro-1,19-dihydroxy-4,4,20-trimethylpicene-23-carboxylate (**8**) isolated from *Crassostreamadrasensis*

Compound 8



Position No.	^{13}CNMR	δ^{1}H NMR (int., mult., J in Hz)[b]	^{1}H-^{1}H COSY	HMBC (^{1}H-^{13}C)
1	71.8	1-H	4.08(dd,1H)	10-H
2	30.58	2-H	1.71(m,2H)	3-H
3	65.57	3-H	4.29(t,1H)	-
4	34.06	-	-	-
5	30.2	5-H	1.62(q,1H)	6-H
6	28.96	6-H	1.31(m,2H)	7-H
7	24.87	7-H	1.15(m,2H)	8-H
8	29.7	8-H	1.28(t,1H)	14-H,9-H
9	33.83	9-H	1.36 (m,1H)	11-H
10	32.71	10-H	2.04 (m,1H)	1-H
11	128.84	11-H	5.80(m,1H)	12-H
12	114.06	12-H	4.96(m,1H)	13-H
13	31.44	13-H	1.96(m,1H)	14-H
14	38.88	14-H	1.06(m,1H)	15-H
15	25.93	15-H	1.85(m,2H)	-
16	33.75	16-H	2.02(m,2H)	17-H,15-H
17	38.88	17-H	1.12(m,1H)	-
18	43.39	18-H	1.61(m,1H)	13-H

19	64.8	19-H	4.12(m,1H) (OH-5.32)	18-H
	56.14	-	-	-
20	30.56	21-H	1.62(m,2H)	22-H
21	29.51	22-H	1.33(m,2H)	17-H
22	173.2	-	-	-
23	51.44	24-H	3.65(s,3H)	-
24	167.67	-	-	-
25	49.57	26-H	2.17(s,3H)	-
26	139.28	-	-	-
27	132.38	28-H	7.56 – 7.51 (m, 1H)	29-H
28	130.9	29-H	7.65 – 7.75 (m, 1H)	-
29	124.05	30-H	7.65 – 7.75 (m, 1H)	-
30	130.29	31-H	7.65 – 7.75 (m, 1H)	32-H
31	132.53	32-H	7.56 – 7.51 (m, 1H)	31-H
32	14.11	33-H	0.97(s,3H)	-
33	13.72	34-H	0.87(s,3H)	-
34	22.69	35-H	0.84(s,3H)	-

[a] NMR spectra were recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometers.
[b] Values in ppm, multiplicity and coupling constants (J¼ Hz) were indicated in parentheses. The assignments were made with the aid of the 1H-1H COSY, HSQC, HMBC and NOESY experiments.

Compound **8** showed close structural similarity with **7** except the appearance of additional hydroxyl group at C-1 position in **8**. The new dihydroxypicene derivative, methyl-3-(26-phenylacetyloxy)-icosahydro-1,19-dihydroxy-4,4,20-trimethylpicene-23-carboxylate (**2**)was isolated as white solid on repetitive chromatographic purification. The olefinic and carbonyl groups were symbolized by the IR bonds at 1642 and 1737 cm^{-1},respectively. The relatively downfield shift of the methine proton at δ 4.30 (H-3) and 4.14 (H-19) referred to the possible oxygenation in its vicinity. An extra proton peak at δ 4.08 was found to be attached with δ 71.8 (C-1), and these attributions were supported by ^{1}H-^{1}H COSY and HMBC correlations, such as δ4.08 (H-1)/2.06 (H-10) and δ 4.08 (H-1) to δ 32.7 (C-10), respectively. The ^{1}H-^{1}H COSY correlations between δ4.08 (H-1)/1.72 (H-2)/4.30 (H-3); δ1.63 (H-5)/1.30 (H-6)/1.17 (H-7)/1.29 (H-8)/1.37 (H-9); δ 1.37 (H-9)/5.82 (H-11)/4.98 (H-12)/1.99 (H-13)/1.04 (H-14)/1.89 (H-15)/2.05 (H-16)/1.11 (H-17)/1.34 (H-22)/1.63 (H-21)and δ 1.63 (H-18)/4.14 (H-19) supported the presence of the pentacyclic ring in **2**. As discussed in the previously described compound **1**, the appearance of aromatic peaks along with a singlet methylene ($-CH_2$) at δ_H 2.18/δ_C 49.57 (C-26position) in the ^{1}H and ^{13}C NMR spectra confirmed the occurrence of 2-phenylacetate moiety. This attributions were

validated by the HMBCs from δ 2.18 (H-26) to δ 139.2 (C-27) and 132.3 (C-28). The methine proton at δ 4.30 (H-3) were showed NOE cross-peak with δ 4.14 (H-19), which indicated that these were in same plane of symmetry (arbitrarily defined as *α*-disposed). The methine proton at H-3 did not exhibit NOE interactions with δ 4.08 (H-1), thereby indicating that these groups were disposed at the opposite direction of the plane and considered as *β*-oriented.

The molecular ion peak at *m/z* 564.2 ($C_35H_{48}O_5$ •+) appeared to undergo elimination of $C_2H_2O_2$ yielded trimethylpicene-2-carboxylate derivative at *m/z* 506.3 ($C_{33}H_{46}O_4$•+).The daughter ion again undergo elimination of phenylacetate ($C_8H_7O_2$, *m/z*: 135.04) to yield another daughter ion at *m/z* 430.3 ($C_{27}H_{44}O_4$•+).

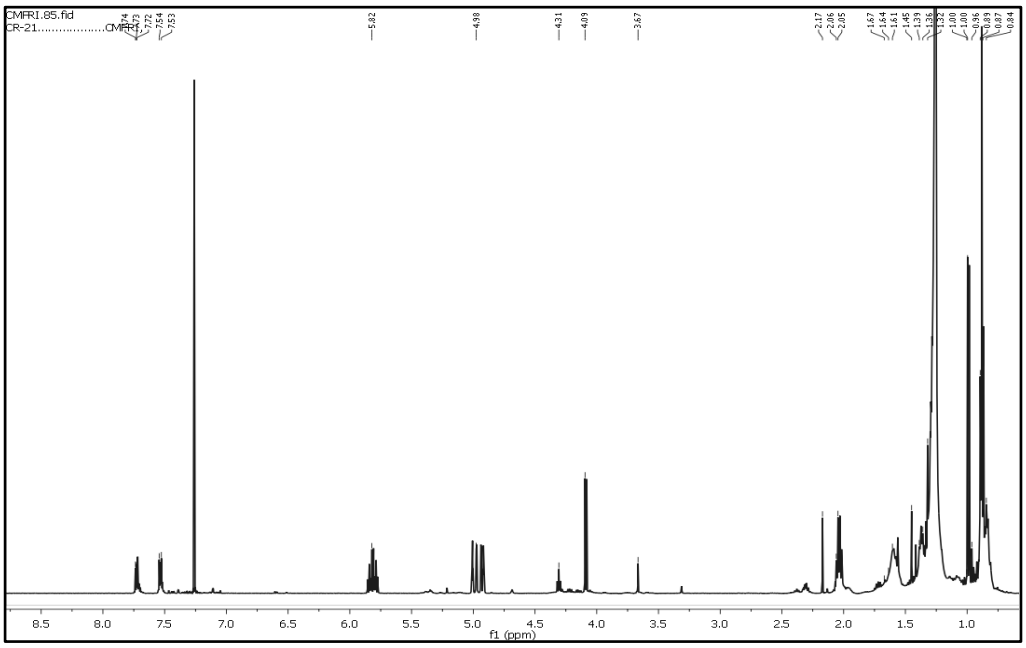


Fig 5.11.A. ^{1}HNMR spectrum of methyl-3-(26-phenylacetyloxy)-icosahydro-1,19-dihydroxy-4,4,20-trimethylpicene-23-carboxylate (8).

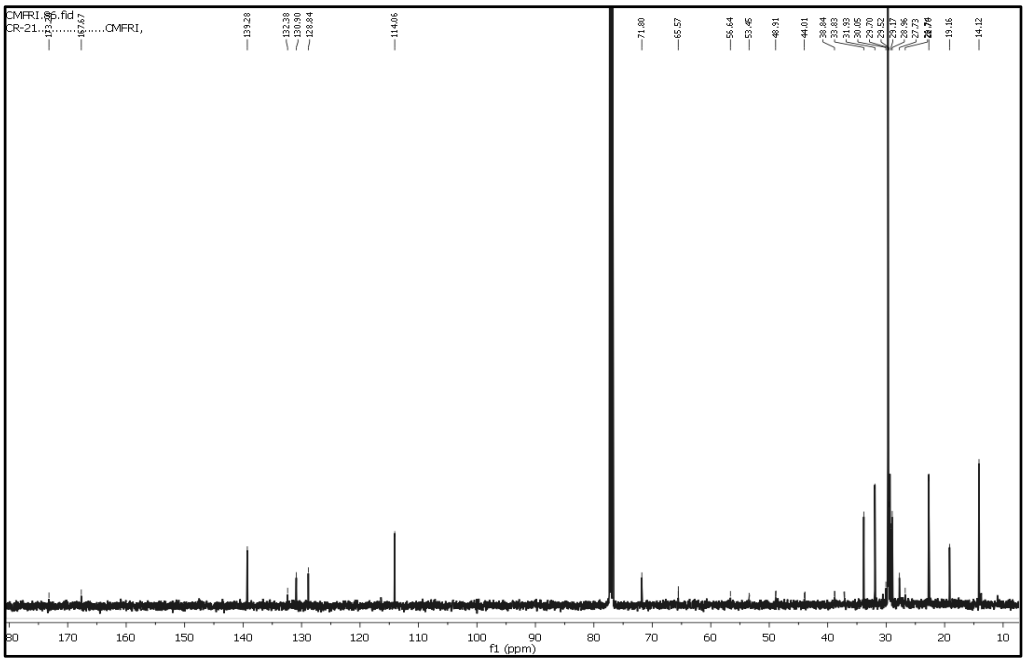


Fig 5.11.B. ^{13}C NMR spectrum of methyl-3-(26-phenylacetyloxy)-icosahydro-1,19-dihydroxy-4,4,20-trimethylpicene-23-carboxylate (8).

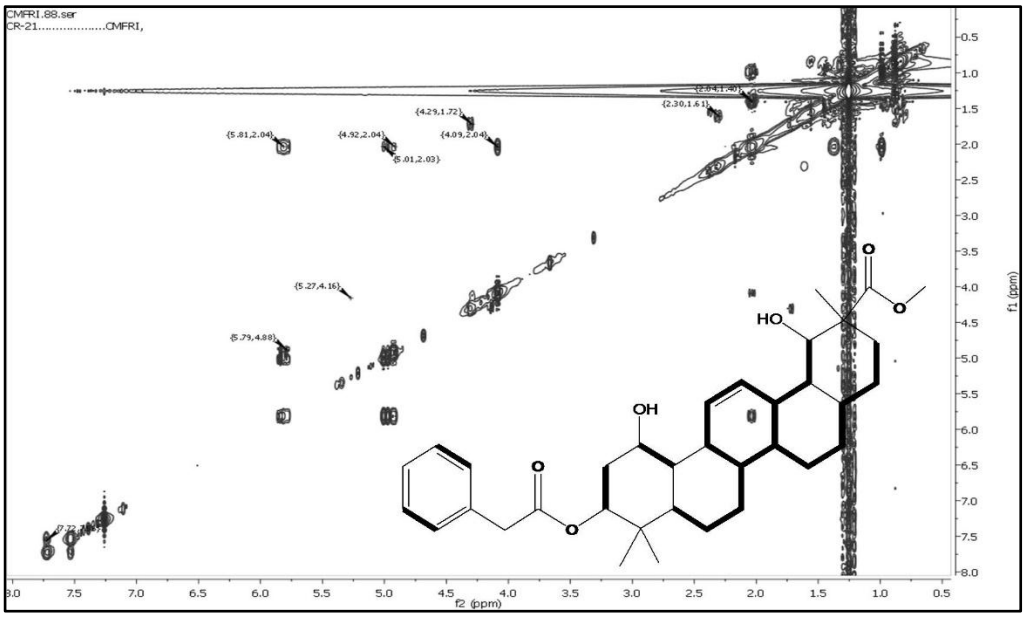


Fig 5.11.C. ^{1}H—^{1}H COSYspectrum of methyl-3-(26-phenylacetyloxy)-icosahydro-1,19-dihydroxy-4,4,20-trimethylpicene-23-carboxylate (8).

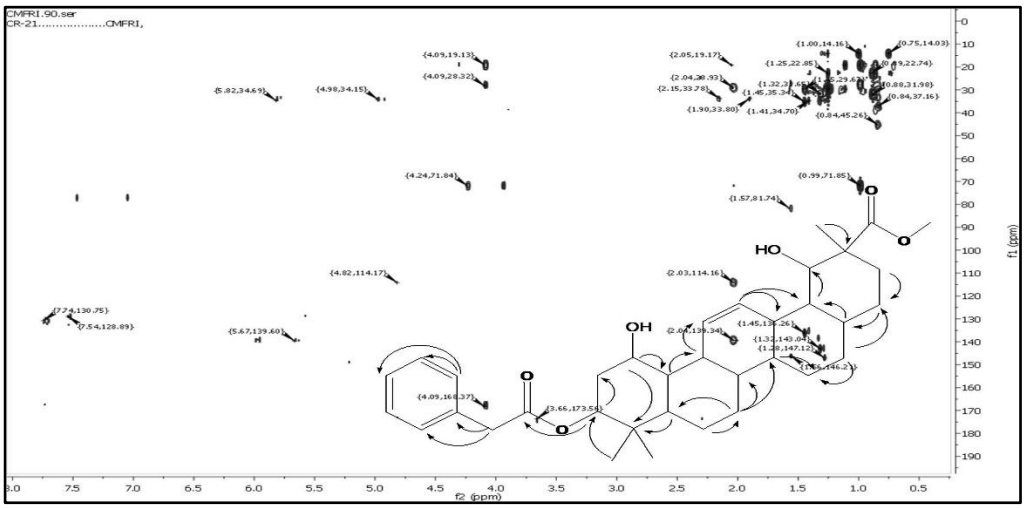


Fig 5.11.D. HMBC spectrum of methyl-3-(26-phenylacetyloxy)-icosahydro-1,19-dihydroxy-4,4,20-trimethylpicene-23-carboxylate (8).

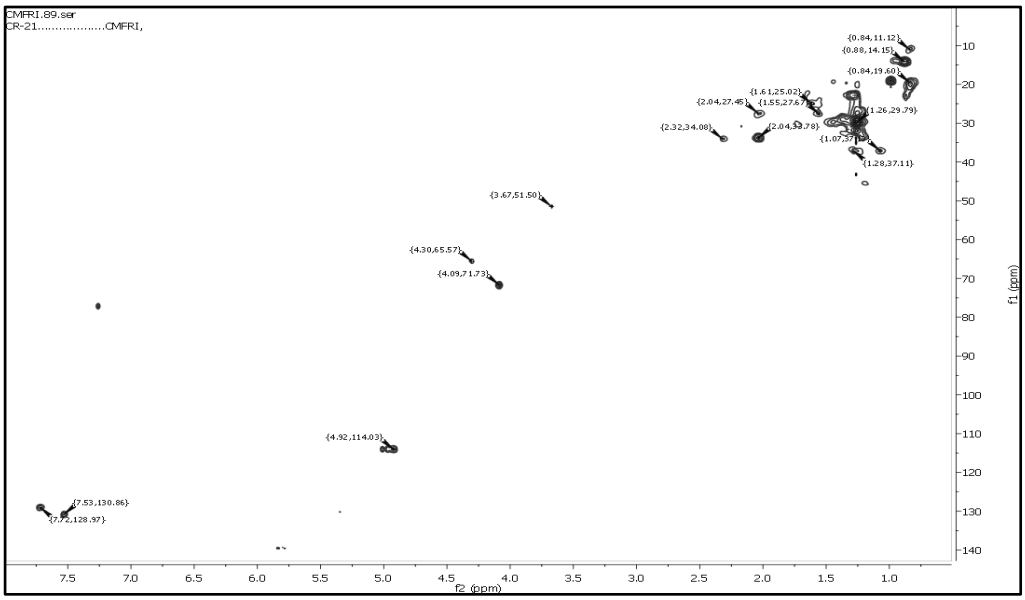


Fig 5.11.E. HSQC spectrum of methyl-3-(26-phenylacetyloxy)-icosahydro-1,19-dihydroxy-4,4,20-trimethylpicene-23-carboxylate (8).

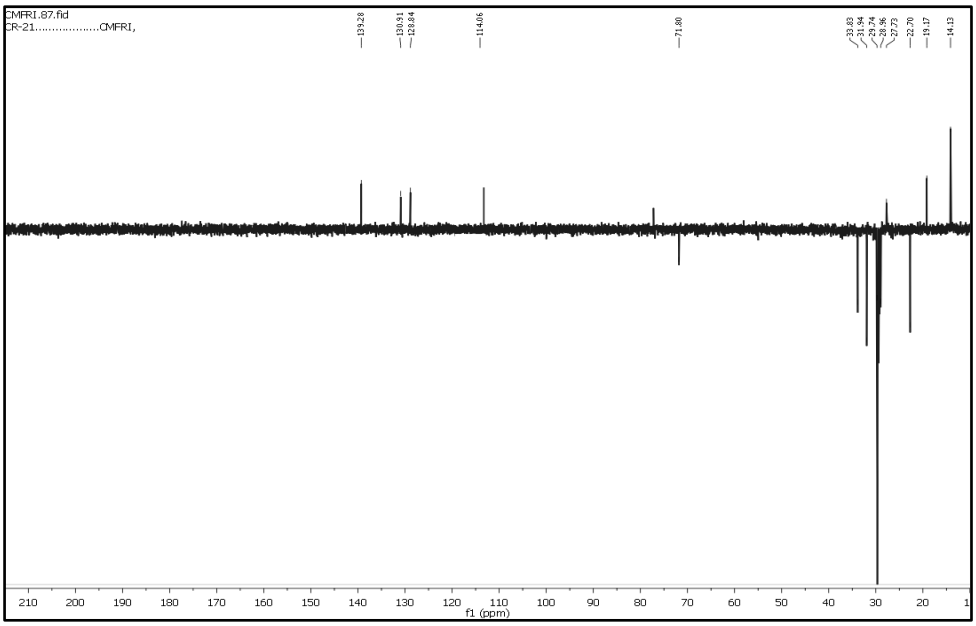


Fig 5.11.F. DEPT spectrum of methyl-3-(26-phenylacetyloxy)-icosahydro-1,19-dihydroxy-4,4,20-trimethylpicene-23-carboxylate (8).

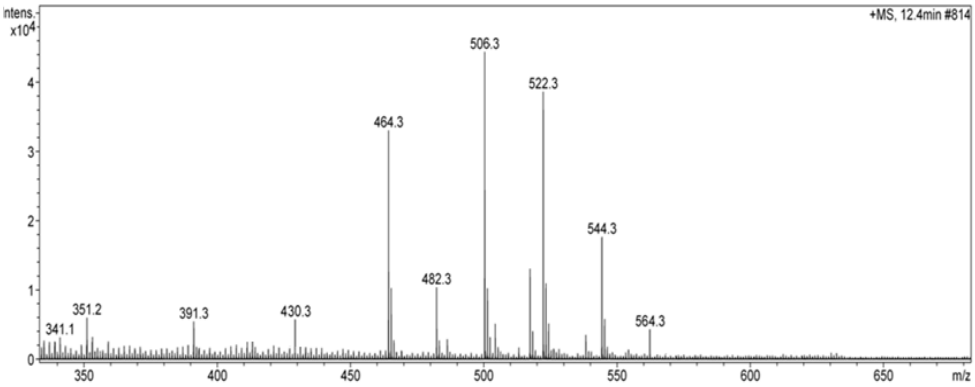


Fig 5.11.G. Mass spectrum of methyl-3-(26-phenylacetyloxy)-icosahydro-1,19-dihydroxy-4,4,20-trimethylpicene-23-carboxylate **8**).

5.2.2.2 Conclusion

Currently, reactive oxygen species and lipid oxidation in food industry are being controlled or minimized by the addition of synthetic antioxidants. The ethylacetate:methanol extract of *Crassostrea madrasensis* was fractionated chromatographically to yield methyl-3-(26-phenylacetyloxy)-icosahydro-19-hydroxy-4,4,20-trimethylpicene-23-carboxylate (7) and methyl-3-(26-phenylacetyloxy)-icosahydro-

1,19-dihydroxy-4,4,20-trimethylpicene-23-carboxylate **(8)**. The structures of the compounds, as well as their relative stereo chemistries, were established by means of spectral data analyses, including 2D-NMR experiments. Among the compounds isolated from *Crassostrea madrasensis*, compound **7** showed maximum DPPH radical-scavenging ability (72.5 percent) than **8**. No significant differences in the $ABTS^{\cdot+}$ radical-scavenging activity, COX-2 and 5-LOX inhibition activities were observed for **7** and **8**.The effective antioxidant property of these compounds indicated that they have potential as natural antioxidant lead molecules in the food industry. The knowledge on the structural features responsible for antioxidative bioactivities will guide us to synthesize the molecules in commercial scale for use as new generation antioxidant leads

5.2.3. Secondary metabolites from *O. dolffusi*

The yield, R_f and antioxidant activities of each column/P-TLC fractions and anti-inflammatory activities of the selected fractions were given in **Table 5.12**.

Table 5.12. Yield, R_f and antioxidant activities of each column/P-TLC fractions and anti-inflammatory activities of the selected fractions of *O.dolffusi*

	Yield (mg)	R_f	DPPH radical scavenging activity (%)	ABTS radical scavenging activity (%)	COX-2 inhibition activity (%)	LOX-5 inhibition activity (%)
OCT EXT	**5000**					
			OCT CC (EA/HX)			
OF-1 (2% EtOAc/HX)	101	0.23	35.43±0.22	21.36±0.31	48.41±0.01	46.40±0.76
OF-2 (5% EtOAc/HX)	97	0.36	54.88±0.14	20.28±0.28	47.39±0.00	46.55±0.57
OF-3 (10% EtOAc/HX)	92	0.45	44.39±0.36	14.73±0.17	36.29±0.01	35.30±0.23
OF-4 (15% EtOAc/HX)	456	0.56	39.42±0.18	15.79±0.25	38.14±0.01	29.21±0.46
OF-5 (20% EtOAc/HX)	922	0.61	53.68±0.08	22.34±0.13	46.29±0.01	45.05±0.39
OF-6 (25% EtOAc/HX)	84	0.67	52.13±0.22	23.42±0.01	47.18±0.00	45.17±0.65
OF-7 (35% EtOAc/HX)	176	0.73	44.84±0.14	17.63±0.20	23.12±0.00	20.09±0.27
OF-8 (50% EtOAc/HX)	906	0.81	56.66±0.32	25.81±0.13	45.58±0.00	44.67±0.47
OF-9 (75% EtOAc/HX)	337	0.85	25.72±0.62	8.577±0.18	40.66±0.00	37.57±0.73
OF-10 (100 %HX)	56	0.91	43.11±0.12	12.11±0.25	36.38±0.01	38.20±0.54
			OF-5 (EA/HX)			
OF-11 (0.5%EtOAc/HX)	71	0.25	35.56±0.14	11.68±0.23	NA	NA
OF-12 (1%EtOAc/HX)	55	0.37	55.34±0.19	25.54±0.17	NA	NA
OF-13 (1.5%EtOAc/HX)	289	0.54	64.52±0.32	26.75±0.28	48.16±0.00	46.56±0.09
OF-14 (2%EtOAc/HX)	92	0.68	39.42±0.15	11.04±0.14	NA	NA
OF-15 (3%EtOAc/HX)	26	0.72	41.46±0.09	17.90±0.24	NA	NA
OF-16 (4%EtOAc/HX)	49	0.86	46.88±0.21	22.73±0.36	NA	NA
OF-16 (5%EtOAc/HX)	250	1.86	46.88±0.22	22.73±0.37	NA	NA
			OF-8 (EA/HX)			
OF-17 (2 %EtOAc/HX)	48	0.24	56.66±0.05	30.81±0.27	NA	NA
OF-18 (5 %EtOAc/HX)	35	0.35	53.84±0.01	31.67±0.18	NA	NA
OF-19 (10 %EtOAc/HX)	49	0.39	52.19±0.10	26.17±0.16	NA	NA

OF-20 (12 %EtOAc/HX)	27	0.48	64.48±0.09	25.11±0.22	NA	NA
OF-21 (15 %EtOAc/HX)	244	0.55	58.58±0.02	27.81±0.26	48.24±0.05	47.56±0.01
OF-22 (20 %EtOAc/HX)	44	0.67	35.24±0.13	11.54±0.14	NA	NA
OF-23 (25 %EtOAc/HX)	168	0.73	56.42±0.42	26.45±0.11	NA	NA
OF-24 (30 %EtOAc/HX)	45	0.84	54.54±0.22	27.46±0.19	NA	NA
OF-25 (50 %EtOAc/HX)	38	0.92	35.24±0.15	14.25±0.57	NA	NA
OF-26 (70 %EtOAc/HX)	54	0.24	58.34±0.01	28.71±0.11	NA	NA
OF-13 (P TLC 10% EA/HX)						
OF-27	36	0.24	61.85±0.25	25.67±0.28	NA	NA
OF-28	61	0.37	65.32±0.41	26.48±0.35	NA	NA
OF-29	55	0.42	70.95±0.13	31.33±0.28	52.32±0.03	50.45±0.26
OF-30	32	0.55	60.15±0.16	22.24±0.27	NA	NA
OF-31	47	0.68	58.55±0.21	21.09±0.15	NA	NA
OF-32	25	0.79	67.49±0.09	27.22±0.23	NA	NA
OF-21 (P TLC 10% MeOH/$CHCl_3$)						
OF-31	54	0.24	68.34±0.01	28.71±0.11	NA	NA
OF-32	45	0.35	69.13±0.29	31.43±0.39	NA	NA
OF-33	61	0.47	58.29±0.00	24.92±0.10	NA	NA
OF-34	35	0.54	71.05±0.10	33.81±0.05	54.26±0.05	52.64±0.06
OF-35	29	0.69	72.21±0.01	30.54±0.22	NA	NA

DPPH and ABTS radical scavenging activities at 0.1 mg/mL was expressed in percent;COX-2 inhibitory activity at 0.1 mg/mL was expressed in percent; LOX-5 inhibitory activity at 0.05 mg/mL was expressed in percent; NA – not assayed,*ie*, the fractions with low yield were evaluated only for DPPH and ABTS radical scavenging activity. CC – column chromatography; P TLC -preparative thin layer chromatography; MeOH - methanol; EtOAc - ethyl acetate; HX - *n*-hexane; $CHCl_3$- chloroform; OCT- EtOAc-MeOH fraction of *O.dolffusi*.

5.2.3.1 Chromatographic purification and spectral analyses of secondary metabolites from *O.dolffusi*

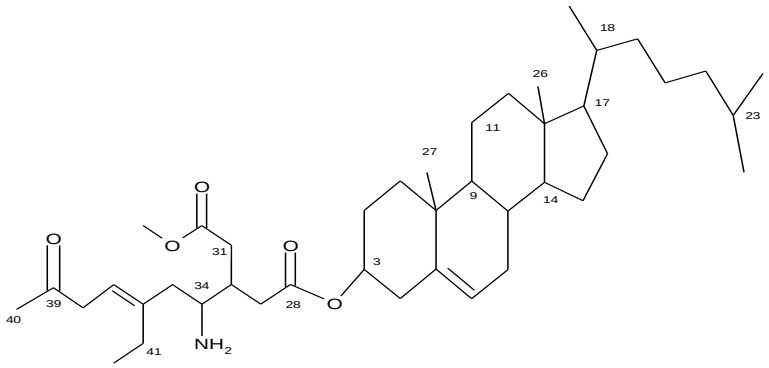
Chromatographic separation led to the isolation of two new derivatives. The molecular structures of the purified compounds were proposed on the basis of comprehensive analysis of the ^{1}H NMR, ^{13}C NMR, including 2D-NMR experiments (^{1}H–^{1}H–COSY, HMQC, HMBC, and NOESY), and mass spectra.

5.2.3.1.A Chromatographic purification and spectral analyses of cholesta-5-en-3-yl-(32-methyl-(30-((*E*)-34-amino-36-ethyl-39-oxohept-36-enyl)-pentanedioate (9).

The compound 9 is cholesta-5-en-3β-yl-(32-methyl-(30-((E)-34-amino-36-ethyl-39-oxohept-36-enyl)-pentanedioate, new phenanthere derivative was isolated as light yellow solid upon repeated chromatography over silica columns/TLC. The olefinic (C=C) and ether (C-O-C) groups were assigned by the absorption bands at 1642 and 1068 cm^{-1} respectively. The UV absorbances at λ_{max} (log ε) 252nm (3.61) were assigned to be a chromophore with carbonyl groups and olefinic system. The mass spectrum exhibited a molecular ion peak at *m/z*667 (HRESIMS m/z 668.0425 [M+1] amu), which in combination with ^{1}H and ^{13}C NMR data **(Table 5.11)** assigned the molecular formula as $C_{42}H_{69}NO_5$with 9 degrees of unsaturation. Five degrees of unsaturation were from the double bonds and the remaining four degrees of unsaturation were due to cyclic ring systems, which was not of aromatic type.

Table 5.13 NMR spectroscopic data of cholesta-5-en-3β-yl-(32-methyl-(30-((E)-34-amino-36-ethyl-39-oxohept-36-enyl)-pentanedioate (**9**) isolated from *Octopus dollffusi*.

Compound 9



Position	^{13}CNMR	δ^{1}H NMR (int., mult., J in Hz)[b]	^{1}H-^{1}H COSY	HMBC(^{1}H-^{13}C)
1	37.01	1-H	1.30(m,2H)	2-H
2	31.87	2-H	1.98(m,2H)	-
3	73.69	3-H	4.52(m,1H)	1-H,2-H,4-H
4	42.31	4-H	2.30(m,2H)	-

5	139.7	-	-	-
6	122.57	6-H	5.35(dd,1H)	7-H
7	31.92	7-H	2.28(m,2H)	8-H
8	31.79	8-H	1.98(t,1H)	14-H
9	50.03	9-H	0.95(m,1H)	11-H
10	36.19	-	-	-
11	21.03	11-H	1.49(m,2H)	12-H
12	39.74	12-H	1.26 (t,2H)	11-H
13	42.31	-	-	-
14	56.14	14-H	1.03(m,1H)	8-H
15	24.28	15-H	1.58(m,2H)	16-H
16	28.23	16-H	1.86(m,2H)	17-H,15-H
17	56.14	17-H	0.99(m,1H)	18-H
18	35.79	18-H	1.27(m,1H)	19-H
19	18.73	19-H	0.98	20-H
20	36.19	20-H	1.83(m,2H)	-
21	23.83	21-H	1.80(m,2H	22-H
22	39.52	22-H	1.85(m,2H	23-H
23	28.01	23-H	0.90 (m,1H)	25-H,24-H
24	22.82	24-H	1.99(m,3H)	23-H
25	22.69	25-H	1.92(m,3H)	23-H
26	11.85	26-H	0.70(s,3H)	-
27	19.32	27-H	1.02(s,3H)	-
28	173.35	-	-	-
29	34.59	29-H	2.34(d,2H)	33-H
30	29.36	30-H	2.04(m,1H)	29-H
31	34.73	31-H	2.23(dd,2H)	-
32	174.36	-	-	-
33	51.42	33-H	3.59(s,3H)	
34	24.96	34-H	1.06(m,1H)	35-H[b]
35	39.76	35-H[a],	1.78(dd,1H)	-
		35-H[b]	1.93(dd,1H)	-
36	155.38	-	-	-
37	129.91	37-H	5.27(td,1H)	38-H
38	29.65	38-H	2.73(d,2H)	37-H
39	195.37	-	-	
40	34.59	40-H	2.23(s,3H)	
41	38.09	41-H	2.21(m,2H)	42-H
42	14.11	42-H	1.54(t,3H)	-

[a] NMR spectra were recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometers.
[b] Values in ppm, multiplicity and coupling constants (J¼ Hz) were indicated in parentheses. The assignments were made with the aid of the 1H-1H COSY, HSQC, HMBC and NOESY experiments.

The molecular ion peak appeared at *m/z*667.5, which undergoes elimination of the methyl groups to afford a daughter ion with *m/z* 625.5 ($C_{39}H_{63}NO_5^+$).The latter appeared to undergo molecular fragmentation to afford the fragment with *m/z*583.4($C_{36}H_{57}NO_5$).The multiple electronegative centres of the compound and electron delocalization served as the potential electronegative centres of the compound resulting in higher antioxidative and anti-inflammatory potential.

The olefinic and ether groups were assigned due to the FTIR absorption bands at 1642 and 1068 cm^{-1}, respectively. The mass spectral data displayed the molecular ion peak at *m/z* 667 (HRESIMS *m/z*: found for$C_{42}H_{69}NO_5$667.5180 [$(M)^+$], cal. 667.5176), with 9 degree of unsaturations. Five degrees of unsaturations were attributed to the double bonds and the remaining four were due to the tetracyclic rings.

The ^{13}C along with DEPT NMR data revealed the occurrence of sixteen methylenes, eleven methines, eight methanes and seven quaternary positions in the molecule. The characteristic olefinic signals were observed at δ 139.7/122.5 in the sterol framework (C5=C6) and δ 155.3/129.9 in the side chain (C36=C37) at C-3. Highly deshielded proton signals at δ 5.35 (H-3) and 5.27 (H-33) were found to correspond to alkenes, whereas δ 4.52 and 3.59 were represented by the carboxylate end of methylene and methyl groups positioned at C-3 and C-33, respectively.

The ^1H NMR spectrum displayed five methyls at δ 0.70 (s), 1.02 (s), 0.98 (m), 0.81 (d) and 0.83 (d), which were connected to the carbons at δ 11.8 (C-18), 19.3 (C-19), 18.7 (C-21), 22.8 (C-26) and 22.6 (C-27), respectively based on the HSQC experiments. These groups were characteristic groups of the sterol framework. The chemical shift value at δ 4.52 (H-3) exhibiting

HSQC correlation with δ 73.6 (C-3) was found to be higher than the usual >CH-OH shift of δ 3.50. This validated that C-3 was directly attached to the oxygen atom of highly electronegative group like carboxylate.

The ^{1}H-^{1}H COSY experiment exhibited five spin systems in the sterol moiety, and were δ 1.30 (assigned to H-1)/1.98 (H-2)/4.52 (H-3)/2.30 (H-4); δ 5.35 (H-6)/2.28 (H-7)/1.98 (H-8)/0.95 (H-9)/1.49 (H-11)/1.26 (H-12) along with δ 1.98 (H-8)/1.03 (H-14); δ 1.58 (H-15)/1.86 (H-16)/0.99 (H-17)/1.27 (H-20)/0.98 (H-21); δ 1.83 (H-22)/1.80 (H-23)/1.85 (H-24) and δ 1.99 (H-25)/0.81 (H-26)/0.83 (H-27), which established the basic C-27 sterol skeleton. The HMBC correlations between δ 1.98 (H-2) to δ 37.8 (C-1), 73.6 (C-3); δ 2.30 (H-4) to δ 37.0 (C-1), 31.8 (C-2), 73.6 (C-3), 139.7 (C-5), 122.5 (C-6); δ 1.49 (H-11) to δ 139.7 (C-5), 50.0 (C-9), 39.7 (C-12) and δ 1.26 (H-12) to δ 50.0 (C-9), 42.3 (C-13) further corroborated the occurrence of sterol skeleton. The deshielded peak at δ 4.52 (H-3) was found to be the junction point of the side chain, 30-(34-amino-36-ethyl-39-oxohept-36-enyl)-pentanedioate.

Three spin system were found in the side chain, δ 1.78, 1.93 (H-35)/1.06 (H-34)/2.04 (H-30)/2.23 (H-31), 2.34 (H-29); δ 5.27 (H-37)/2.73 (H-38) and δ 2.21 (H-41)/1.54 (H-42) .The ^{13}C peaks at δ 173.3 (C-28) and 174.3 (C-32) represented ester carbonyl and the peak at δ 195.3 (C-39) was related to carbonyl moiety in the side chain. The long range C-H couplings from δ 2.34 (H-29) to δ 34.7 (C-31), 51.4 (C-33); δ 2.04 (H-30) to δ 174.3 (C-32); δ 2.23 (H-31) to δ 155.3 (C-6), 39.7 (C-35); δ 5.27 (H-34) to δ 195.3 (C-39) and δ 2.21 (H-41) to δ 155.3 (C-36), 39.7 (C-35), 24.9 (C-34) attributed to the side chain fragment. The proton at δ 4.52 (H-3) displaying $J_{1,3}$=4.1 indicated that the proton was situated in the axial position thereby indicating that the C-3 attached carbonyl was at the equatorial position.

The spatial correlations at the chiral centers, δ 4.52 (H-3) and δ 2.04 (H-30) were showed NOE correlation, which implied that they were in the same plane and considered as α-protons. The methine proton at C-3 group did not show NOE signal with H-18 and H-19, which indicated that these were situated at the opposite plane and β-oriented. There was no NOESY correlation was found between δ 5.27 (H-37) and δ 2.73 (H-38), 2.21 (H-41). However, NOESY relation was apparent among δ 2.73 (H-38) and δ 2.73 (H-38) which suggested the *trans* (*E*) orientation of trisubstituted alkene >C(36)=C(37) in the side chain of **3**.

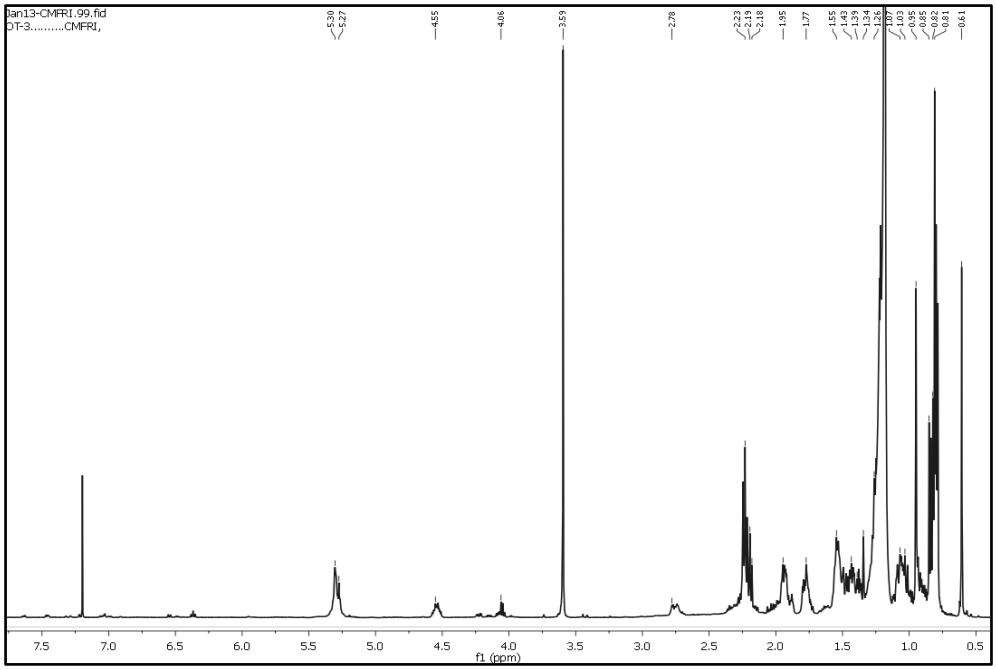


Fig 5.12.A. ^{1}H NMR spectrum of cholesta-5-en-3β-yl-(32-methyl-(30-((E)-34-amino-36-ethyl-39-oxohept-36-enyl)-pentanedioate (**9**).

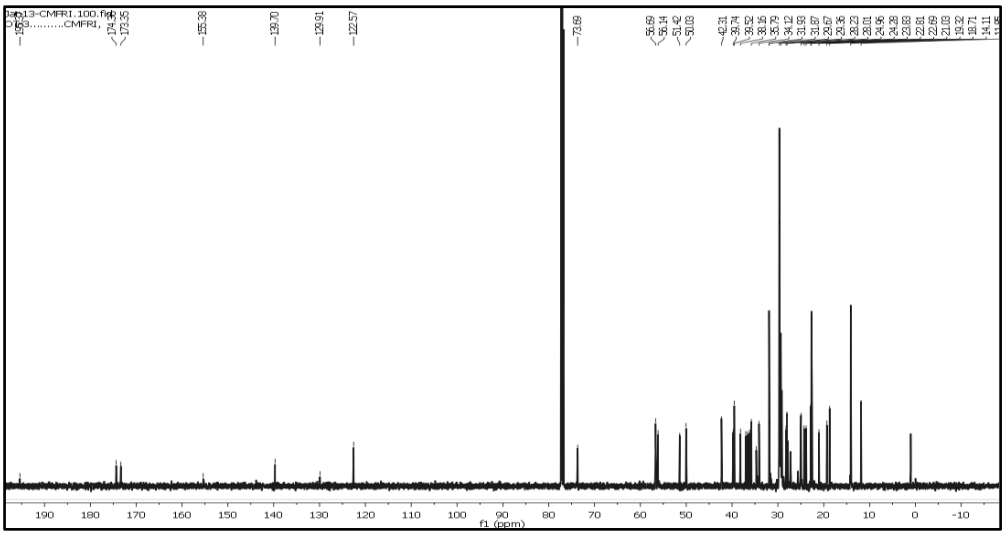


Fig 5.12.B. ^{13}C NMR spectrum of cholesta-5-en-3β-yl-(32-methyl-(30-((E)-34-amino-36-ethyl-39-oxohept-36-enyl)-pentanedioate **(9)**.

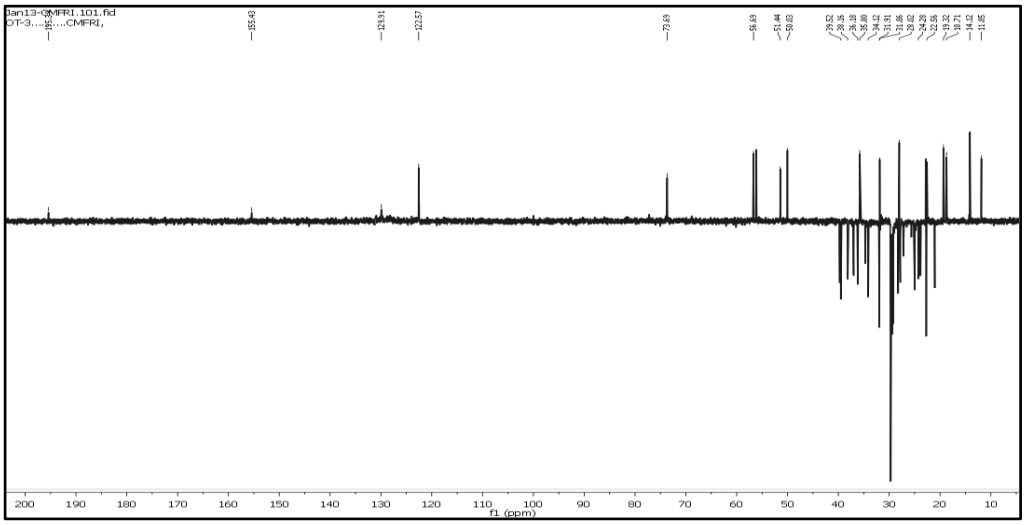


Fig 5.12.C. DEPT NMR spectrum of cholesta-5-en-3β-yl-(32-methyl-(30-((E)-34-amino-36-ethyl-39-oxohept-36-enyl)-pentanedioate (**9**).

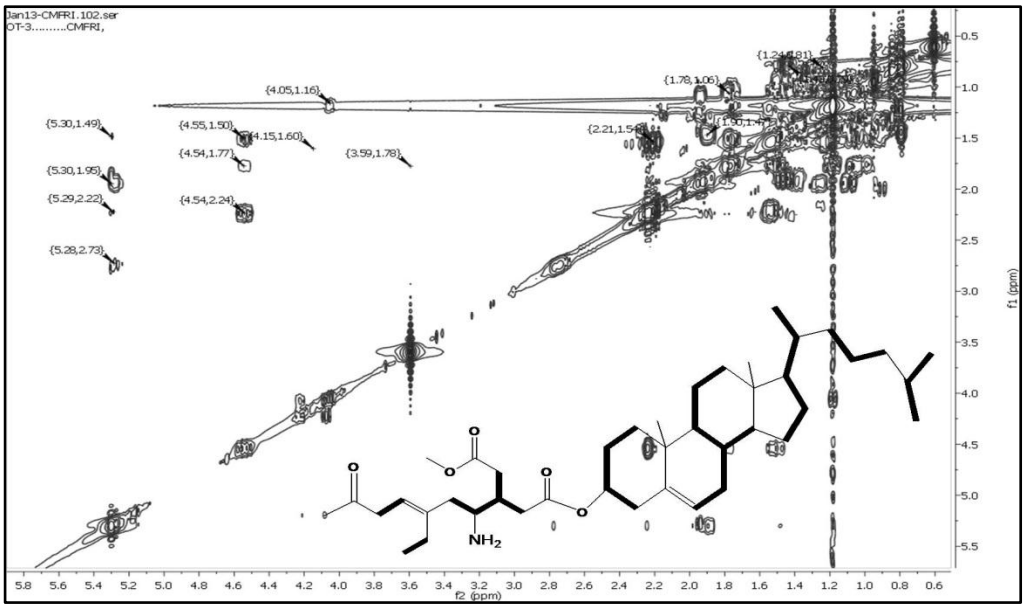


Fig 5.12.D. $^{1}H–^{1}H$ COSYspectrum of cholesta-5-en-3β-yl-(32-methyl-(30-((E)-34-amino-36-ethyl-39-oxohept-36-enyl)-pentanedioate (**9**).

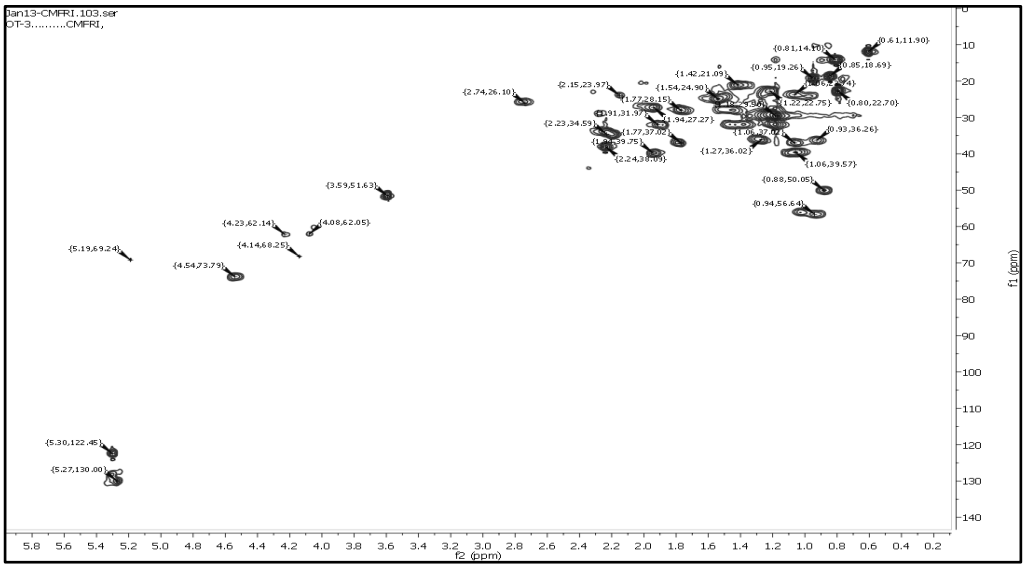


Fig 5.12.E. HSQC spectrum cholesta-5-en-3β-yl-(32-methyl-(30-((E)-34-amino-36-ethyl-39-oxohept-36-enyl)-pentanedioate (**9**).

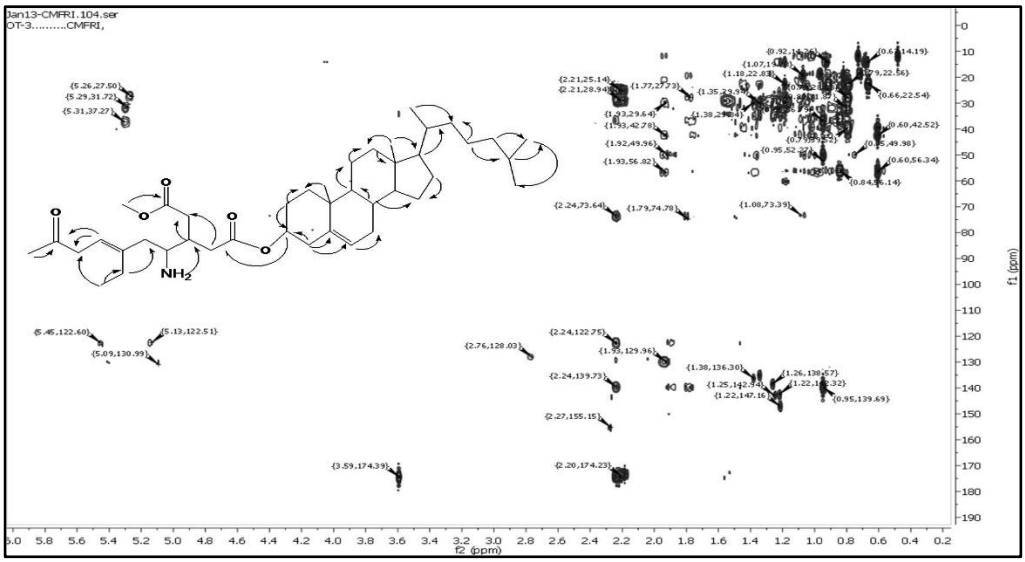


Fig 5.12.F. HMBC spectrum cholesta-5-en-3β-yl-(32-methyl-(30-((E)-34-amino-36-ethyl-39-oxohept-36-enyl)-pentanedioate (**9**).

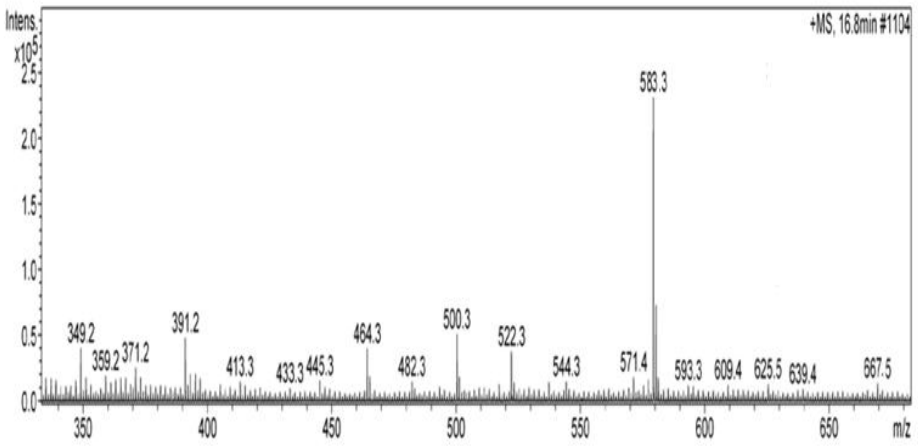


Fig 5.12.G Mass spectrum of cholesta-5-en-3β-yl-(32-methyl-(30-((E)-34-amino-36-ethyl-39-oxohept-36-enyl)-pentanedioate (**9**).

5.2.3.1.B Chromatographic purification and spectral analyses of 7-ethyl-9-vinyl-octahydroazuleno[1,8-*bc*]pyran-3,12-dione (10)

The compound 7-ethyl-9-vinyl-octahydroazuleno[1,8-bc]pyran-3,12-dione (**9**), a new pyranderivative, was isolated as white solid upon

repeatedchromatography over silica columns/TLC. The presence of 4-ethyl-6-vinyl-2,3,6,7,7a,8,9,9a-octahydroazulene and pyran-2,9-dione, groups were confirmed by detailed NMR and mass spectroscopic experiments.

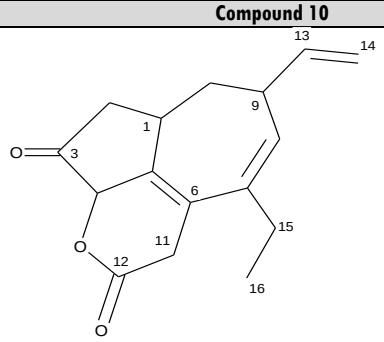
The compound 7-ethyl-9-vinyl-octahydroazuleno[1,8-*bc*]pyran-3,12-dione (**10**), a new derivative of substituted pyran was isolated as a yellow solid. It exhibited 8 degrees of unsaturation which includes five double bonds and three cyclic ring system. The 1D (^{13}C and DEPT) NMR data demonstrated the presence of five each of methylenes, methines and quaternary carbons (Table S2). The alkenic signals at δ 5.81 (H-13) and 4.97 (H-14) appeared significantly deshielded due to its position at the terminal (-C<u>H</u>=C<u>H</u>$_2$) part of the alkenic side chain, which was directly attached to the ring system at C-9. The quaternary carbon appeared at δ 148.0 (C-5) was established as the junction point between the pyran ring system and octahydroazulene ring. The peaks at δ 148.0 (C-5), 131.3 (C-6), 139.2 (C-7) and 122.2 (C-8) indicated the presence of olefinic groups, which deduced the presence of tricyclic skeleton by the HMBC experiments. The ^{13}C signals at δ 196.4 (C-3) and 173.0 (C-12) were found to correspond to ketone group in the cyclopentanone ring and ester carbon of pyranone ring, respectively. The proton δ 1.46 (H-1) showed HMBC correlations with δ 28.9 (C-2) and 22.6 (C-10). Likewise, the proton δ 3.69 (H-4) afforded HMBC relation with δ 148.0 (C-5) and 196.4 (C-3), which indicated that the pyran ring was attached to the substituted azulene ring in the compound. The ^{1}H-^{1}H COSY exhibited two spin systems, which were due to δ 2.01 (assigned to H-2)/1.46 (H-1)/1.38 (H-10)/2.04 (H-9)/5.37 (H-8) along with δ 2.04 (H-9)/5.81 (H-13)/4.97 (H-14) corresponding to the octahydro azuleno ring attached to vinyl moiety at C-9 and δ 1.29 (H-15)/0.87 (H-16) for terminal ethyl side chain at C-7. The structure and its side chain attachments were further corroborated by HMBC correlations. The long range couplings from δ 3.69 (H-4) to δ 148.0 (C-5), 196.4 (C-3); δ 5.37 (H-8) to δ 139.2 (C-7); δ 2.04 (H-9) to δ 137.0 (C-13), 114.0 (C-14); δ 2.33 (H-11) to δ 173.0 (C-12), 131.3 (C-6); δ 4.94 (H-14) to δ 33.8 (C-9) and δ 1.29 (H-15) to δ 139.2 (C-7)

validated the structural attributions of the compound **10**. The relative sterochemistries at the chiral centres, δ 1.46 (H-1) were exhibited NOESY cross-peaks with δ 2.04 (H-9), which implied that these were in the same plane and denoted as *β*-protons. Therefore, the vinyl group at C-9 was placed at the opposite face of *α*-protons. The proton at δ 3.69 (H-4) did not show NOE correlation with H-1, thereby indicating its *α*-disposition with reference to the molecular plane of symmetry. The olefinic, carbonyl and ether groups were symbolized by the absorption bands at 1541, 1736 and 1070 cm^{-1},respectively. The characteristic IR absorption spectra at 721 (C-H rocking), 1736 (C-CO-C stretching) and 2923 cm^{-1} (C-H stretching of alkane) supported the structural attributions of 7-ethyl-9-vinyl-octahydroazuleno[1,8-*bc*]pyran-3,12-dione (**10**).

The LCMS spectrum of the compound **10,** showed an sodium adduct (2M+1) peak at *m/z* 281.1 ($C_{16}H_{18}O_3Na$ •+).The mass spectrum exhibited a molecular ion peak at *m/z* 258.1, which in combination with its ^{1}H and ^{13}C NMR data (**Table 5.14**) indicated the elemental composition of $C_{16}H_{18}O_3$.The molecular ion peak at *m/z* 258.1 appeared to undergo elimination of C_2H_2 (*m/z* 26) to yield *m/z* 232.1($C_{14}H_{16}O_3$.The mass spectrum of 7-ethyl-9-vinyl-octahydroazuleno[1,8-bc]pyran-3,12-dione were presented in **Fig 5.10**.

Table 5.14. NMR spectroscopic data of and 7-ethyl-9-vinyl-octahydroazuleno[1,8-bc]pyran-3,12-dione (**10**) isolated from *Octopus dollffusi*.

Compound 10



Position No.	^{13}C NMR	δ^{1}H NMR (int., mult., J in Hz)[b]	^{1}H-^{1}H COSY	HMBC(^{1}H-^{13}C)
1	1	29.62	1-H	1.46(m,1H)
2	2	28.96	2-H	2.01(d,2H)

3	3	196.4	-	-
4	4	55.9	4-H	3.69(s,1H)
5	5	148.08	-	-
6	6	131.3	-	-
7	7	139.28	-	-
8	8	122.28	8-H	5.37(d,1H)
9	9	33.82	9-H	2.04(m,1H)
10	10	22.69	10-H	1.38(t,2H)
11	11	51.23	11-H	2.33(s,2H)
12	12	173.09	-	-
13	13	137.01	13-H	5.81(m,1H)
14	14	114.05	14-H	4.97(m,2H)
15	15	31.93	15-H	1.29(m,2H)
16	16	14.11	16-H	0.87(t,3H)

[a] NMR spectra were recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometers.
[b] Values in ppm, multiplicity and coupling constants (J¼ Hz) were indicated in parentheses. Assignments were made with the aid of the 1H-1H COSY, HSQC, HMBC and NOESY experiments.

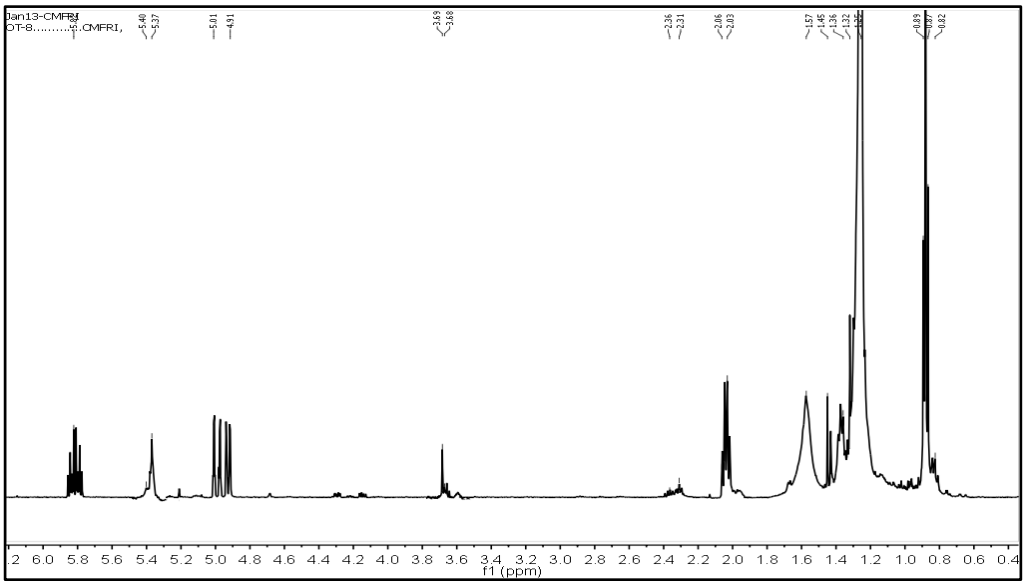


Fig 5.13. A. ^{1}H NMR spectrum of 7-ethyl-9-vinyl-octahydroazuleno[1,8-bc]pyran-3,12-dione (**10**).

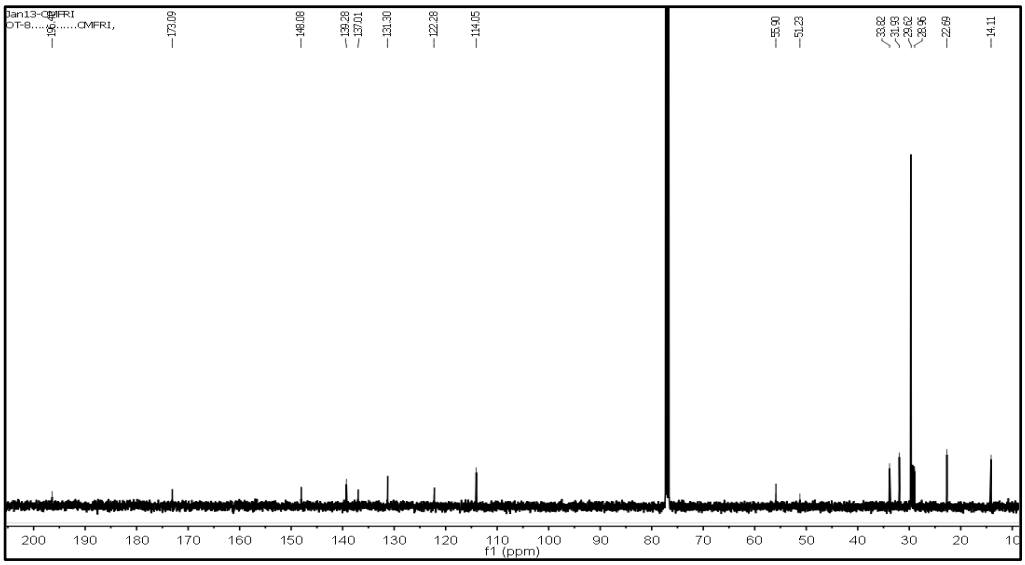


Fig 5.13.B. ^{13}C NMR spectrum of 7-ethyl-9-vinyl-octahydroazuleno[1,8-bc]pyran-3,12-dione (**10**).

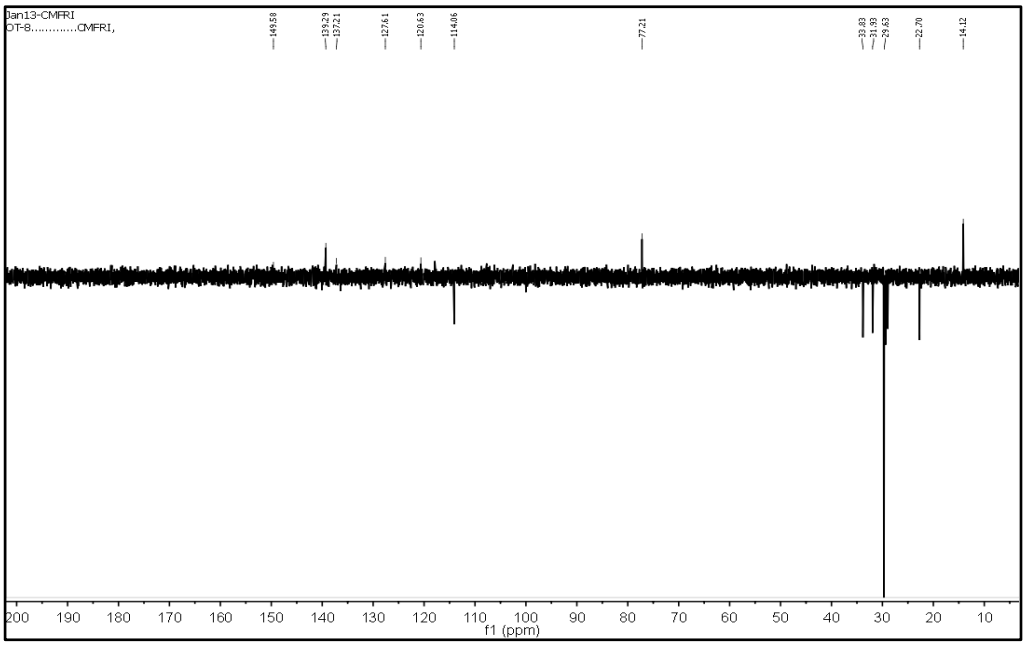


Fig 5.13.C. DEPT NMR spectrum of 7-ethyl-9-vinyl-octahydroazuleno[1,8-bc]pyran-3,12-dione (**10**).

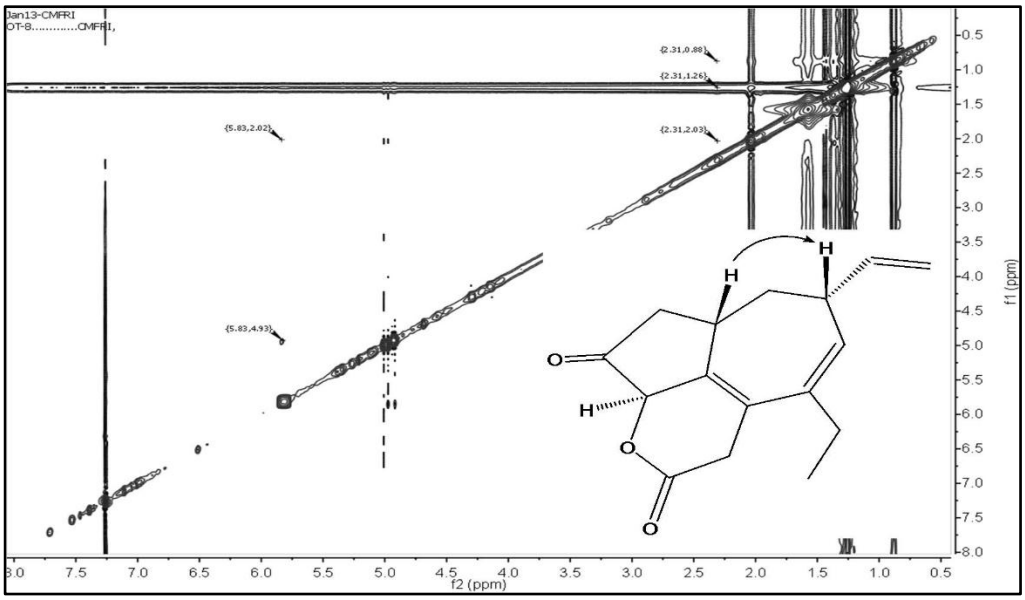


Fig 5.13.D. NOE spectrum 7-ethyl-9-vinyl-octahydroazuleno[1,8-bc]pyran-3,12-dione (**10**).

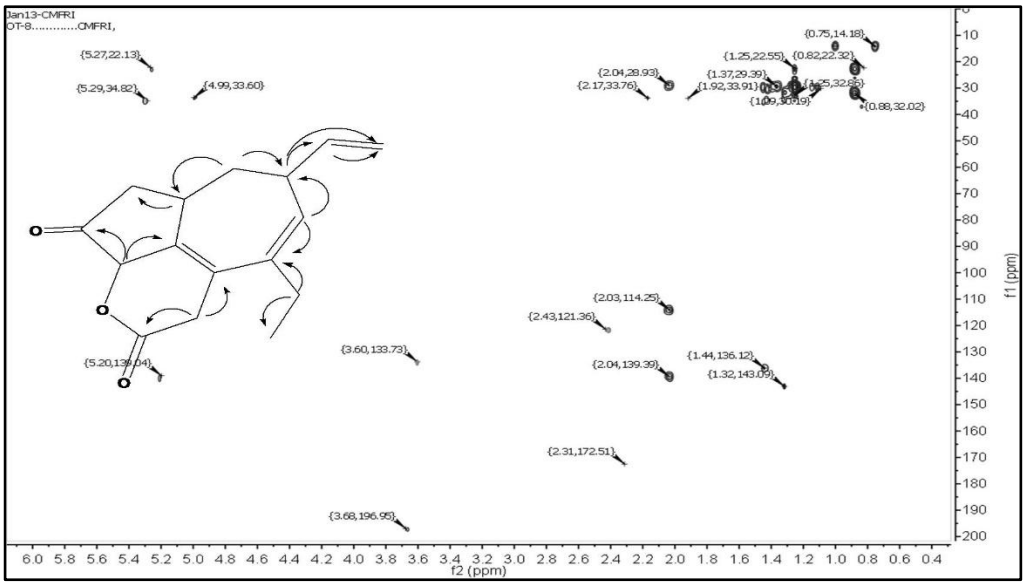


Fig 5.13.E. HMBC spectrum of 7-ethyl-9-vinyl-octahydroazuleno[1,8-bc]pyran-3,12-dione (**10**).

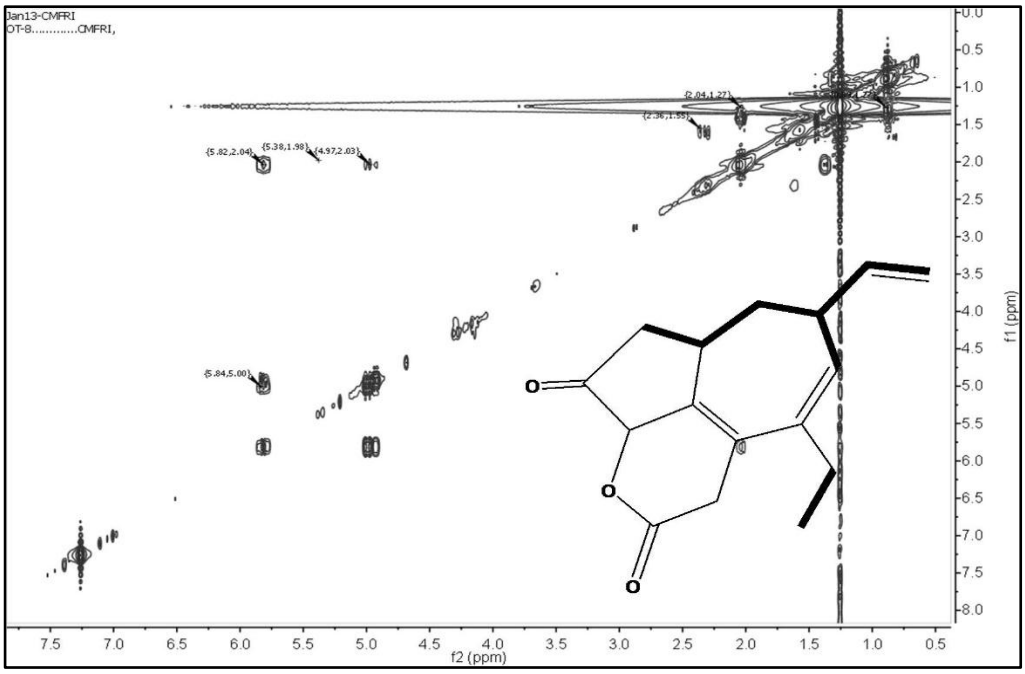


Fig 5.13.F. $^{1}H-^{1}H$ COSYof 7-ethyl-9-vinyl-octahydroazuleno[1,8-bc]pyran-3,12-dione (**10**).

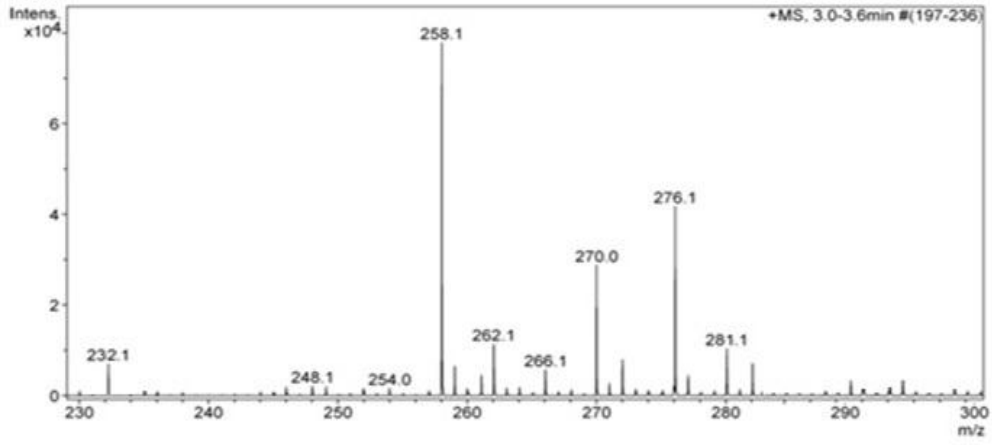


Fig 5.13.G. Mass spectrum of 7-ethyl-9-vinyl-octahydroazuleno[1,8-bc]pyran-3,12-dione (**10**).

5.2.3.2 Conclusions

The ethyl acetate-methanol extract of *Octopus dolffusi*yielded two pure compounds, namely, cholesta-5-en-3β-yl-(32-methyl-(30-((E)-34-amino-36-

ethyl-39-oxohept-36-enyl)-pentanedioate (**9**) and 7-ethyl-9-vinyl-octahydroazuleno[1,8-bc]pyran-3,12-dione (**10**). The structures of the compounds, as well as their relative stereo chemistries, were established by means of spectral data analyses, including 2D-NMR experiments. Both compounds (**9** and **10**) isolated from *Octopus dolffusi*showed similar effect in inhibiting the DPPH and $ABTS^{\cdot+}$radicals. The *in vitro* anti-inflammatory properties with respect to COX-2 and 5-LOX inhibitory activities were also found to be similar for **9** and 10. This study established the potential of marine derived natural alternatives as potential lead antioxidative molecules to the synthetic antioxidants for use in pharmaceutical and food industries. The knowledge on the structural features responsible for antioxidative bioactivities will guide us to synthesize the molecules in commercial scale for use as new generation antioxidant leads.

The compounds belonging to phenanthrenone and chromene derivatives, isolated from the extract of *P. viridis*, demonstrated higher anti-inflammatory and antioxidative properties compared to the compounds isolated from *C. madrasensis* and *O. dolffusi*.The effective antioxidant and anti-inflammatory property of these compounds indicated that they have potential as natural lead molecules in the food industry. The anti-inflammatory potential of these compounds are proved to be comparable to the synthetic anti-inflammatory agents, aspirin and indomethacin. It is therefore, the *P. viridis having* anti-inflammatory potential is used for the preparation of an anti-inflammatory concentrate, for use as nutraceuticals and/or functional foods, and candidates in combating carcinogenesis, oxidative stress-induced and inflammatory diseases which can offer solutions to the drawbacks of the adverse effects of synthetic painkillers.

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Chapter 6

FUNCTIONAL FOOD ENRICHED WITH NATURAL INGREDIENTS FROM GREEN MUSSEL *P. VIRIDIS*

Contents

The increasing knowledge regarding the impact of diet on human health along with state-of-the-art technologies has led to significant nutritional discoveries and product innovations on an unprecedented scale. In particular, the naturally occurring bioactive extracts or single compounds thereof, that are believed to benefit human health, have spawned an important and dynamic new area of research resulting in substantial advances in nutritional knowledge. There is also growing awareness that dietary source and form of food may affect overall health. Suitably, the role of food as an agent for improving health has been recognized, initiating the development of new classes of food, known as functional foods. However, proving that these naturally occurring bioactive substances have a defined health benefit poses a dilemma in nutritional research as investigating preventive activity can be difficult when effect is only moderate. This means that the effect of the compounds on the human body may be very small over relatively short periods, but could contribute significantly to health when they are consumed throughout life as part of the daily diet.

The marine environment is an untapped source of functional ingredients that can be applied to various aspects of food processing, storage, and fortification. The marine world, due to its phenomenal biodiversity, is a rich natural resource of many biologically active compounds, such as, polyunsaturated fatty acids (PUFAs), sterols, proteins, polysaccharides, antioxidants and pigments. Marine-derived nutrients and other marine bioactive components have great potential as functional food ingredients as they possess advantageous physiological effects, with medicinal characteristics and added health benefits, such as, anticancer or anti-inflammatory activities. As reactive oxygen species (ROS) are important mediators that initiate and propagate inflammatory responses by stimulating release of pro-inflammatory mediators the mechanisms for nutritional defense and disease prevention includes ROS scavenging, reduction of peroxides and repair of peroxidized biological membranes. Marine-derived bioactive food ingredients can be derived from a vast array of sources, and several marine invertebrates based bioactive compounds interfere with the pathogenesis of diseases. Among the marine invertebrates, the mollusks are potential sources of bioactive substances. Therefore, to facilitate the nutritional defense and disease prevention the chapter describes suitability of marine-derived bioactive compounds as functional food ingredients for the prevention and treatment of chronic diseases. The chapter is divided into four sections.

6A) Preparation of potential anti-inflammatory combination stabilized with natural anti-oxidants from *P. viridis.*

6B) Enrichment of the potential anti-inflammatory combination by anti-inflammatory principles isolated from *P. viridis*

6C) Response of pro-inflammatory prostaglandin contents in anti-inflammatory supplements from green mussel *P. viridis*

6D) Toxicity profile of the nutraceutical formulation derived from green mussel *P. viridis*

6A) Preparation of potential anti-inflammatory combination stabilized with natural antioxidants from *P. viridis*

Background of the study

There has been a growing interest in functional foods in recent years and the functional foods, enriched with natural ingredients are able to provide or promote a beneficial action for human health. The bioactive compounds in functional foods exist as natural constituents or as fortificants in food having the potential to provide health benefits beyond the basic nutritional value of the product. Marine-derived nutrients and other marine bioactive components have excellent potential as functional food ingredients as they possess advantageous physiological effects, with medicinal characteristics and added health benefits, such as, anticancer or anti-inflammatory activity.

Inflammation is a process involved in the pathogenesis of several disorders *viz*., arthritis and cardiovascular diseases (Calder, 2006). Cycloxygenase (COX) and lipoxygenase (LOX) are the two major enzyme families that catalyze the rate-limiting step in the formation of prostanoids, prostaglandins, and thromboxane A_2 by the COX pathway, and leukotrienes by the LOX pathway, whose products are significant mediators of pain, fever and inflammation (Coy *et al*., 2009). COX-1 is constitutive, and biosynthesize prostaglandins (PGs) that protect the stomach and kidney from damage. COX-2 is induced by inflammatory stimuli, such as cytokines, and produces prostaglandins (PGs) that contribute to the pain and inflammatory disorders. The systemic inhibition of the COX-1 leads to a subsequent reduction in cytoprotective PGs required for an effective mucosal defense.

Current symptomatic treatment of inflammation is mainly by non-steroidal anti-inflammatory drugs (NSAIDs), which exert anti-inflammatory

actions by inhibiting cycloxygenase enzymes (Jaggi *et al*., 2004). NSAIDs produce intestinal tract ulcers (with potential internal bleeding) in 10-30 percent of long-term users, and erosions of the stomach lining and intestinal tract in 30-50 percent of cases (McPhee *et al*., 2007). Selective COX-2 inhibitors (Coxibs) have come to attention in recent years, although they too exhibit side effects, most notably in relation to cardiovascular disturbances. It is of note that the simultaneous inhibition of COX-2/5-LOX is important to allow synthesis of lipoxins to resolve inflammation and attenuate any remaining leukotriene effect. As a result, medical researchers are looking for safer, more efficacious alternatives to both the traditional NSAIDs and the Coxibs. It is therefore, necessary to find suitable medications that can inhibit 5-LOX and COX-2 simultaneously while maintaining COX-1/COX-2 ratio below the threshold limit (preferably lesser than 1.0) for targeted and selective activities against the inflammatory response.

Traditionally, indigenous people, notably in Western Mexico and throughout the South Pacific use shellfish supplements as a remedy for arthritis (Whitehouse *et al*., 1997). The commercially available products, such as, freeze-dried extract (Seatone) and CO_2 extracted oil (Lyprinol), obtained from *Perna canaliculus* were reported to inhibit experimentally induced inflammation in the treatment of rheumatoid arthritis and osteoarthritis (McPhee *et al*., 2007). Okinawan mollusk *Pinna muricata* contains a pinnatoxin A, which is reported to have Ca^{2+} channel activating and anti-inflammatory properties (Uemura *et al*., 1995). The green lipped mussel, *P. canaliculus* is restricted to the temperate waters around New Zealand, whereas *Perna viridis* occurs widely in tropical waters throughout the Indo-Pacific region (Spencer, 2002).

Mussels constitute about 14% of total bivalve landing in India (CMFRI, 2003), and are distributed all along the east and west coast. Marine mussels are

abundant in the low tide level to a depth of 10 meters, and being used as food for thousands of years. *P. viridis* considered to be an economically important bivalve mollusk, and is abundantly available in the coastal and estuarine waters of the Indo-Pacific region. *P. viridis* was reported to grow and reproduce at a temperature range between 11-32°C, and a wide-ranging salinity of about 18-33% at about 2m below water surface, rich in phytoplankton. *P. viridis* is harvested in the Indo-Pacific region as a food source due to its exceptionally fast growth and high nutritional properties. *P. viridis* supports a traditional fishery in the Peninsular India, particularly at Malabar and Konkan areas, accounting for the bulk of the mussel production (Kripa and Mohammed, 2008)**.** In recent years, the *n*-3 PUFAs from bivalve mollusks provide an unlimited variety of long chain PUFAs with reported beneficial roles in human health, such as, alleviating symptoms of thrombosis, inflammatory conditions (James *et al*., 2000), and asthma (Emelyanov *et al*., 2002). The extract from *P. viridis* was reported to be active against inflammatory joint diseases, influenza, herpes, HIV, and hepatitis viral strains (Mitra and Chatterji, 2004). Although the coastline of South Indian subcontinent is bestowed with large assemblage of *P. viridis,* they have not been explored in detail regarding their potential to be used as nutritional supplement.

Considering the importance and easy availability of *P. viridis*, compared with other species, an anti-inflammatory concentrate from *P. viridis,* for use as nutraceuticals or functional foods, in combating oxidative stress-induced and inflammatory diseases, has been formulated. Specifically a nutraceutical formulation that can simultaneously inhibit 5-LOX and COX-2 with COX-1/COX-2 ratio below the safety threshold limit leading to selective activity against inflammatory mediators has been prepared.

However it is to be noted that the active ingredients of *P. viridis* extract are lipidic in nature, and therefore, are susceptible to peroxidation (or oxidation) due to the presence of olefinic bonds in their structure, resulting in low molecular weight aldehydes, ketones, and deleterious free radical species under shelf, and are major risk factors for cardiovascular and inflammatory diseases. The product, which is not of sufficient stability, can result in changes in physical (like color, flavor, odor etc.) as well as chemical characteristics (formation of high risk decomposition substances, such as, free radicals, small molecular weight aldehydes, and ketones. The harmful reactive oxygen species (ROS), which attack the biological macromolecules, leading to the formation of lipid hydroperoxides produced either through non-enzymatic ways, or through the action of ROS on polyunsaturated fatty acids, or as specific products of LOX and COX activities. It is therefore, necessary to arrest the oxidation of the mussel active principles by antioxidants, and to retain its anti-inflammatory qualities for a longer period on shelf.

The ROS in food industry is being controlled or minimized by the addition of commercial synthetic antioxidants like BHA (butylated hydroxyanisol), BHT (butylated hydroxytoluene) and TBHQ (*tert*-butyl hydroquinone). Recently, there has been an increasing interest in the use of natural antioxidants, such as, tocopherols and flavonoids for the preservation of food materials because these natural antioxidants avoid the toxicity problems, which arise due to the synthetic antioxidants. These compounds can trap the free radicals directly or scavenge them through a series of coupled reactions with antioxidant enzymes thereby having antioxidant, anti-inflammatory and anticancer activities.

In recent years, the use of antioxidants of natural origin is considerably enhanced, by the concern about the adverse side effects of these popularly

used synthetic antioxidants and other analogues. The present study, therefore, explored natural alternatives to arrest the degradation of vital nutrients with respect to the LC-PUFAs of the *P. viridis* concentrate. The different compositions of oleoresins (*Rosmarinusofficinalis* (ROO) and *Curcuma longa* (CLO) and other natural antioxidant additives in different proportions were blended with the extract (FDPE) in different combinations (GME_1, GME_2, GME_3 and GME_4). These experimental combinations were subjected to accelerated shelf life study for a period of 90 days (d_{90}) to find their individual and synergistic effects, which were able to reduce the free radicals causing oxidation reactions thereby deteriorating the nutritional compositions of the mussel extract.

6A.1 Materials and methods

6A.1.1 Reagents and instrumentation

All solvents (E-Merck, Darmstadt, Germany) were of analytical or high performance liquid chromatography (HPLC) grade as required. All glasswares were rinsed with $CHCl_3/CH_3OH$ (2:1 v/v), and dried under N_2. All other reagents were of analytical grade, and purchased from E-Merck. DPPH (1, 1-diphenyl-2-picrylhydrazyl), Folin–Ciocalteau reagent, bovine serum albumin, trichloroacetic acid and gallic acid are obtained from HiMedia. Standards of fatty acid methyl esters (Supelco^{TM} 37 Component FAME Mix, Catalog No. 47885-U), tert-butyl hydroxyquinone (TBHQ), and boron trifluoride/methanol (14% BF_3/CH_3OH, w/v) were procured from Sigma-Aldrich Chemical Co. Inc. (St. Louis, MO). Unless otherwise stated, starting material, reactants and solvents were used as such, or purified and dried by standard means.

GLC data were recorded on a Perkin-Elmer AutoSystem XL gas chromatograph (HP 5890 Series II, Perkin Elmer, and USA). Carnosic acid,

carnosol, curcuminoids and vitamin content were quantified using reverse-phase isocratic HPLC system (Shimadzu LC-20AD) fitted with a C_{18} column (Phenomenex, 250 mm length, 4.6 mm i.d, 5µ particle size) and photo diode array detector (PDA). HPLC data of amino acids were analyzed using Waters (Waters Corp., Milford, Massachusetts, USA) equipped with a column oven, on-line degasser, and detector (Waters 2487 dual absorbance detector). The spectrophotometric measurements were performed using Varian Cary 50 conc UV-visible spectrophotometer. A benchtop refrigerated centrifuge (Heraeus Biofuge Stratos benchtop centrifuge, Thermo Scientific, Waltham, MA 02454 USA) was used for centrifugation. The distillation under reduced pressure was performed using rotary vacuum evaporator (Heidolph, Germany; Hei-VAP series). The samples for shelf life studies were kept in an incubator (Labline, India). A laboratory Freeze Dryer (Alpha 1-4 LD plus, Germany) was used for freeze-drying the samples.

6A.1.2. Collection of samples

Green mussel *P. viridis* (10 kg) were collected from their natural habitat at Elathur (Lat: 11054'11.6"N; Long: 750 12'.21.8''E), in Kozhikode district in Kerala**.** The shell-on samples of *P. viridis* were thoroughly washed in running distilled water, and the meat (3.0 kg) was manually removed without applying heat. The samples were thereafter homogenized by a grinding machine, kept overnight under -80ºC for freezing, and were freeze-dried in a laboratory freeze dryer (Alpha 1-4 LD plus, Germany) for 36 h to get the freeze-dried green mussel extract (FDPE, 213.6 g; yield 7.1 g/100g). This was then powdered and stored in vacuum packed polyethylene biohazard autoclave bags (Fisherbrand^{TM}, Fischer Scientific) at -80 ºC until further processing.

Standards of curcumin (from *Curcuma longa* ≥ 80%), carnosic acid and carnosol (from *Rosmarinus officinalis* ≥ 91%) were procured from Sigma-

Aldrich Chemical Co. Inc. (St. Louis, MO). The leaves of rosemary were purchased from a local company (BOS Natural Flavors Pvt. Ltd, Cochin, Kerala). Rhizomes of turmeric (*Curcuma longa*) and ginger (*Zingiber officinale*; Zingiberaceae); fruits of tamarind (*Tamarindus indica*; Leguminosae), Indian gooseberry (*Emblica officinalis*; Phyllanthaceae), lemon (*Citrus limon*; Rutaceae) and pineapple (*Ananascomosus*; Bromeliaceae) were collected freshly from the local farms (Cochin, Kerala). The brown seaweeds, *Turbinaria conoides* and *Sargassam myriocystum* (Sargassaceae) were collected from the Gulf of Mannar of Mandapam region on the south east coast of India.

6A.1.3 Preparation and analysis of rosemary and turmeric oleoresins

The air dried (50°C) leaves of *R. officinalis* (50 g) were subjected to soxhlet extraction (70°C) in EtOAc (250 mL), and evaporated to dryness in *vacuo* (50°C; Heidolph, Germany, 5.6 g). The EtOAc extract thus obtained was stirred with *n*-hexane (16 mL) for 1 h. The *n*-hexane solubles were centrifuged (Superspin Plasto Crafts, Mumbai, India) and the centrifugate was concentrated *in vacuo* to obtain semi-purified oleoresin of *R. officinalis* (2.2 g). This was further emulsified with polysorbate 80 (Tween 80; 1:1 w/w) and designated as ROO. Likewise, air-dried (50°C), powdered rhizomes of *C. longa* (100 g) was extracted with EtOAc (800 mL x 3) and concentrated to 50 mL, and to this concentrate, *n*-hexane (100 mL) was added. The mixture was kept at room temperature for 8 h and centrifuged. The residue obtained was dried *in vacuo* to furnish the semi-purified oleoresin of *C. longa* (9 g). This was further emulsified with polysorbate 80 (Tween 80; 1:1 w/w) and designated as CLO.

The quantification of carnosic acid, carnosol in ROO, and curcuminoids in CLO were performed using reverse-phase binary gradient

HPLC system (Shimadzu LC -20AD) fitted with a C_{18} column (phenomenex, 250 mm length, 4.6 mm i.d, 5µ particle size) and photo diode array detector (PDA). Carnosic acid and carnosol were detected at 230 nm by using acetonitrile: 1% acetic acid in HPLC water (55:45, v/v) as mobile phase. The curcuminoids were detected at 420 nm using acetonitrile: 0.1% citric acid in HPLC water (60:40, v/v) as mobile phase.

6A.1.4 Preparation of aqueous extracts of natural additives

The air dried (50°C), pulverized samples of *Z. officinale, T. indica*, *E. officinalis*, *C. limon,* and *A. comosus* (100 g each) were separately refluxed (100°C, 2h) with distilled water (100 mL x 2), filtered, freeze-dried in a laboratory freeze drier (Alpha 1-4 LD plus, Germany) to obtain aqueous extracts of *Z. officinale* (ZOE, 3.5 g), *T. indica* (TIE, 14.1 g), *E. officinalis* (PEE, 3.8 g), *C. limon* (CLE, 3.9 g), and *A. comosus* (ACE, 5.5 g). The shade dried, powdered brown seaweeds, *T. conoides* and *S. myriocystum* (Sargassaceae) (100 g) were refluxed (100°C, 2 h) with distilled water (400 mL x 3) centrifuged, freeze-dried to furnish the aqueous extract of *T. conoides* (TCE, 15 g) and *S. myriocystum* (SME, 13.2 g).

6A.1.5. Preparation of *P. viridis* extract, experimental combinations and designing of stability studies.

The *P. viridis* meat (3.0 kg) acquired manually from the shell-on samples was freeze dried to furnish *P. viridis* extract (FDPE; 213.6 g; yield - 7.1%). By assigning FDPE as the base material, four different combinations of GME with antioxidative oleoresins (ROO an CLO) and aqueous extracts of the additives were prepared according to **Table 6A.1**

Table 6A.1 Compositions (in g/100g) of different treatments (GME_{1-4}) along with control GME_0 (FDPE)

Treatments	Composition (in g/100g)								
	CLO	ROO	ZOE	PEE	CLE	TIE	TCE	SME	ACE
GME_0	0	0	0	0	0	0	0	0	0
GME_1	0.4	0.8	0.05	0.05	0.05	0.05	0.025	0.025	-
GME_2	0.8	0.4	0.05	0.05	0.05	0.05	0.025	0.025	-
GME_3	0.8	0.8	0.05	0.05	0.05	0.05	0.025	0.025	-
GME_4	0.8	0.4	0.05	0.05	0.05	0.05	0.025	0.025	0.05

CLO: *C. longa* oleoresin (water-dispersible); ROO: *R. officinalis* oleoresin (water-dispersible); ZOE: *Z. officinale* lyophilized aqueous extract; PEE: *E. officinale* lyophilized aqueous extract; CLE: *C. limon* lyophilized aqueous extract; TIE: *T. indica* lyophilized aqueous extract; TCE: *T. conoides* lyophilized aqueous extract; SME: *S. myriocystum* lyophilized aqueous extract; ACE: *A. comosus* lyophilized aqueous extract. The additives were dissolved in 100 mL double distilled water. Mussel extract, FDPE (GME_0) was added to this antioxidant solution, stirred and then lyophilized to get different treatments, GME_{1-4}.

In order to find the most effective antioxidant combination, the stability of different biochemical characteristics of these four treatments compared with GME_0 were tested by time dependent accelerated shelf life study in a thermostatically controlled incubator at an elevated temperature of 50°C. The samples (10 g each; GME_{1-4} and GME_0) were kept in screw capped flat bottom culture tubes (Vensil; 30 mL capacity, 20 mL relative volume; 10 mL head space), and were evaluated for the changes in anti-inflammatory and anti-oxidative status (at day 0, 15, 30, 60 and 90), and nutritional assays in relation to the various nutritional indicators namely fatty acids (at day 0, 1, 2, 4, 5, 8, 15, 30, 60 and 90); amino acids and total protein (at day 0 and 90); vitamins and carotenoids (at day 0, 15, 60 and 90).

6A.1.6 Evaluation of antioxidative status of the green mussel extract spiked with various combinations of additives

The capability of the samples to scavenge hydrogen peroxide (H_2O_2) was determined according to the established method (Ruch *et al.*, 1989). A solution of H_2O_2 (10mM) was prepared in phosphate buffer (pH 7.4). Reaction mixtures contained 10mM of H_2O_2 and 5 mg/mL of test samples, and

absorbance values were measured at 0 min after 10 min using wavelength of 240nm. Ascorbic acid was used as the standard and H_2O_2 scavenging activity was expressed as % [H_2O_2] scavenged = $\{(A_0 - A_1)/A_0\}$ 100, where A_0 was the absorbance of the control, and A_1 was the absorbance in the presence of the sample. The amount of total phenolics, 2, 2-diphenyl -1- picrylhydrazyl (DPPH) radical scavenging activity and degree of lipid peroxidation were determined as described earlier (Tai *et al*., 2011). Total phenolic contents of $GME_{0\text{-}4}$ (5mg) were estimated using Folin-Ciocalteu reagent and gallic acid standard. The absorbance was measured at 760 nm and the result was expressed as gallic acid equivalent in mg (mg GAE)/g sample. The lipid peroxidation ability was determined through thiobarbituric acid reactive substances (TBARS) formation inhibitory activity assay. The samples (10 mg) were incubated with AcOH (2 mL, 1.03 g/mL) and an aqueous solution of thiobarbituric acid (TBA, 2 mL, 0.78 g/100 mL) at 95 °C for 45 min. The resultant mixture (5 mL) was cooled to room temperature and clarified by centrifugation (8 000 r/min, 10 min) to get the supernatant. The absorbance of the supernatant was recorded at 532 nm and the antioxidant capacity was expressed as equivalent mmol of malonaldehyde (MDA)/kg of sample. TBARS concentration was calculated using a standard curve based on MDA and expressed as mM malondialdehyde equivalents (mM MDAEQ)/kg of the sample. For ABTS (2, 2′-azino-bis-3-ethylbenzothiozoline-6-sulfonic acid diammonium salt) assay, the procedure followed the method of Arnao *et al*. (Arnao *et al*., 2001) with suitable modifications.

6A.1.7 Nutritional parameters and time series stability studies

Lipid compositions of the samples (0.1g each) were determined using established methods (Bligh and Dyer, 1959) and the fatty acid composition of the total lipids was determined as described elsewhere (Chakraborty and

Paulraj, 2009).Results were expressed as percent of total fatty acids (% TFA). The total digestible protein contents in the samples (0.1g each) were estimated by the established method (Lowry *et al*., 1951) using a UV-Visible spectrophotometer (Varian Cary, USA) and expressed as mg/100g sample. Estimation of amino acid in samples (0.1g each) was carried out using the Pico-Tag method (Heinrikson and Meredith, 1984) using reversed-phase binary gradient HPLC (Waters reversed-phase PICO.TAG amino acid analysis system), fitted with a packed column (dimethylocatadecylsilyl- bonded amorphous silica; Nova-Pak C_{18}, 3.9 X 150 mm) maintained at 38 ± 1°C in a column oven to be detected by their UV absorbance (λ_{max} 254 NM; Waters 2487 dual absorbance detector), and the results were expressed in mg/100g sample. Estimation of fat soluble vitamins (A, D_3, E and K) was carried out by the method of Salo-Vaananen *et al*. (Salo-Vaananen *et al*.,2000) using HPLC equipped with a C_{18} column (Phenomenex, 250 mm length, 4.6 mm I.D., 5µm) in column oven (32 °C) and photo diode array (PDA) detector. Vitamin C was determined based upon the quantitative discoloration of 2, 6-dichlorophenol indophenol titrimetric method as described (AOAC, 2005). The vitamins A, D_3, E, K_1 and C were expressed as µg/100g sample. The total carotenoid content was determined using the method described by Jensen (Jensen, 1978) at 414 nm against 95% EtOH as blank.

6A.1.8. *In vitro* and *in vivo* anti-inflammatory assays

Cyclooxygenase (COX) inhibition assay was performed using established 2, 7-dichlorofluorescein method (Larsen *et al*., 1996) as described in the section 4.1.2.2.1. The 5-lipoxygenase (5-LOX) inhibition assay was carried out using the principle of 1-4 diene (linoleic acid) oxidation to 1- 3-diene (Baylac and Racine, 2003) with modification as illustrated in the section 4.1.2.2.2. The *in vivo* carrageenan-induced mice paw edema experiment was

carried out as previously described (Winter *et al*., 1962) as explained in the section 4.1.2.3.

6A.1.9. Spectroscopic analyses

Fourier Transform Infra Red spectrometer (FTIR) spectra of the compounds under KBr pellets were recorded in a Thermo Nicolet, Avatar 370 explained in **5.1.1.2A.**

6A.1.10 Statistical analyses

One-way analysis of variance (ANOVA) was carried out with the Statistical Program for Social Sciences 13.0 (SPSS, USA, ver. 13.0) to assess for any significant differences between the means. Differences between means at the 5% (P<0.05) level were considered as significant.

6A.2 Results and discussion

6A.2.1 Antioxidant and lipoperoxidation inhibitory activities of the natural additives to the green mussel extract

The antioxidative activities of different compositions of turmeric and rosemary oleoresins when blended with freeze-dried mussel extract (GME_0) were studied in order to find their individual and synergistic effects (**Table 6A.2**). The HPLC chromatograms of carnosic acid, carnosol in ROO, and curcuminoids in CLO were represented in **Fig.6A.1**.The maximum DPPH radical scavenging activity was observed for the ratios in the order: CLO (0.8 g/100g): ROO (0.4 g/100g) > CLO (0.8 g/100g): ROO (0.8 g/100g) > CLO (0.4 g/100g): ROO (0.8 g/100g) (**Table 6A.2**). The TBARS formation inhibitory activity maxima were observed for CLO (0.8 g/100g): ROO (0.4 g/100g) followed by CLO (0.4 g/100g): ROO (0.8 g/100g) (**Table 6A.2**). It is evident that CLO and ROO added individually (1g/100g each) to GME_0 showed

significantly low (P<0.05) TBARS formation inhibitory activity (2.55 & 2.38 mM MDAEQ/kg, respectively). The high antioxidant activity obvious for the mixed combinations compared to individual combinations inferred to be due to the synergistic effects of the individual components (Koduru *et al*., 2007). This study showed that the effective oleoresin combinations imparting anti-oxidative stability to GME_0 are CLO (0.8 g/100g): ROO (0.4 g/100g); CLO (0.8 g/100g): ROO (0.8 g/100g) and CLO (0.4 g/100g): ROO (0.8 g/100g), and therefore, these ratios of oleoresins were selected for making the four experimental combinations. The antioxidant activity of rosemary extract can be attributed to two major phenolic components, carnosic acid and carnosol (Cheung and Tai, 2007). Curcuminoids from *C. longa* rhizomes had been reported to inhibit the activity of the free radicals, and are inhibitors of lipid peroxidation. An equal concentration of water extracted freeze-dried powder samples from different natural sources (ZOE, TIE, PEE, CLE, TCE, SME) were added with the base green mussel extract formulation containing antioxidant oleoresins of ROO and CLO (GME_1 through GME_4), and the respective composition of each, as their individual activity did not influence the results of this study, with each sample treated the same. The study on the DPPH free radical scavenging activities of the trace amounts of natural additives determined individually were in the order: SME ~ TCE ~ PEE (IC_{50} <0.15 mg/mL) > ZOE ~ TIE ~ ACE ~ CLE (IC_{50} 0.3-1.1 mg/mL). The addition of these natural compounds has been demonstrated to enhance the antioxidative stability of the control mussel extract blended with antioxidant oleoresins. Since these natural additives have been added with equal proportion to the base formulation, their individual activities don't have any influence on the results in the comparison of the differing ratios of ROO and CLO. ZOE was reported to contain gingerols, which inhibit the formation of free radicals. CLE contains ascorbic acid as a primary antioxidant component in

addition to citric acid, which make up about 5-6% of the fluid (Gironés-Vilaplana *et al*., 2012). TIE and PEE extracts primarily contain tartaric acid and ascorbate, respectively, the reported antioxidants in addition to the tannins, *viz.*,emblicanin A, emblicanin B (or β-glucogallin), and mucic acid (1,4-lactone 5-O-gallate) (Scartezzini *et al*, 2006). *A. comosus* contains anti-inflammatory enzyme, bromelain that was proved to alleviate various inflammatory conditions (Taussig and Batkin, 1998).

Table 6A.2 Free radical scavenging (DPPH· scavenging activity) and lipid peroxidation inhibitory activities (TBARS formation inhibition activity) with different ratios of oleoresins spiked to the control (GME_0).

Spiked antioxidant (g/100g)	DPPH· scavenging activity*	TBARS formation inhibitory activity **
GME_0(100g)	13.00±0.24^{a}	3.94±0.04^{a}
GME_0(99.9)+ CLO (0.1)	15.60±0.16^{be}	2.85±0.03^{b}
GME_0(99.6)+ CLO(0.4)	18.84±3.13^{ce}	2.99±0.05^{c}
GME_0(99.2)+ CLO(0.8)	22.23±0.47^{df}	2.75±0.02^{d}
GME_0(99)+ CLO(1)	22.26±0.23^{d}	2.72±0.05^{e}
GME_0(99.9)+ ROO(0.1)	16.93±1.81^{e}	2.95±0.13^{c}
GME_0(99.6)+ ROO(0.4)	18.35±0.64^{ce}	2.79±0.09^{d}
GME_0(99.2)+ ROO(0.8)	19.02±0.39^{ce}	2.58±0.02^{e}
GME_0(99)+ ROO(1)	19.03±1.16^{ce}	2.58±0.04^{e}
GME_0(99.7)+ CLO(0.2) + ROO(0.1)	23.12±0.46^{d}	2.31±0.07^{g}
GME_0(98.8)+ CLO(0.8) + ROO (0.4)	26.35±0.44^{g}	2.18±0.07^{h}
GME_0(99.7)+ CLO(0.1) + ROO (0.2)	19.56±0.45^{c}	2.43±0.07^{i}
GME_0(98.8)+ CLO(0.4) + ROO(0.8)	23.61±0.59^{d}	2.19±0.02^{hj}
GME_0(98.4)+ CLO(0.8) + ROO (0.8)	26.18±0.05^{g}	2.23±0.05^{i}
GME_0(98)+ CLO(1) + ROO(1)	26.18±0.15^{g}	2.21±0.07^{hj}

GME_0: freeze-dried mussel extract without additives (control). a-j: Column wise values with different superscripts of this type indicate significant differences (P<0.05) within different ratios. *DPPH activities were expressed as percentage of total antioxidant radical scavenging activity (% TARSA). **TBARS inhibition activity expressed as the equivalence of millimolar malondialdehyde formed per kilogram (mM MDAEQ/kg) of the sample. The oleoresins were added to GME_0 in different concentrations (g/100g) which are given in the parentheses.

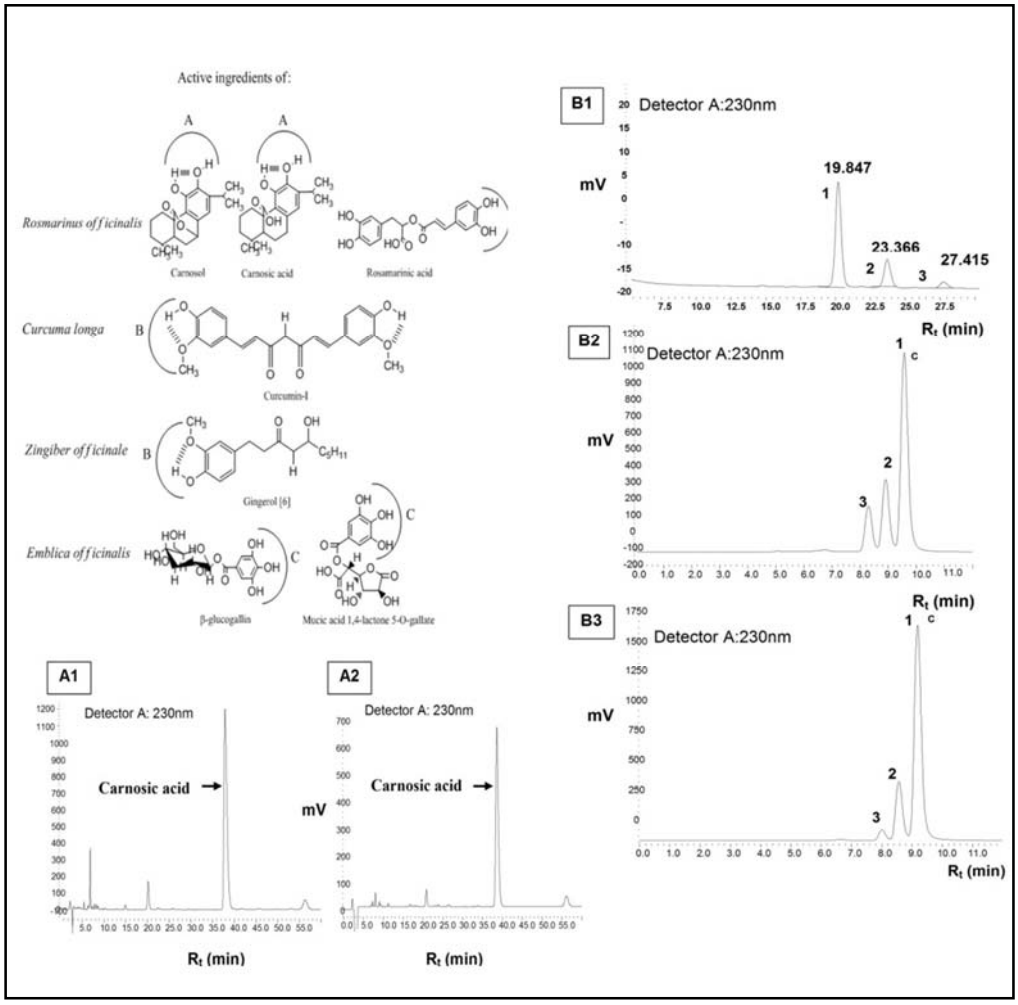


Fig 6A.1 Active antioxidant principles of the additives used to supplement the mussel extract and their structural features responsible for anti-oxidative activities. (**A**) Rosemary phenolic antioxidants bearing *o*-hydroxyphenolic functionalities that produce stable *o*-quinone material, which play a key role as a radical scavenger; (**B**) Curcumin-I bearing ortho-methoxyphenolic functionalities as representative antioxidant principles of turmeric. HPLCchromatograms of (**A1**) standard carnosic acid and (**A2**) rosemary oleoresin. Mobile phase: Acetonitrile: 1% acetic acid (55:45, v/v). HPLC chromatograms of (**B1**) standard curcuminoids and (**B2**) turmeric oleoresin. Mobile phase: Acetonitrile: 0.1% citric acid (60:40, v/v).

6A.2.2 Time series studies of antioxidative activities of various treatments of green mussel extract (GME_0 in relation to GME_{1-4})

The accelerated stability study has been designed using a time and temperature matrix, which can result in robust models to quickly and reliably predict the shelf life period. In this study, accelerated testing is performed at elevated temperatures in an attempt to obtain information on the shelf life of the product in a relatively short time. The accelerated shelf life study of 90 days was carried out in this experiment by following the International Conference on Harmonization (ICH) guidelines (ICH, 2003). The duration of the shelf life period under the accelerated stability conditions (50°C) with acceptable stability for 60-120 days is equivalent to that of two years stability at room temperature. The shelf-life of the product rich in lipidic ingredients (as in green mussel extract) is primarily determined by the oxidative status at various time intervals during the storage period as described below.

6A.2.2.1 Hydrogen peroxide scavenging ability

H_2O_2 formed can release $H^{\cdot}$, $O^{\cdot}$, $HO^{\cdot}$, or $HOO^{\cdot}$ free radicals, which can start a sequel of oxidative chain reaction. Initially the treatments GME_2 and GME_4 registered higher H_2O_2 scavenging abilities than other treatments. A marginal reduction (with respect to d=0, 0^{th} day) in the H_2O_2 scavenging ability of GME_2 and GME_4 were more apparent after 90 days (d=90) of accelerated stability study than in GME_3, GME_1, and GME_0, which exhibited a significant ($P<0.05$) reduction in activity during the same time period. The high H_2O_2 scavenging activity of these treatments is attributed to the

polyphenolic compounds present in the extracts of *R. officinalis*, *Z. officinalis*, and *P. emblica*to scavenge $HOO^{\cdot}$, superanions and $HO^{\cdot}$ radical **Table 6A.3**.

6A.2.2.2 Total polyphenolic content

The total phenolic content was observed to be significantly higher in the treatments GME_1 - GME_4 than GME_0 ($P<0.05$) (**Table 6A.3**), and this is due to the presence of phenolic compounds present in the natural additives. GME_0 exhibited accelerated degradation in its total polyphenolic content due to the absence of antioxidant additives leading to photodegradation of phenolic groups into aromatic radicals. The accelerated storage conditions (50°C) resulted in incremental degradation of polyphenolics in GME_0 at various time intervals during the storage period by auto-oxidation and epimerization evidently due to the absence of antioxidant additives (**Table 6A.3**). The addition of natural extracts with phenolic moieties (as in GME_{1-4}) as additives apparently shielded the mussel active principles against thermally induced autoxidation at elevated temperatures as evident by decelerated degradation rate of GME_1 through GME_4.

6A.2.2.3 DPPH and ABTS radical scavenging activities

GME_2 and GME_4 showed a significantly higher DPPH scavenging activity as compared to other treatments and GME_0 ($P<0.05$) (**Table 6A.3**). The ability of GME_0 to stabilize the free radical was significantly reduced ($P<0.05$) after d=90 of accelerated shelf life study with respect to its initial activity (d=0). The significant reduction of total polyphenolic content in storage might be one of the primary factors causing the reduction in radical scavenging activity of GME_0. The DPPH scavenging activity of GME_2 and GME_4 showed decelerated reduction of its initial activity than those shown by GME_1 and GME_3 at d=90. The reduction in

ABTS radical scavenging activity was significant for GME_0 compared with GME_2 and GME_4 ($P<0.05$) after accelerated oxidation of 90 days. GME_1 and GME_3 showed significant reduction of its initial activity than GME_2 and GME_4 after d=90. It is interesting to note that GME_3 has higher concentrations of phenolics (ROO and CLO = 0.8 g/100g each) than in GME_4, GME_2 (CLO and ROO of 0.8 and 0.4 g/100g, respectively) and GME_1 (CLO and ROO of 0.4 and 0.8 g/100g, respectively). However, the presence of higher phenolic content in GME_3 has no direct impact on antioxidant activities of that particular treatment. This may be explained by the fact that a threshold level of CLO: ROO of 0.8 g/100g: 0.4 g/100g is sufficient for optimum antioxidant activity, and further addition of endogenous antioxidants (ROO to 0.8 g/100g as in GME_3) does have a negative impact on the stability of the active principle (GME_0). A possible explanation for this synergistic effect would be that a heterogeneous activation of a hydroxyl group on the antioxidant principles of other antioxidants to enhance the formation of H radical, which rapidly reacts with exogenous free radical (DPPH.) to quench it (Vaya and Michael, 2001). A phenolic antioxidant bearing ortho-dihydroxyl groups as in carnosic acid (with 1-(1, 1-dimethylperhydro-4-naphthalenyl)-1-ethanone) and carnosol (with perhydro-1-isochromenone and 3-isopropyl-1, 2-benzenediol) in rosemary was investigated as efficient antioxidants, and the produced stable *o*-quinone material plays a key role in its antioxidant activity (Wei and Ho, 2006) (**Fig. 6A.2**).

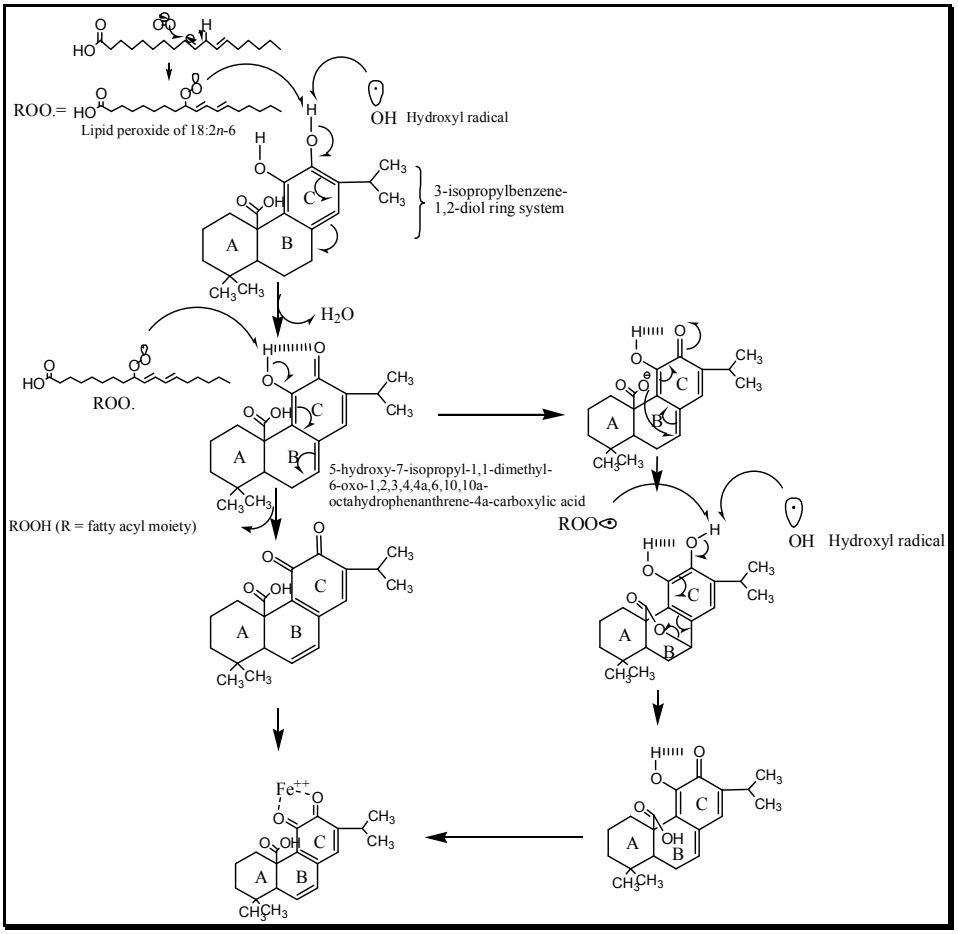


Fig. 6A.2 Schematic diagram illustrating lipid peroxidation inhibitory and antioxidant mechanism of rosemary active principles carnosic acid with 1-(1, 1-dimethylperhydro-4-naphthalenyl) -1-ethanone) and carnosol with perhydro-1-isochromenone and 3-isopropyl-1, 2-benzenediol, which together are responsible for ~90% of antioxidant activity. HAT (H-donation) and chain breaking mechanism, as in phenolic antioxidants, is the mode of action of CA and carnosol. Another mechanism by that these polyphenolic compounds exhibit lipid peroxidation is of metal (Fe^{2+}) ion chelation by phenoxyl radical and free hydrogen on -OH group. The polyphenols with *o*-dihydroxy groups provide improved stability to polyphenol radical by electron delocalization through the system accompanying radical formation.

Curcumin-I and II, the major curcuminoids of *C. longa* were reported to possess phenolic 2-methoxy-1-methyl-4-[(*E*)-1-propenyl]benzene and 1-methyl-4-[(*E*)-1-propenyl] benzene moieties with an additional 2,4-pentanedione system, which are efficient to scavenge free radicals. Ortho-methoxyl group as in curcumin-I can form intramolecular hydrogen bonds

with the phenolic hydrogen, making the H-atom abstraction from the ortho-methoxyphenols easy (de Heer *et al*., 2000). The antioxidant action of curcuminoids was also found to be associated with the β-dicarbonylic system in the heptadienone link of curcumin-I, which has conjugated double bonds (dienes) **(Fig.6A.3)**. H-atom abstraction from the CH_2 group in the heptadienone link of curcumin-I also may be involved in antioxidant activity.

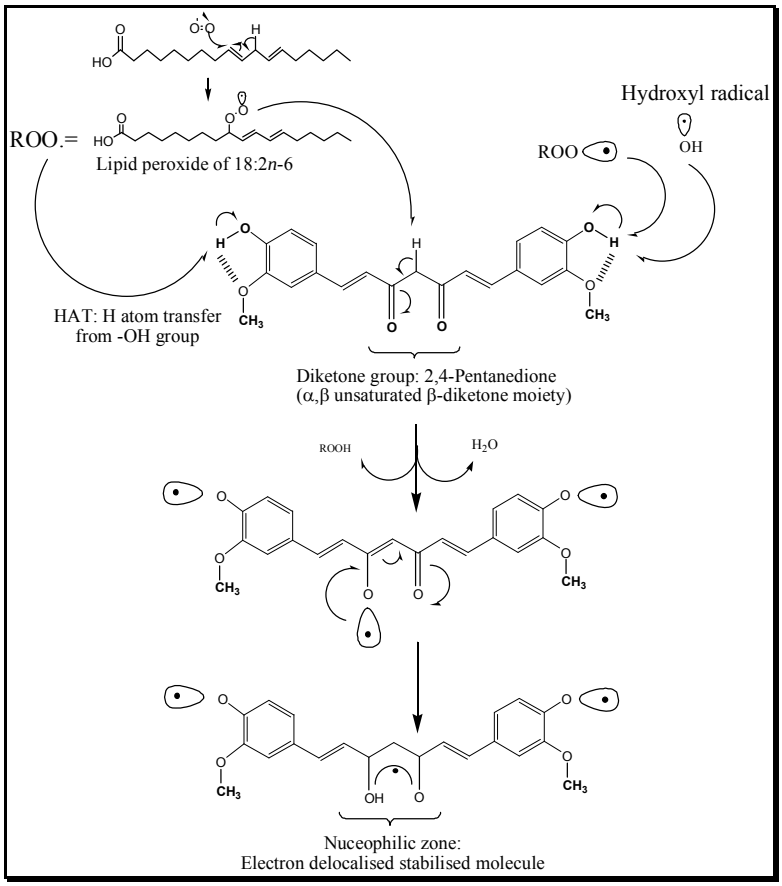


Fig.6A.3. Schematic diagram illustrating lipid peroxidation inhibitory and antioxidant mechanism of curcumin-I. Ortho-methoxyl group of curcumin-I can form intramolecular hydrogen bonds with the phenolic hydrogen, making the H-atom abstraction from the o-methoxyphenols easy. The antioxidant efficiency of phenolics is enhanced by the introduction of a second OH group and is increased by one or two methoxy substitutions in positions ortho to the OH group as in curcuminoids. The antioxidant action of curcuminoids was also found to be associated with the β-dicarbonylic system in the heptadienone link of curcumin-I, which has conjugated double bonds (Dienes). H-atom abstraction from the -CH_2 group in the heptadienone link of curcumin-I also may be involved in antioxidant activity

6A.2.2.4 Lipid peroxidation assay

Lipid peroxidation is a free radical (peroxides and hydroperoxides) mediated process in biological systems due to the oxidation of unsaturated fatty acids. Anti-oxidative substances are able to trap free radicals, and terminate the chain reaction leading to lipid oxidation. As evident from **Table 6A.3**, the TBARS content of GME_0 increased significantly during the 90 days of accelerated oxidation study as compared to those in GME_2 and GME_4 ($P<0.05$). The effective inhibition of lipid peroxidation in the treatments GME_2 and GME_4 may be attributed to the presence of phenolic antioxidants in the additives that were reported to disrupt free-radical chain reaction by donating a proton to fatty acid radicals to terminate chain reactions, and have roles to inhibit lipid peroxidation. Though carnosic acid is the primary antioxidant in rosemary, and it metabolized to other compounds like carnosol, rosamanol and rosamariqionone, which too were reported to possess antioxidant and lipid peroxidation inhibitory properties. Rosmarinic acid possesses 1, 2-dihydroxybenzene (catechol) ring system, contributing to its antioxidant activity. Furthermore, the presence of four phenolic hydrogens in 4-[(*E*) -1-propenyl] -1, 2-benzenediol moiety in rosmarinic acid significantly increased the polarity of the catechol ring system. These phenolic hydrogens undergo intramolecular H-bonding thereby improving its radical stability, and ease of H-atom abstraction from catechol forming phenoxyl radical. Another mechanism by that these polyphenolic compounds exhibit lipid peroxidation is of metal (Fe^{2+}) ion chelation by phenoxyl radical and free hydrogen on -OH group (Wei and Ho, 2006; Seeram and Nair, 2002). The polyphenols with ortho-dihydroxy groups provide improved stability to polyphenol radical by electron delocalization through the system accompanying radical formation (de Heer *et al*., 2000). Curcuminoids, besides harboring two aromatic groups (two 2-methoxy-1-methyl-4-[(*E*) -1-propenyl] benzene in curcumin-I and one each of 2-methoxy-1-methyl-4-[(*E*) -1-propenyl] benzene and 1-methyl-4-[(*E*) -1-propenyl] benzene in curcumin-II, also possess a diketone group (2,4-pentanedione moiety) that can react with and H_2O_2 and OH radicals **(Fig.6A.3).**

Table 6A.3 *In vitro* antioxidative activity of different treatments compared with GME_0 during accelerated shelf life study

Days	GME_0	GME_1	GME_2	GME_3	GME_4
H_2O_2 radical scavenging capacity (%)					
0	48.64 ± 0.25^{ap}	51.24 ± 0.56^{abp}	55.17 ± 0.05^{cp}	52.15 ± 1.92^{bp}	58.42 ± 0.53^{dp}
15	46.61 ± 0.61^{ar}	51.04 ± 0.18^{abp}	51.71 ± 1.15^{abq}	51.16 ± 1.51^{br}	53.27 ± 0.09^{br}
60	34.33 ± 0.33^{as}	49.21 ± 0.65^{bq}	49.84 ± 0.81^{bq}	50.65 ± 0.08^{cs}	51.63 ± 0.04^{cs}
90	31.79 ± 1.23^{at}	44.32 ± 1.09^{bcr}	49.32 ± 1.38^{bdq}	45.73 ± 0.52^{cdt}	52.73 ± 1.24^{et}
x	34.6	13.5	10.6	12.3	9.7
Total phenolic content (mg GAE/g)					
0	2.70 ± 0.03^{ap}	4.02 ± 0.01^{bp}	4.84 ± 0.07^{cp}	5.11 ± 0.01^{dp}	4.92 ± 0^{pe}
15	1.77 ± 0.02^{as}	3.52 ± 0.03^{br}	4.25 ± 0.13^{cs}	4.25 ± 0.22^{cs}	4.41 ± 0.14^{dr}
60	1.60 ± 0.02^{au}	2.94 ± 0.03^{bu}	3.98 ± 0.14^{cu}	4.16 ± 0.01^{cu}	4.15 ± 0.05^{cs}
90	0.80 ± 0.02^{av}	2.72 ± 0.02^{bv}	3.82 ± 0.02^{cv}	3.18 ± 0.02^{cv}	3.93 ± 0.02^{ct}
x	70.4	32.3	21.1	37.8	20.1
DPPH scavenging activity (% TARSA)					
0	13.0 ± 0.03^{ap}	25.6 ± 0.02^{bp}	29.2 ± 0.23^{cp}	23.1 ± 0.03^{dp}	29.5 ± 0.14^{ep}
15	9.04 ± 0.02^{ar}	20.9 ± 0.07^{br}	26.3 ± 0.24^{cr}	21.4 ± 0.02^{dr}	26.6 ± 0.44^{eq}
60	2.07 ± 0.02^{au}	17.1 ± 0.09^{bt}	21.9 ± 0.06^{cu}	17.9 ± 0.02^{du}	22.4 ± 0.01^{es}
90	1.03 ± 0.01^{av}	16.8 ± 0.04^{bu}	21.6 ± 0.15^{cu}	16.3 ± 0.15^{du}	21.8 ± 0.03^{et}
x	92.1	34.4	26	29.4	26.1
ABTS radical scavenging activity (% TEAC)					
0	0.21 ± 0.01^{ap}	0.42 ± 0^{bp}	0.66 ± 0.02^{cp}	0.45 ± 0.01^{bp}	0.67 ± 0^{cp}
15	0.19 ± 0^{apq}	0.4 ± 0.02^{bp}	0.62 ± 0.04^{cp}	0.41 ± 0.01^{bp}	0.63 ± 0.03^{cpq}
60	0.15 ± 0.01^{aq}	0.38 ± 0.01^{bp}	0.61 ± 0^{cp}	0.4 ± 0.02^{bp}	0.61 ± 0.01^{cq}
90	0.14 ± 0^{aq}	0.37 ± 0.02^{bp}	0.61 ± 0.06^{cp}	0.38 ± 0.01^{bp}	0.61 ± 0.01^{cq}
x	35	11.2	7.9	14.9	9
TBARS formation inhibitory activity (mM MDAEQ/kg)					
0	3.94 ± 0.01^{ap}	2.00 ± 0.05^{bp}	2.01 ± 0.02^{bp}	2.08 ± 0.04^{bp}	2.06 ± 0.05^{bp}
15	4.62 ± 0.06^{aqr}	2.69 ± 0.15^{bq}	2.52 ± 0.02^{cr}	2.33 ± 0.03^{cpq}	2.35 ± 0.04^{cpq}
60	8.2 ± 0.09^{at}	4.0 ± 0.09^{bs}	3.39 ± 0.02^{bct}	4.43 ± 0.02^{bs}	3.23 ± 0.01^{cr}
90	10.4 ± 0.22^{au}	4.14 ± 0.07^{bs}	3.42 ± 0.17^{ct}	4.85 ± 0.04^{dt}	3.32 ± 0.09^{cr}
x	164	107	70.1	133.2	61.2

a-e: Row wise values with different superscripts of this type indicate significant differences ($p< 0.05$) within different samples. p-w: Column wise values with different superscripts of this type indicate significant differences ($P< 0.05$) within different days. The values are expressed as mean standard deviation, $n=3$. H_2O_2 radical scavenging capacity is in %. The total phenolic content was expressed as gallic acid equivalent in mg (mg GAE/g sample). DPPH radical scavenging activity was expressed as Total antioxidant radical scavenging activity (% TARSA). $ABTS^+$ radical scavenging activity was expressed relative to trolox in terms of % TEAC (Trolox equivalent antioxidant capacity). Lipid peroxidation inhibitory activity expressed as mMmalondialdehyde (MDA) equivalents/ kg (mM MDAEQ/kg) of the sample. 'x' represents the percent reduction in activity on 90^{th} day (d=90) with respect to 0^{th} day (d=0).

6A.2.3 Effect of various combinations of natural additives on nutritional parameters in different treatments of green mussel Extract

6A.2.3.1 Fatty acids

This study used different treatments supplemented with anti-oxidative ingredients (GME_{1-4}) to arrest the oxidation of these fatty acids under accelerated shelf storage for a period of 90 days (d=90). The addition of natural extracts of additives with polyphenolic moieties (in GME_{1-4}) apparently shielded the mussel PUFAs against oxidation as evident by higher PUFAs in GME_{1-4} as compared to GME_0 after 90 days (d=90) of accelerated shelf life study. PUFAs in GME_0 experienced a significant reduction in their total content from the initial composition after d=90 as compared to the treatments (GME_{1-4}), which point towards the beneficial effect of natural additives added to it. The total *n*-3 PUFA content in GME_0 (19.5%) was found to be lesser than recorded in GME_0 supplemented with additives at d = 0, thereby indicating the added *n*-3 fatty acid pool to GME_0 by the additives. The content of the *n*-3 fatty acid 22:6*n*-3 in GME_2 and GME_4 (8.7 and 7.4%) were found to be higher than in GME_1 (8%) and GME_3 (7.6%) at d=0. This fatty acid registered a significant reduction (>35%, $P<0.05$) in GME_0, after d=90. The treatments GME_2 and GME_4 were able to effectively protect this important fatty acid from oxidative degradation after d=90 than did GME_1 and GME_3. The comparatively poor shelf-life of GME_3 than GME_2 in degradation of DHA to a minimum in spite of higher concentration of antioxidant principles can be explained by the fact that a threshold level of CLO: ROO of 0.8:0.4 g/100g is sufficient for optimum antioxidant activity, and further addition of antioxidants (as 0.8 g/100g ROO in GME_3) does have negative impact on stability of the fatty acid.

Table.6A.4 Fatty acid profile of GME_0 and different treatments (GME_{1-4}) during accelerated shelf life study of 90 days.

Fatty acids	0	1	2	4	5	8	15	30	60	90 days
					GME$_0$					
20:5*n*-3	6.98±0.63^{a}	5.95±0.54^{ab}	5.9±0.53^{ab}	5.83±0.52^{ab}	5.06±0.46^{ab}	5.16±0.46^{ab}	5.3±0.48^{ab}	5.33±0.48^{ab}	4.74±0.43^{b}	4.53±0.41^{b}
22:6*n*-3	7.92±0.71^{a}	6.42±0.58^{a}	6.08±0.55^{a}	5.59±0.5^{a}	5.52±0.5^{a}	5.15±0.46^{a}	5.21±0.47^{a}	5.19±0.47^{a}	5.15±0.46^{a}	5.11±0.46^{a}
∑ PUFA	28.4±1.01^{a}	25.91±2.33^{ab}	26.98±1.25^{ab}	25.02±1.01^{ab}	23.59±2.01^{b}	23.74±2.14^{b}	24.74±1.47^{ab}	25.38±1.56^{ab}	24.64±1.02^{ab}	24.62±2.01^{ab}
∑ *n*-3	19.45±1.75^{a}	16.43±1.48^{b}	15.77±1.42^{b}	14.99±1.35^{b}	13.23±1.19^{b}	12.76±1.15^{b}	13.9±1.25^{b}	13.79±1.24^{b}	13±1.17^{b}	12.52±1.13^{b}
∑ *n*-6	8.49±0.76^{a}	8.93±0.8^{a}	10.81±0.97^{ab}	9.57±0.56^{ab}	9.9±0.58^{ab}	10.47±0.94^{ab}	10.42±0.85^{ab}	11.15±0.59^{b}	11.05±0.99^{b}	11.49±1.03^{b}
*n*3/*n*6	2.29±0.21^{a}	1.84±0.17^{a}	1.46±0.13^{a}	1.57±0.14^{a}	1.34±0.12^{a}	1.22±0.01^{a}	1.33±0.12^{a}	1.24±0.11^{a}	1.18±0.11^{a}	1.09±0.1^{a}
					GME$_1$					
20:5*n*-3	6.79±0.61^{a}	6.12±0.55^{a}	5.98±0.54^{a}	5.91±0.53^{a}	5.66±0.51^{a}	5.46±0.49^{a}	5.37±0.48^{a}	5.44±0.49^{a}	5.16±0.46^{a}	5.12±0.46^{a}
22:6*n*-3	7.96±0.72^{a}	6.93±0.62^{a}	6.95±0.63^{a}	5.82±0.05^{a}	5.78±0.52^{a}	5.79±0.52^{a}	5.67±0.51^{a}	5.49±0.49^{a}	5.55±0.5^{a}	5.36±0.48^{a}
∑ PUFA	28.95±2.61^{a}	28.04±2.52^{a}	28.09±0.12^{a}	26.72±2.4^{ab}	26.22±2.36^{ab}	25.32±2.28^{b}	25.32±2.28^{b}	24.74±2.23^{b}	24.6±2.21^{b}	25.34±2.28^{b}
∑ *n*-3	19.27±1.01^{a}	18.81±1.02^{a}	18.55±1.01^{a}	17.21±1.55^{ab}	16.59±0.69^{b}	15.95±1.44^{b}	15.67±0.89^{b}	15.5±1.02^{b}	15.03±1.35^{b}	15±1.02^{b}
∑ *n*-6	9.21±0.83^{a}	8.81±0.79^{a}	8.91±0.8^{a}	9.07±0.82^{a}	9.15±0.82^{a}	8.97±0.81^{a}	8.45±0.76^{a}	8.41±0.76^{a}	9.16±0.82^{a}	9.31±0.84^{a}
*n*3/*n*6	2.09±0.19^{a}	2.14±0.19^{a}	2.08±0.19^{a}	1.9±0.17^{a}	1.81±0.16^{a}	1.78±0.16^{a}	1.85±0.17^{a}	1.84±0.17^{a}	1.64±0.15^{a}	1.61±0.14^{a}
					GME$_2$					
20:5*n*-3	7.85±0.71^{a}	7.83±0.7^{a}	7.81±0.7^{a}	7.78±0.7^{a}	7.79±0.7^{a}	7.72±0.69^{a}	7.69±0.69^{a}	7.67±0.69^{a}	7.63±0.69^{a}	7.61±0.68^{a}
22:6*n*-3	8.7±1.02^{a}	8.65±0.78^{a}	8.57±0.77^{a}	8.57±0.77^{a}	8.48±1.32^{a}	8.25±0.74^{a}	8.11±0.73^{a}	8.05±0.72^{a}	7.85±0.71^{a}	7.84±0.71^{a}
∑ PUFA	30.36±1.21^{a}	30.46±2.74^{a}	30.1±2.11^{a}	30.01±2.7^{a}	30.61±2.75^{a}	29.97±2.14^{ab}	27.8±2.5^{b}	27.29±2.46^{b}	26.65±2.4^{b}	26.67±2.4^{b}
∑ *n*-3	22.58±2.03^{a}	22.52±1.02^{a}	22.37±2.01^{a}	22.2±2^{a}	22.21±2^{a}	21.76±1.01^{a}	21.45±1.93^{a}	21.29±1.02^{a}	20.86±0.23^{a}	20.71±1.86^{a}
∑ *n*-6	7.5±0.68^{a}	7.62±0.69^{a}	7.37±0.66^{a}	7.37±0.66^{a}	7.25±0.65^{a}	7.5±0.68^{a}	5.78±0.52^{a}	5.41±0.49^{a}	5.3±0.48^{a}	5.43±0.25^{a}
*n*3/*n*6	3.01±0.27^{a}	2.96±0.27^{a}	3.04±0.27^{a}	3.01±0.27^{a}	3.06±0.28^{a}	2.9±0.26^{a}	3.71±0.33^{a}	3.94±0.35^{a}	3.94±0.35^{a}	3.81±0.34^{a}
					GME$_3$					
20:5*n*-3	7.89±0.71^{a}	7.85±0.71^{a}	7.82±0.7^{a}	7.76±0.7^{a}	7.74±0.7^{a}	7.72±0.69^{a}	7.72±0.69^{a}	7.68±0.69^{a}	7.64±0.69^{a}	7.63±0.69^{a}
22:6*n*-3	7.58±0.68^{a}	7.43±0.67^{a}	7.32±0.66^{a}	6.9±0.62^{a}	6.51±0.59^{a}	6.16±0.55^{a}	6.12±0.55^{a}	6.15±0.55^{a}	5.38±0.48^{a}	5.24±0.47^{a}
∑ PUFA	30.64±1.01^{a}	30.27±0.14^{a}	29.85±1.98^{a}	29.12±0.21^{a}	28.68±1.14^{acd}	28.09±2.53^{acd}	28.51±1.56^{acd}	27.93±2.51^{bcd}	26.86±2.42^{cd}	25.58±2.3^{d}
∑ *n*-3	21.41±1.93^{a}	21.18±1.91^{a}	21.16±1.9^{a}	20.4±1.84^{a}	19.97±1.8^{a}	19.47±1.75^{a}	19.52±1.76^{a}	19.36±1.74^{a}	18.36±1.65^{a}	18.2±1.64^{a}
∑ *n*-6	8.89±0.8^{a}	8.73±0.79^{a}	8.27±0.35^{a}	8.26±0.74^{a}	8.27±0.74^{a}	8.14±0.73^{a}	8.47±0.76^{a}	7.94±0.71^{a}	8.04±0.72^{a}	6.91±0.62^{a}
*n*3/*n*6	2.41±0.22^{a}	2.43±0.22^{a}	2.56±0.23^{a}	2.47±0.22^{a}	2.41±0.22^{a}	2.39±0.22^{a}	2.3±0.56^{a}	2.44±0.22^{a}	2.28±0.21^{a}	2.63±0.24^{a}
					GME$_4$					
20:5*n*-3	7.74±0.7^{a}	7.64±0.69^{a}	7.5±0.68^{a}	7.21±0.65^{a}	7.14±0.64^{a}	7.07±0.64^{a}	6.83±0.61^{a}	6.8±0.61^{a}	6.78±0.61^{a}	6.74±0.61^{a}
22:6*n*-3	7.43±0.67^{a}	7.21±0.65^{a}	7.19±0.65^{a}	7.17±0.65^{a}	7.11±0.64^{a}	7.18±0.65^{a}	7.15±0.64^{a}	7.11±0.64^{a}	7.05±0.63^{a}	7±0.63^{a}
∑ PUFA	29.81±1.69^{a}	29.61±1.54^{a}	28.99±1.25^{a}	28.19±1.36^{a}	28.12±1.02^{a}	28.19±2.54^{a}	26.75±0.21^{a}	25.97±2.34^{a}	25.49±1.02^{a}	25.44±2.29^{a}
∑ *n*-3	20.95±0.99^{a}	20.62±1.86^{a}	20.25±1.82^{a}	19.79±1.78^{a}	19.48±1.75^{a}	19.43±1.75^{a}	19.06±1.72^{a}	18.85±1.7^{a}	18.56±1.67^{a}	18.47±1.66^{a}
∑ *n*-6	8.44±0.76^{a}	8.64±0.78^{a}	8.38±0.75^{a}	8±0.72^{a}	8.12±0.73^{a}	8.33±0.75^{a}	7.08±0.64^{a}	6.49±0.58^{a}	6.36±0.57^{a}	6.61±0.59^{a}
*n*3/*n*6	2.48±0.22^{a}	2.39±0.22^{a}	2.42±0.22^{a}	2.47±0.22^{a}	2.4±0.22^{a}	2.33±0.21^{a}	2.69±0.02^{a}	2.9±0.26^{a}	2.92±0.26^{a}	2.79±0.25^{a}

Data are expressed as mean ± standard deviation of three replicates. ΣPUFA Total polyunsaturated fatty acids. Σ*n*-3-Total *n*-3 fatty acids; Σ*n*-6-Total *n*-6 fatty acids;Means with different superscripts (a, b, c, d) in the same row indicates a statistical difference (P<0.05).

The antioxidant additives serve as oxidation "chain breakers" for the treatments (GME_{1-4}) by intercepting the free radicals generated from unsaturated fatty acids during accelerated storage. The treatments GME_2 and GME_4 were found to exhibit maximum *n*-3/*n*-6 ratio than recorded in GME_3, GME_1, and GME_0 *n*-3/*n*-6 ratio was increased within the treatments GME_{2-4} over a period of 90 days of accelerated shelf-life study than in GME_0. Phenolic compounds present in the additives have been demonstrated to exhibit a scavenging effect of free radicals present in the system. It is apparent that ROO and CLO contain polyphenolic compounds with multiple –OH groups/centers of unsaturation that are capable of deactivating free radical to initiate a chain breaking peroxidation of olefinic bonds in *n*-3 PUFAs. The addition of natural extracts of additives with polyphenolic moieties (as in GME_{1-4}) apparently shielded the mussel active ingredient against oxidation as evident by higher *n*-3/*n*-6 ratio as compared to GME_0 after d=90 **(Table 6A.4)**.

6A.2.3.2 Protein and amino acid contents

The total digestible protein contents during shelf life study were determined and are presented in **Table 6A.5**.It is of note that the degradation of the total digestible proteins after 90 days of accelerated shelf life study (d=90) were significantly higher ($P<0.05$) in GME_0 (16.4%) with respect to d=0 than in treatments with antioxidant additives GME_2 and GME_4, which showed a decelerated degradation of total digestible protein (6.6, and 6.7% decrease at d=90 with respect to those at d=0) throughout the period during shelf life study. It is, therefore, apparent that the treatments GME_2 and GME_4 were efficient to reasonably arrest the degradation of total digestible protein throughout the period under the shelf (d=90) with respect to that recorded at

d=0. The total essential amino acid (EAA) was found to be significantly higher in the treatments supplemented with additives (GME_3> GME_1> GME_2> GME_4) than in GME_0. The most abundant essential amino acids of GME_{1-4} were found to be arginine (average - 400 mg/100 g) followed by lysine (average - 264 mg/100 g), and leucine (average - 242 mg/100 g) at d=0. Among the non-essential amino acids, negatively charged amino acid glutamate and aspartate were observed to be the predominant amino acids in the treatments (GME_{1-4}). The ratio of essential amino acid (EAA/NEAA) was recorded to be 0.9in the treatments (GME_{1-4}). It is interesting to note that the treatments GME_2 and GME_4 recorded an increase of this ratio after d=90 than in GME_0. These differences also appeared to be due to proteolysis of amino acids that might have occurred to a higher extent in the samples without any added additives. In the present study, the essential and non-essential amino acids in GME_0 experienced a maximum reduction in terms of their total content from the baseline composition (d=0) during the accelerated shelf life study for 90 days (d=90) than did the treatments (GME_{1-4}), which point towards the beneficial effect of natural additives. It is, therefore, evident that the preparations GME_2,GME_3 and GME_4 were found to be more effective to prevent the essential amino acids from degradation after 90 days (d=90) of accelerated storage than GME_1 seemingly due to comparatively less titre of curcuminoids in GME_1 (CLO 0.4 g/100g) than in GME_2 and GME_4 (CLO 0.8 g/100g).

Table 6A.5 Variation in the composition of essential and non-essential amino acids (expressed as mg/100 g edible meat) and total protein (mg/100g edible meat) of different treatments (GME1-4) compared with control (GME0) during accelerated shelf life study of 90 days. (d=90).

	GME_0	GME_1	GME_2	GME_3	GME_4	GME_0	GME_1	GME_2	GME_3	GME_4
Protein	Baseline (day zero, d=0)					End of the experiment (day ninety, d=90)				
(mg/100g)	116.7±0.55	138.97 ±2.35	139.01 ±1.02	139.25 ±1.05	139.00 ±1.36	97.55±1.06	128.94±2.56	129.85±0.02	127.55±0.98	129.68±0.09
Essential aminoacids (EAA)										
His	62.70±0.32	80.92±1.2	78.11±0.21	84.02±0.19	74.93±0.98	17.86±2.31*	78.03±2.31	53.07±0.03	47.68±2.31*	46.35±0.50*
Arg	322.8±1.2	396.7±1.3	393.6±0.07	419.7±0.07	389.7±0.07	144.1±2.17*	184.0±2.17*	249.3±1.58	247.2±2.17*	240.8±0.17*
Thr[b]	122.1±0.56	150.9±1.56	151.6±0.12	161.9±0.12	150.2±0.12	58.67±2.98*	69.72±2.98*	90.10±0.6*	98.21±2.98*	104.5±0.18*
Val[b]	149.9±0.26	173.7±2.5	167.3±0.04	176.8±0.04	162.1±0.04	75.54±0.66*	77.49±0.23*	97.65±2.21*	115.9±0.66*	129.1±0.56*
Met[b]	68.18±2.3	81.28±3.2	79.76±0.04	88.39±1.04	80.97±0.05	41.27±2.64*	47.62±2.64	60.00±1.56	67.19±2.64	77.29±0.62
Ile[b]	149.7±1.2	166.6±1.2	160.3±0.50	169.7±0.65	156.2±0.68	75.85±0.66*	86.96±0.66*	112.1±0.72	121.1±0.66	124.9±0.17
Leu[b]	212.0±1.4	248.2±2.0	239.0±0.01	250.8±0.1	230.7±0.10	118.3±0.88	128.0±0.88	168.2±2.21	183.7±0.88	180.5±0.26
Phe[b]	123.8±0.89	134.6±2.1	132.1±0.05	136.5±0.12	127.4±0.21	77.10±1.56	97.50±1.36	129.9±3.29	123.3±0.96	116.3±2.35
Lys[b]	236.5±1.5	277.3±0.5	258.8±0.88	270.9±0.12	249.9±0.56	105.1±0.48*	93.10±0.48*	108.4±0.40*	142.8±0.48*	92.22±0.37*
Σ EAA	**1447.68**	**1710.2**	**1660.57**	**1758.71**	**1622.1**	**713.79***	**862.42***	**1068.72***	**1147.08***	**1111.96***
Non-essential amino acids (NEAA)										
Asp	323.4±2.3	408.7±1.2	390.5±0.94	420.0±0.65	388.8±0.09	127.3±1.11*	125.4±1.11*	149.3±0.86*	201.2±1.11*	132.7±2.08*
Glu	465.5±2.01	572.5±1.1	555.1±0.01	607.9±0.01	557.9±0.09	198.2±0.05*	204.8±0.05*	253.1±1.32*	310.7±0.05*	234.9±1.25*
Ser	148.3±1.2	188.2±0.26	185.4±1.01	200.9±0.98	187.5±1.01	64.12±3.04*	80.06±3.04	100.9±1.2	96.32±3.04	106.4±2.3
Gly	221.0±1.1	227.6±2.3	222.6±0.02	228.6±0.02	212.7±0.02	86.00±1.56*	111.5±1.06*	172.4±2.6	180.7±1.23	171.7±.56
Ala	190.5±1.5	207.4±1.2	200.1±0.02	210.4±0.02	192.8±0.02	84.41±0.77*	78.47±0.77*	99.42±1.5*	120.6±0.77*	100.0±2.63*
Pro	121.4±2.6	122.1±2.6	117.0±0.96	120.8±1.02	112.2±0.52	72.03±2.13*	93.58±2.13	134.2±5.5	119.7±2.13	132.9±.96
Tyr	70.70±0.25	74.13±1.25	72.06±0.01	75.21±0.26	70.02±0.21	43.40±3.2*	43.93±3.6*	63.29±5.5	62.12±3.2	70.87±2.27
Cys	23.20±1.3	32.69±1.3	33.80±0.01	39.17±0.23	34.21±0.01	11.28±1.14	12.35±1.14	21.15±3.2	23.64±1.14	20.28±0.89
Σ NEAA	**1564**	**1833.32**	**1776.56**	**1902.98**	**1756.13**	**686.74***	**750.09***	**993.76***	**1114.98***	**969.75***
EAA/NEAA	**0.926**	**0.933**	**0.935**	**0.924**	**0.924**	**1.039**	**1.150**	**1.075**	**1.029**	**1.147**

Results are expressed as mean ± SD (n = 3). Σ EAA represents total essential amino acids; Σ NEAA represents total non-essential amino acids * represents the significant difference ($p<0.05$) of each treatments at 90^{th} day with respect to the 0^{th} day.

6A.2.3.3 Vitamins and carotenoids

The variation in the stability of vitamins and carotenoids during the accelerated shelf life study is shown in **Table 6A.6.**The total vitamin C content in GME_0 was found to be lesser than recorded in the treatments at d = 0, thereby indicating the added vitamin C pool to GME_0 by *C. limon, P. emblica,* and *T. indica* aqueous extract (0.05 g/100g each). Vitamin C, associated with antioxidant activity by scavenging free radicals, registered a significant reduction (91.4%) in GME_0, after d = 90 with respect to d=0 (P<0.05) than in treatments with antioxidant additives GME_{1-4}, which showed a decelerated reduction of the same (**Table 6A.6**). This is due to the presence of polyphenolic compounds present in the natural additives that prevent degradation of ascorbic acid in shelf. Vitamin E which reacts with peroxyl radicals produced from PUFAs thereby reducing harmful lipid free radicals was found to be higher in the treatments GME_{2-4} than in GME_0 at d=0. A significant decrease of vitamin E (46% with respect to the initial value, P <0.05) was observed in GME_0 after d=90 than in GME_1 through GME_1, which experienced an insignificant reduction with respect to the baseline value (d=0) (P >0.05). Likewise the treatments GME_2 and GME_4 were able to arrest the degradation of vitamin D_3 after d = 90 than did GME_1, GME_3 and GME_0. The treatments GME_2 and GME_4 effectively arrested the degradation of vitamin K_1, which have been supported by a moderate reduction than GME_0 (>94%) without added additives. A 67% reduction in vitamin K_1 during 90^{th} day as compared to d=0 was observed within GME_1, apparently due to comparatively less titre of curcuminoids which were reported to possess potential to arrest oxidation of the vitamins. GME_0 is susceptible towards oxidation by free radicals, thereby reducing the content of these vitamins, if stored for a longer time period under shelf.

Table 6A.6 Variation in fat soluble vitamins A, D_3, E, K_1 and total carotenoid content of different treatments compared with GME_0 during the accelerated shelf life study of 90 days (d=90).

Nutrients	0	15	60	90	X
Retinol (Vit A IU/100g)					
GME_0	479.28 ± 0.34^{ap}	99.25 ± 0.40^{aq}	70.57 ± 0.63^{ar}	41.4 ± 0.86^{as}	91.36
GME_1	495.49 ± 3.73^{bp}	479.52 ± 1.00^{bq}	443.82 ± 1.33^{br}	384.48 ± 1.23^{bs}	22.40
GME_2	770.20 ± 1.85^{cp}	722.15 ± 1.09^{cq}	656.31 ± 0.31^{cr}	615.22 ± 0.72^{cs}	20.12
GME_3	856.25 ± 3.04^{dp}	798.46 ± 0.09^{dq}	711.21 ± 1.34^{dr}	613.32 ± 0.81^{ds}	28.37
GME_4	771.56 ± 2.63^{ep}	762.36 ± 4.15^{ed}	674.90 ± 0.59^{er}	602.94 ± 0.59^{es}	21.85
Cholecalciferol (Vit D_3 IU/100g)					
GME_0	21553.5 ± 1.5^{ap}	15925.5 ± 0.5^{aq}	20692 ± 1.1^{ar}	12411.5 ± 1.0^{as}	42.42
GME_1	32093.12 ± 4^{bp}	27543 ± 3.0^{bq}	21949 ± 7.0^{br}	20151 ± 1.2^{bs}	37.21
GME_2	28325.5 ± 0.5^{cp}	27741.5 ± 4.5^{cq}	23363 ± 7.3^{cr}	21400 ± 2.2^{cs}	24.45
GME_3	33594.5 ± 4.5^{dp}	29098.5 ± 0.5^{dq}	26701 ± 1.02^{dr}	21088 ± 0.5^{ds}	37.23
GME_4	28356.2 ± 2^{ep}	27562 ± 1.01^{dq}	23212 ± 5.5^{eq}	21411.0 ± 0.5^{es}	24.49
α-Tocopherol (Vit E IU/100g)					
GME_0	1.94 ± 0.02^{ap}	1.70 ± 0.14^{aq}	1.89 ± 0.07^{ar}	1.67 ± 0.98^{as}	13.92
GME_1	1.77 ± 0.01^{bp}	1.54 ± 0.02^{bq}	1.045 ± 0.85^{br}	0.95 ± 0.21^{bs}	46.33
GME_2	2.56 ± 0.07^{cp}	2.12 ± 0.02^{cq}	1.81 ± 2.71^{cr}	1.71 ± 0.11^{cs}	33.20
GME_3	3.345 ± 0.21^{dp}	2.805 ± 0.17^{dq}	2.505 ± 0.14^{dr}	2.36 ± 0.19^{dr}	29.34
GME_4	2.73 ± 0.15^{er}	2.5 ± 0.04^{eq}	2.64 ± 0.34^{er}	2.12 ± 0.05^{es}	22.34
Phylloquinone (Vit K_1 µg/100g)					
GME_0	57.03 ± 0.03^{ap}	27.73 ± 0.76^{aq}	7.35 ± 0.09^{ar}	3.27 ± 0.28^{as}	42.47
GME_1	67.535 ± 2.31^{bp}	45.545 ± 1.00^{bq}	27.433 ± 1.11^{br}	22.48 ± 0.05^{bs}	94.27
GME_2	69.24 ± 0.09^{cp}	67.45 ± 0.82^{cq}	52.8 ± 0.74^{cr}	43.92 ± 0.61^{cs}	36.57
GME_3	69.83 ± 0.06^{dp}	58.79 ± 0.50^{dq}	50.61 ± 0.11^{dr}	40.17 ± 0.12^{ds}	66.71
GME_4	71.81 ± 0.50^{ep}	69.2 ± 0.63^{cq}	55.47 ± 0.08^{er}	47.29 ± 0.25^{es}	34.15
Total carotenoids (µg/g)					
GME_0	$459.51^{ap}\pm0.48$	$387.74^{aq}\pm0.14$	$265.92^{ar}\pm0.39$	$201.10^{as}\pm0.12$	56.22
GME_1	$784.40^{bp}\pm0.15$	$691.58^{bq}\pm0.02$	$530.69^{br}\pm0.24$	$510.49^{bs}\pm0.24$	34.88
GME_2	$1426.3^{cp}\pm0.03$	$1389.1^{cq}\pm0.12$	$1093.5^{cr}\pm0.32$	$955.36^{cs}\pm0.41$	33.04
GME_3	$1385.6^{dp}\pm0.13$	$1282.5^{dq}\pm0.12$	$887.48^{dr}\pm0.32$	$735.45^{ds}\pm0.21$	46.94
GME_4	$1451.2^{ep}\pm0.03$	$1233.6^{eq}\pm0.12$	$922.41^{er}\pm0.32$	$960.8^{es}\pm0.01$	33.80

Ra-e: Row wise values with different superscripts of this type indicate significant differences ($P<0.05$) within different samples; p, q, r: Column wise values with different superscripts of this type indicate significant differences ($P<0.05$) within different days of shelf life study. Results are expressed as mean ± SD (n = 3). X represents the percent change of vitamins and carotenoids at the end of the experiment (d=90) with respect to baseline (d=0)

In the present study, the total carotenoid content in GME_0 (460 µg/g) was found to be significantly lower than recorded in GME_0 supplemented with additives ($GME_{2\text{-}4}$ ~1400 µg/g, d=0) thereby indicating the added carotene pool to GME_0 by the additives ($P<0.05$). The carotenoid content in GME_0 registered a significant reduction ($P<0.05$) at d=90 (**Table 6A.6**) than in the

treatments (GME_{1-4}), which point towards the beneficial effect of natural additives. Interestingly enough, though an inverse correlation was observed between higher ROO and CLO concentrations and efficacy to contain carotenoid degradation in GME_2 and GME_4 at d=0 and at the end of the study (d=90), this does not hold true for GME_3, which showed an accelerated degradation of carotenoids. As obvious, the treatments GME_2 and GME_4 recorded a decelerated degradation of carotenoids (~33%) after 90 days (d=90) of accelerated storage than did GME_3 (46.9%).

6A.2.4 Time series studies of *in vitro* anti-inflammatory activities of various combinations of green mussel extract (GME_1 - GME_4)

6A.2.4.1 Inhibition of cycloxygenase-2 (COX-2)

The COX-2 inhibition assay used in this study is based on the oxidation of (1-DCF) by the hydroperoxide formed in the COX reaction. Time series studies of *in vitro* anti-inflammatory activities of various combinations of green mussel extract are given **Table 6A.7.** The treatment GME_4 was significantly ($P<0.05$) effective in inhibiting COX-2 than other treatments. The COX-2 inhibition rate of GME_0 was significantly reduced ($P<0.05$) on d=30, and a significant reduction in activity was apparent after 90 days (d=90, $P<0.05$) with respect to the baseline activity (d=0). In general, the reduction in the inflammatory response is directly proportional to the active ingredients in GME_0 as apparent from the potential of the same to inhibit COX-2. Apparently, among the treatments, GME_4 and GME_2 showed a marginal reduction with respect to initial activity as compared to GME_1 and GME_3. GME_2 and GME_4 exhibited dose dependent inhibition of COX-1 and COX-2. At higher concentration (1 mg/mL) the treatments GME_2 and GME_4 inhibited COX-2 to the tune of 49.9 and 46.7%, respectively, than did indomethacin.

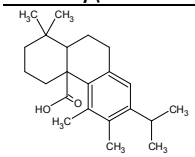
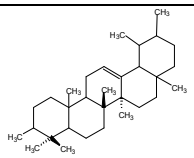
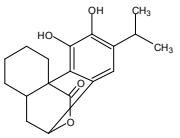
Table 6A.7 *In vitro* anti-inflammatory activities of different treatments (GME_1- GME_4) compared with control (GME_0) (5mg/ml) during the accelerated shelf life study.

Inhibitory activity of COX-2 (percent)					
Days	GME_0	GME_1	GME_2	GME_3	GME_4
0	$48.4^{apq} \pm 0.03$	$50.02^{bp} \pm 1.02$	$52.1^{cp} \pm 0.59$	$51.6^{dp} \pm 0.23$	$52.9^{ep} \pm 0.56$
2	$48.4^{aq} \pm 1.56$	$50.23^{bq} \pm 2.05$	$52.0^{cp} \pm 2.0$	$51.4^{dq} \pm 1.2$	$52.9^{eq} \pm 0.9$
15	$47.69^{ap} \pm 1.25$	$49.89^{br} \pm 1.65$	$51.89^{cp} \pm 1.35$	$51.0^{dr} \pm 0.59$	$52.01^{cr} \pm 0.45$
60	$43.02^{ar} \pm 0.25$	$47.06^{bs} \pm 0.38$	$49.56^{cq} \pm 1.97$	$49.56^{ct} \pm 0.89$	$51.02^{ds} \pm 1.4$
90	$37.23^{as} \pm 2.36$	$46.56^{bt} \pm 0.56$	$49.02^{cr} \pm 0.56$	$47.56^{du} \pm 0.74$	$51.0^{es} \pm 1.45$
x	23.1	6.9	5.9	7.8	3.6
Inhibitory activity of 5-LOX (percent)					
0	$51.9^{ap} \pm 0.56$	$51.26^{bp} \pm 0.45$	$58.2^{cp} \pm 1.25$	$57.325^{dp} \pm 0.32$	$62.3^{ep} \pm 1.56$
2	$51.23^{aq} \pm 0.45$	$51.02^{bq} \pm 0.89$	$57.89^{cq} \pm 0.98$	$57.36^{dq} \pm 1.56$	$61.89^{eq} \pm 1.45$
15	$49.56^{ar} \pm 0.08$	$49.38^{br} \pm 0.23$	$55.68^{cr} \pm 0.14$	$57.022^{dr} \pm 1.78$	$60.56^{er} \pm 0.1$
60	$48.65^{as} \pm 0.25$	$47.89^{as} \pm 0.36$	$54.0^{cs} \pm 1.56$	$56.25^{cs} \pm 0.89$	$58.36^{ds} \pm 0.45$
90	$42.25^{at} \pm 0.02$	$45.25^{bt} \pm 0.25$	$53.56^{ct} \pm 0.89$	$52.01^{dt} \pm 0.45$	$57.56^{et} \pm 1.5$
x	18.6	11.7	8.0	9.3	7.6

Results are expressed as percent activity with respect to the samples, and as mean ± SD (n = 3). 'x' represents percent reduction in activity on 90^{th} day (d=90) with respect to 0^{th} day (d=0).a, b, c, d: Row wise values with different superscripts of this type indicate significant differences ($P<0.05$) within different samples; p, q, r, s: column wise values with different superscripts of this type indicate significant differences ($P<0.05$) within different days of shelf life study.

It is known that COX-2 catalyzes the two-step conversion of ARA to PGH_2, and other inflammatory PGs. The CA and RA in rosemary share a closer physicochemical status with ARA, and appeared to mimic the latter as a competitive COX-2 inhibitor. ARA was found to have tPSA and Pl as 37.3 and 38.25 among electronic variables, and are closely matched with those calculated for CA (**Table 6A.8**) thereby indicating that CA may competitively inhibit with ARA for COX-2 by efficient complexation (salt bond) with the guanidinium group of charged amino acid residue Arg_{120} in the COX active site (Kurubail, 1998).

Table 6A.8 Physicochemical descriptor variables of natural *vis a vis* synthetic antioxidants and some commercially available anti-inflammatory compounds

Compounds	Physicochemical descriptor variables							
	Electronic		Hydrophobic		Steric			
	tPSA	PI	Log P	Clog P	MV	P	MR	CMR
Arachidonic acid	**37.3**	**38.25**	**6.16**	**7.392**	**327.7**	**798.4**	**100.35**	**9.5406**
Rosemary (*Rosmarinus officinalis*)								
 Carnosic acid								
Rosmarinic acid								
 Ursolic acid								
 Carnosol								
Carnosic acid	77.76	39.12	5.14	3.132	316.2	789.2	98.70	9.3220
Rosmarinic acid	144.52	36.21	2.07	1.0996	232.8	705.6	90.45	9.2512
Ursolic acid	-	53.50	9.69	13.269	440.5	1077.5	135.43	13.6428
Carnosol	66.76	32.12	3.78	2.125	228.8	638.5	83.54	8.217
Turmeric (*Curcuma longa*)								
Curcumin-I		Curcumin-II			Aryl turmerone		Curlone	
Curcumin-I	52.6	43.58	4.31	4.7222	323.5	826.8	113.6	11.2364
Curcumin-II	43.37	40.93	4.44	4.8032	299.5	770.1	106.71	10.6195
Aryl turmerone	17.07	25.26	3.52	3.511	211.5	508.8	66.46	6.4853
Curlone	17.07	27.21	3.26	3.1422	237.2	559.3	72.19	7.0685
Indian gooseberry (*Emblica officinalis* Gaertn.)								
β-glucogallin					Mucic acid 1,4-lactone 5-O-gallate			
β-glucogallin	177.14	28.27	-1.42	-1.1735	179.5	603.9	71.48	7.1714
Mucic acid 1,4-lactone 5-O-gallate	191.05	28.20	-1.53	-4.0155	178.0	599.3	70.25	7.0897
***Zingiber officinale* Roscoe L.**								
Zingiberene [6] Shogaol							Gingerol [6]	
Gingerol [6]	66.76	32.88	3.59	2.943	271.7	693.0	83.32	8.2854
(6) Shogaol	46.53	32.31	4.01	3.9112	267.4	666.1	83.79	8.1829
[6] Paradol	46.53	32.29	4.69	4.2452	273.8	678.8	81.86	8.1323
Zingiberene	-	27.16	4.8	5.02	241.0	554.2	73.25	6.8808
Zingerone	46.53	21.27	1.95	1.0712	174.8	440.1	54.12	5.3495
Commercially available antioxidants								
BHT: butylated hydroxyl toluene	20.23	27.64	5.54	5.426	237.5	556.0	72.34	7.0159
BHA: butylated hydroxyl anisole	29.46	21.09	3.22	3.3002	178.5	426.9	54.01	5.3138
TBHQ	40.46	19.18	2.96	2.534	152.9	385.3	48.58	4.85
Gallic acid	97.99	15.39	0.47	0.4255	97.2	314.4	37.53	3.8005
Commercially available NSAIDs								
Salicylates (aspirin)	63.6	17.65	1.21	1.0235	139.5	370.9	43.29	4.4576
Propionic acid derivatives (ibuprofen)	37.3	24.09	3.75	3.679	200.3	497.6	61.2	6.124
Acetic acid derivatives (indomethacin)	66.84	37.49	3.58	4.18	269.5	707.6	93.56	9.5053

Pl: Polarisability (cm^3/mol), P: Parachor (cm^3); tPSA: Calculation of polar surface area based on fragment contributions; CLogP to calculate *n*-octanol/water partition coefficient (log P_{ow}); MR: molar refractivity (cm^3/mol); CMR to calculate Molar Refractivity; MV: molar volume (cm^3).

The hydrophobic and steric parameters of CA too enjoy a close relationship with the substrate fit, eliminating ARA to occupy the desired active site to synthesize inflammatory PGs. The active principles (Curcumin-I, II) in CLO were found to have higher charges (electronic) and π-electron interactions with the amino acyl residues at the COX-2 active site (e.g., guanidinium group of Arg120 and aromatic ring of Phe/Tyr355 residues, respectively) than others due to higher electronic values (tPSAcurcumin-I 52.6, curcumin-II 43.37; Pl 43.58 and 40.93, respectively) (**Fig.6A.4**). No significant differences (P>0.05) in hydrophobic and steric/bulk parameters indicate the similarities in molecular properties of curcuminoids and substrate (ARA) thereby indicating the potential of the former to mimic the enzyme (COX-2) inhibiting the biosyntheses of inflammatory PGs **(Fig.6A.4 and Fig.6A.5).** It is interesting to note that the molecular descriptor variables of the active principles of these natural additives realized distinctly different properties as compared to the NSAIDs thereby indicating the different anti-inflammatory mode of action **(Table 6A.8)**. The ability of *Z. officinale* to reduce inflammation is due to its neutralizing action upon free radicals by phenolic compounds viz., gingerol, zingerone, and shogaol (with 2-methoxy-4-propylphenol moiety). The active ingredients in *Z. officinale* and *E. officinalis* contributed towards efficient ion pair with the amino acid residues in the enzyme active site apparently due to higher electronic effects **(Table 6A.8)** than hydrophobic or steric effects. These compounds reported in the additives appeared to ion pair with the guanidinium group of Arg_{120}, which also ion pairs with the carboxylate of ARA thereby mimicking the substrate to competitively inhibit the production of inflammatory PGs **(Fig.6A.6).**

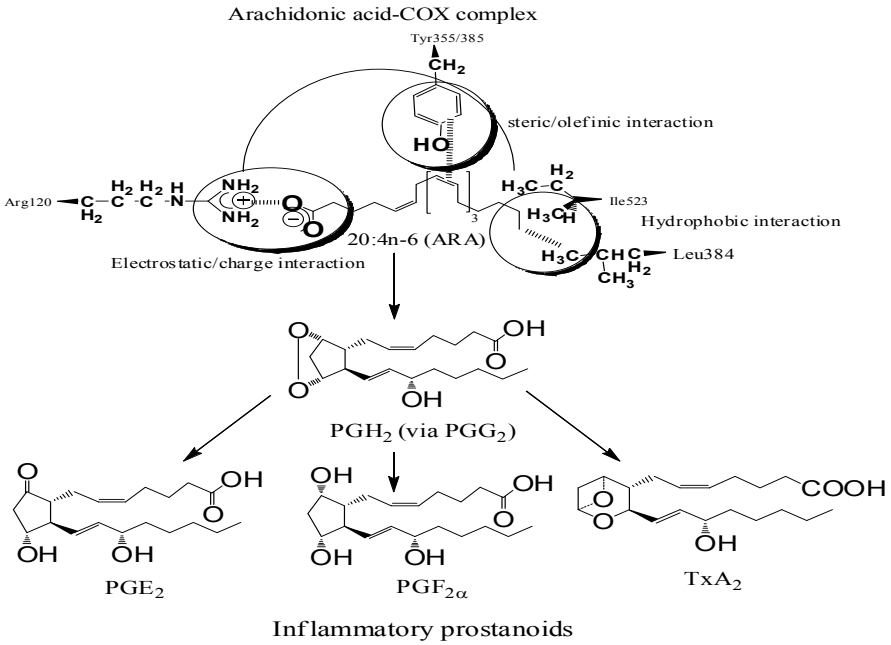


Fig.6A.4 Schematic representation of the COX-2 active site amino acyl residues with the substrate (ARA) forming the inflammatory PGH_2, PGE_2, $PGF_{2\alpha}$, and TxA_2

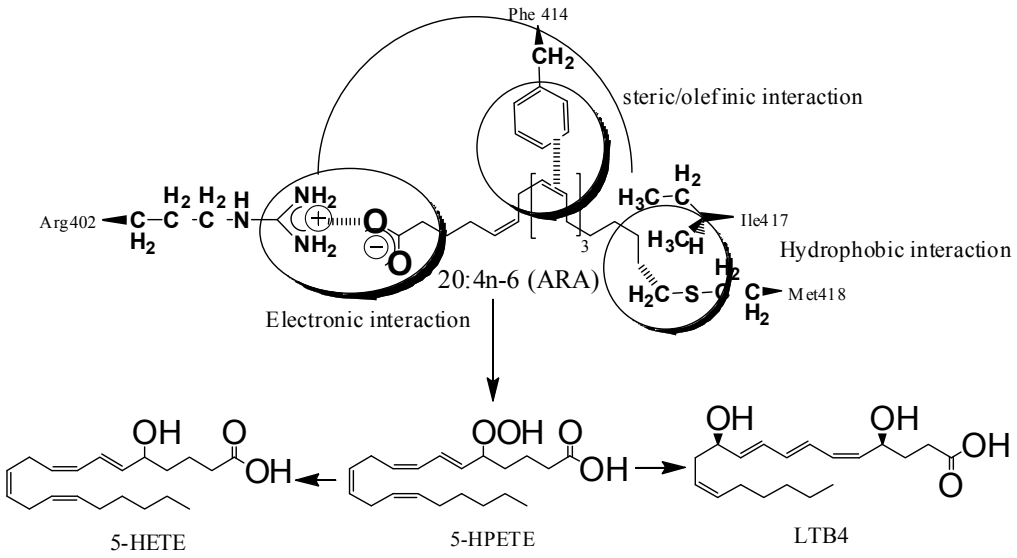


Fig.6A.5. Schematic representation of the 5-LOX active site amino acyl residues with the substrate forms the inflammatory leukotrienes.

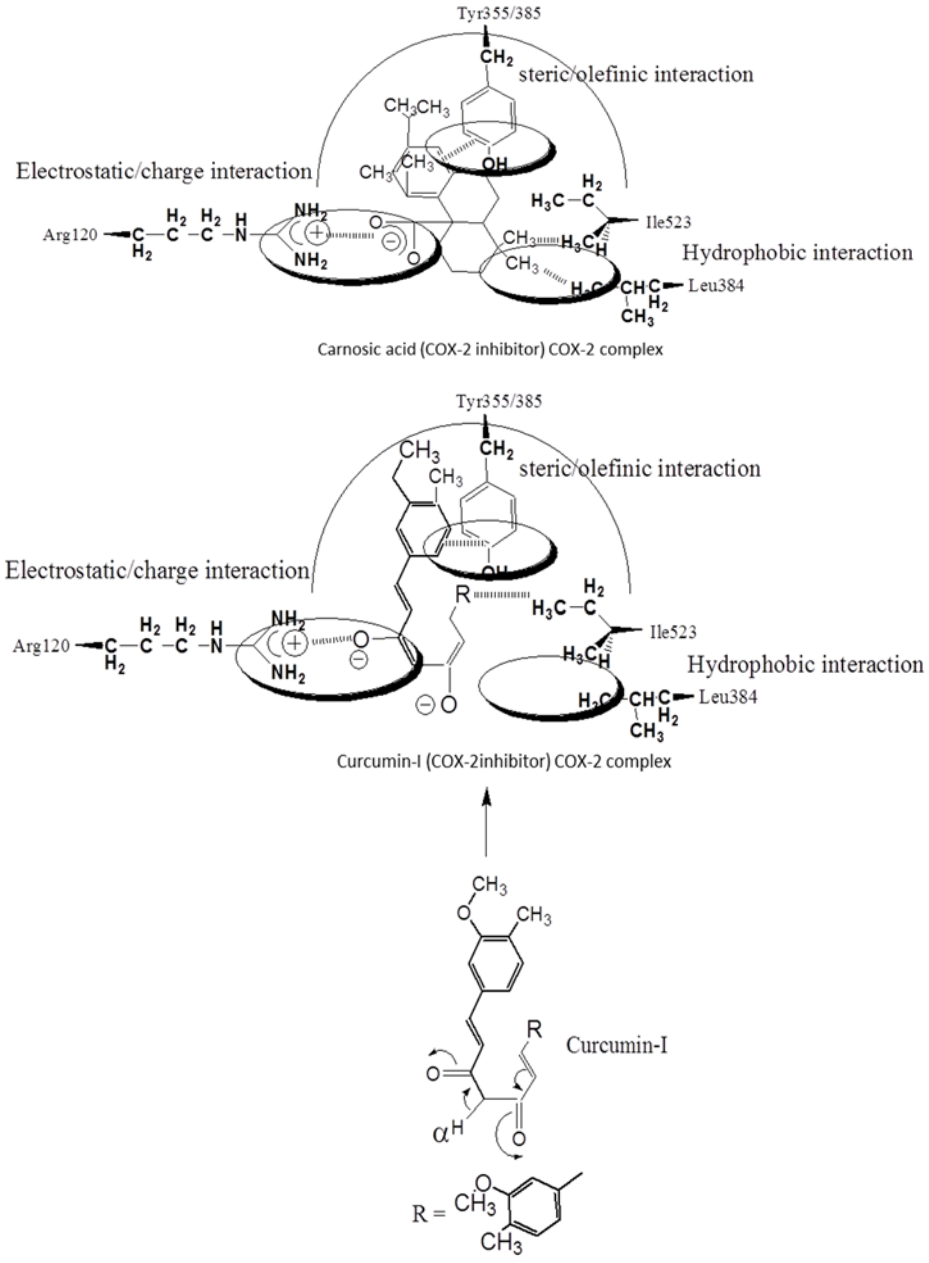


Fig.6A.6 Schematic representation of the COX-2 active site amino acyl residues with the competitive inhibitiors, Carnosic acid and curcumin-I inhibiting the formation of inflammatory prostanoids (PGH_2, PGE_2, $PGF_{2\alpha}$, and TxA_2).

6A.2.4.2. Inhibition of lipoxygenase-V (5-LOX)

Lipoxygenases catalyze the dioxygenation of polyunsaturated fatty acids (PUFAs), such as, ARA (20:4*n*-6), which contain one or more 1, 4-*cis*, *cis*-pentadiene units to yield hydroperoxide products. Though 5-LOX, 12-LOX, and 15-LOX were known to be involved in leukotriene biosynthesis, 5-LOX appears to be the key enzyme. . In this study, GME_4 showed higher inhibition of 5-LOX during the initial hours (d=0) than other treatments (**Table 6A.7)**. Bromelain enzyme escharase present in *A. cosumus* used as a supplement in GME_4 is attributable to the significant activity that supports higher anti-inflammatory action than other combinations (Brien *et al.*, 2006). The anti-inflammatory activities were realized for GME_0 as apparent from the potential of the same to inhibit 5-LOX. Decelerated 5-LOX inhibition by GME_4 (7.6%), GME_1 (7.8%), GME_2 (8.0%) and GME_3 (9.3%) than in GME_0 (18.6%) after 90 days of accelerated storage can be explained due to the absence of antioxidant additives in the latter. As in COX-2, charge/π-electron and hydrophobic interactions in the catalytic domain of lipoxidase play a major role in catalyzing ARA to yield hydroperoxide products. The active ingredients present in the additives used in the present work shares comparable structural/molecular features with the substrate (ARA) as established by calculating the molecular descriptors (**Table 6A.8**). The active ingredients in GME_0 along with the major active principles in the additives appeared to hinder the desired 5-LOX catalyzed formation of hydroperoxide products thereby combating the inflammatory response.

6A.2.5 IR Spectroscopic analyses of the mussel extract supplemented with the natural additives (GME_1-GME_4) in a time series accelerated shelf-life study at various intervals of incubation

One of the principle active ingredients responsible for anti-inflammatory activities in green mussel *P. viridis* was found to be polysaccharides and polysaccharide complexes. These biopolymers in shellfish received considerable attention in recent years because of their anti-inflammatory, antitumor and immunoregulatory activities. The IR spectrum of the mussel extract indicates the presence of polysaccharide units and glycosidic bonds along with common functional groups present in polysaccharides. The absorbance in the range of 1037–1239 cm^{-1} indicates pyranose ring structure. Strong and wide absorption peaks in the region of 3196-3509 cm^{-1} are due to a large amount of -OH stretching vibration characteristic of the polysaccharides. C-H stretching vibrations were characterized by the strong absorption peaks at 2854-3073 cm^{-1}. The absorption bands appeared at 1644-1686 cm^{-1} is due to water associated with polysaccharides (Zhang *et al*., 2010). About other bands, there were intense absorbance with a peak of around 1629-1686 cm^{-1}(C=O asymmetric stretching vibrations of the carboxylic groups), and bands of about 1403 and 1456 cm^{-1}(C=O symmetric stretching vibrations), indicated that there were carboxyl groups in the active principle. The band near 1543 cm^{-1} was assigned to the amide–II band resulting from the coupling between the NH_2 deformation mode and -CN stretching vibration. The spectrum shows absorption at around 845-933 cm^{-1} representing glycosidic linkages of polysaccharides. The spectra (**Fig 6A.7**) have been reported principally as a fingerprint for any future identification, since the absorption bands for such complex compounds are usually broad and diffuse.

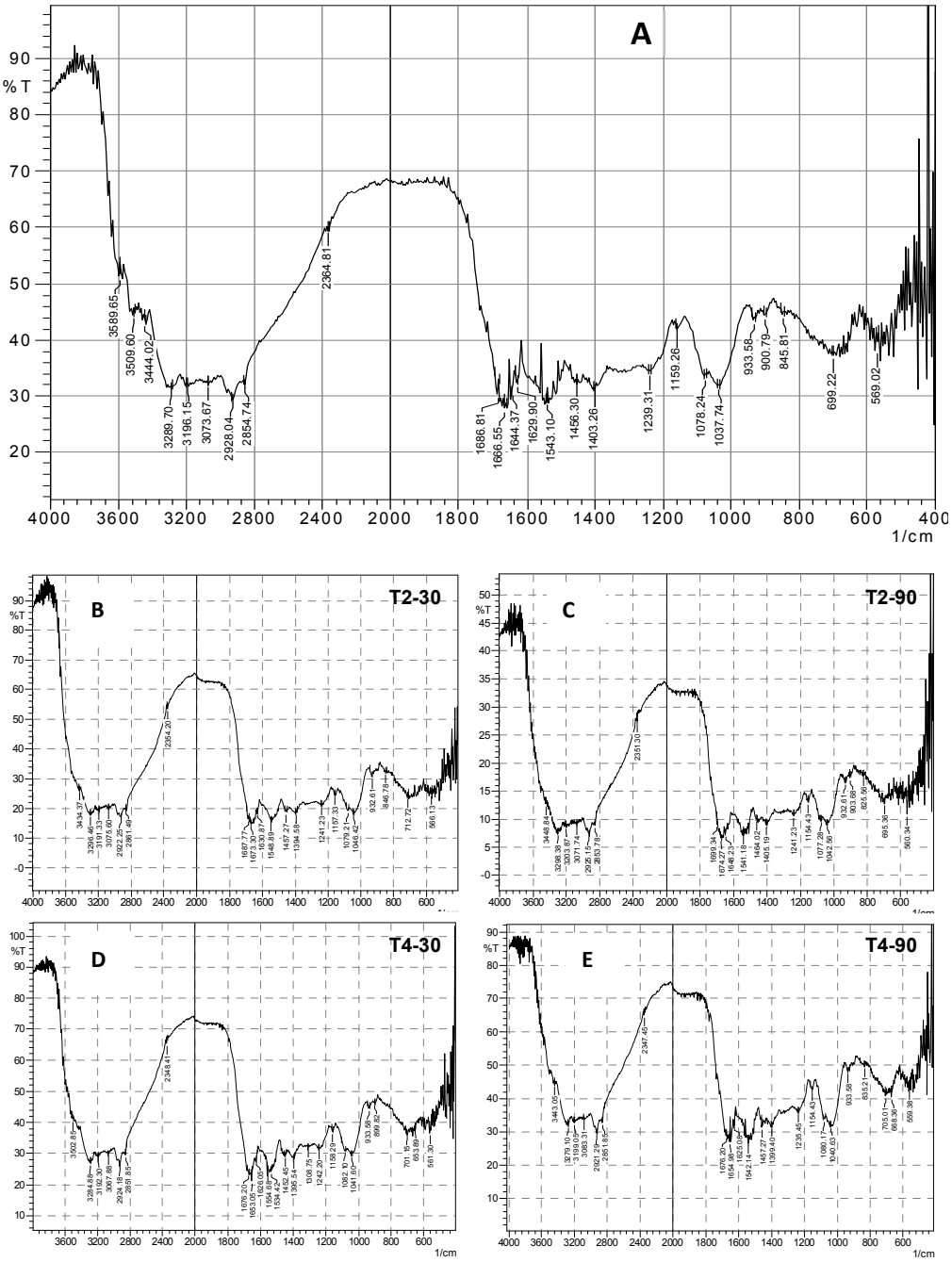


Fig 6A.7(A) Infrared spectra of mussel crude extract at baseline (d=0) *vis-à-vis* GME_2 and GME_4 during **(B & D)** 30 (d=30) and **(C & E)** 90 days (d=90) of accelerated shelf-life study.

The FT-IR spectra of GME_2 and GME_4 combinations at various time intervals are shown in **Fig 6A.7** to understand the possible degradation/retention of these anti-inflammatory principles (polysaccharides) in a time-dependent manner till the end of 90 days of accelerated shelf life study. In this context it is important to note that the treatments GME_2 and GME_4 showed significant stability towards the polysaccharide complexes of mussel extract as evident by the presence of characteristic peaks of pyranose ring structure (1100–1200 cm^{-1}), OH moieties (3200-3500 cm^{-1}), C–H stretching vibrations (2900-3000 cm^{-1}), and glycosidic linkages of polysaccharides (825-950 cm^{-1}). Also of note is the presence of characteristic bands at 1235-1400 cm^{-1} (symmetric CH_3 bending as in polysaccharides) and 1660-1670 cm^{-1} (water associated with polysaccharides) appeared at throughout the period in shelf after 90 days (d=90) of accelerated shelf life study in GME_2 and GME_4 with respect to those recorded at baseline (d=0) and at d=30 (**Fig 6A.7**). GME_2 and GME_4 exhibited the signals at 1600–1700 cm^{-1}, 1400 cm^{-1}, and 1540-1554 cm^{-1} indicating the presence of –COOH and –NH groups at d=90. Hence, the present investigation demonstrated that the combinations GME_2 and GME_4 have the potential to retain the active principles, particularly the polysaccharide fraction.

6A.2.6 *In vitro* and *in vivo* anti-inflammatory studies of the potential anti-inflammatory treatments (GME_2 and GME_4)

The *in vitro* stability studies of anti-inflammatory activity in time series accelerated shelf life study showed that GME_2 and GME_4 retained the anti-inflammatory activity of GME_0, and, therefore, these were shortlisted for further evaluation of *in vitro* and *in vivo* anti-inflammatory activities, and were compared to synthetic NSAIDs.

6A.2.6.1 *In vitro* anti-inflammatory activities

GME_2 and GME_4 exhibited dose dependent inhibition of COX-1 and COX-2. At higher concentration (1 mg/mL) the treatments GME_2 and GME_4 inhibited COX-2 to the tune of 49.9 and 46.7%, respectively, than did indomethacin. Particularly, GME_4 showed excellent inhibitory activity against COX-1, COX-2, and 5-LOX, respectively, and its inhibitory activities were well balanced for these enzymes. It is worth mentioning that a comparable inhibition of these treatments (GME_2 and GME_4) with indomethacin ($\geq$ 58%, 5 mg/mL) was found on inhibiting LOX_V, and was higher than aspirin. This discovery preceded the observation that dual inhibitors of COX-2 and 5-LOX may have a better therapeutic profile and have fewer side effects than NSAIDs.

COX-1 is termed as the housekeeper enzyme due to its presence in most tissues, particularly gastrointestinal tract lining. COX-2 is inducible by the inflammatory response, and increases in response to inflammation including arthritis. It is desirable to inhibit this pro-inflammatory enzyme to deter inflammation. COX-1 maintains normal gastric mucosa, and, therefore, it is undesirable to inhibit this enzyme in an exclusive manner. COX-1/COX-2 ratio apparently provides us with the selectivity of the anti-inflammatory agents. Analysis of COX-1/COX-2 ratio and side effects of the NSAIDs show that the higher the ratio, the lower the selectivity towards inhibiting inflammation, and consequently higher are a side effect. Earlier studies reported that (Engelhardt *et al*., 1995) tenoxicam, diclofenac, tenidap and piroxicam possess higher COX-1/COX-2 ratio (15, 2.2, 122 and 33, respectively), which are undesirable indicators of an ideal anti-inflammatory agent. In the recent context, selective COX-2 inhibitors are gaining importance as potential new generation selective anti-inflammatory agents. In the present study, the treatments GME_2 and GME_4 realized the COX-1/COX-2 ratios less than 1.0, as compared to the NSAIDs (COX-1/COX-2 > 1.0). A lower COX-1/COX-2 ratio of GME_2 and GME_4 relates to less gastrointestinal side-effect profiles, and effectively manages the manifestations of pain and arthritis.

In recent times, interest has been focused towards LOX inhibitors, which were reported to block the synthesis of leukotriene metabolites. Though 5-LOX, 12-LOX, and 15-LOX were known to be involved in leukotriene biosynthesis, 5-LOX appears to be the key enzyme. Accordingly, a lower COX-1/5-LOX (<1.0) indicates higher selectivity and lower side-effect profiles of the anti-inflammatory agents GME_2 and GME_4. Ideally, the simultaneous inhibition of COX-2and 5-LOX maximize the anti-inflammatory effects, and, therefore, the pharmaceutical companies are in search of anti-inflammatory agents with lower COX-1/COX-2 and COX-1/5-LOX due to their higher selectivity against inflammatory response and lower undesirable side effects or toxicity.

Table 6A.9 Percentage inhibition of COX-1, COX-2 and 5-LOX enzymes and ratios of pro-inflammatory enzyme inhibition by anti-inflammatory formulations (GME_2 and GME_4) *vis-à-vis* standard anti-inflammatory drugs, aspirin and indomethacin at different concentrations (0.5 mg/mL, 1 mg/mL, 5 mg/mL).

	Aspirin	Indomethacin	GME_2	GME_4
Inhibition (percent) of COX-1				
0.5 mg/mL	44.6 ± 0.55^{p}	45.6 ± 0.80^{p}	28.6 ± 0.61^{q}	28.1 ± 0.94^{q}
1 mg/mL	52.0 ± 1.83^{p}	54.0 ± 1.05^{p}	41.1 ± 0.95^{q}	42.6 ± 0.52^{q}
5 mg/mL	70.0 ± 0.10^{p}	72.1 ± 0.92^{p}	44.2 ± 0.41^{q}	44.5 ± 1.41^{q}
COX-1/COX-2	1.16 ± 0.94^{p}	1.32 ± 0.14^{q}	0.96 ± 0.24^{p}	0.90 ± 0.14^{p}
COX-1/5-LOX	1.42 ± 0.04^{p}	1.39 ± 0.01^{p}	0.65 ± 0.02^{q}	0.62 ± 0.03^{q}
Inhibition (percent) of COX-2				
0.5 mg/mL	38.5 ± 0.09^{p}	34.6 ± 0.59^{p}	29.9 ± 0.10^{p}	31.2 ± 0.19^{p}
1 mg/mL	52.2 ± 0.10^{p}	46.3 ± 0.49^{q}	49.9 ± 0.30^{pq}	46.65 ± 0.13^{q}
5 mg/mL	58.6 ± 0.60^{p}	59.0 ± 1.0^{p}	52.1 ± 1.10^{q}	52.9 ± 0.12^{q}
COX-1/COX-2	1.00 ± 0.01^{p}	1.17 ± 0.02^{p}	0.82 ± 0.04^{q}	0.91 ± 0.05^{p}
COX-1/5-LOX	1.29 ± 0.01^{p}	1.14 ± 0.04^{p}	0.87 ± 0.07^{q}	0.86 ± 0.03^{q}
Inhibition (percent) of 5-LOX				
0.5 mg/mL	31.3 ± 0.55^{p}	32.9 ± 0.90^{p}	43.8 ± 0.61^{q}	45.1 ± 0.94^{q}
1 mg/mL	40.2 ± 1.83^{p}	47.2 ± 1.05^{q}	47.1 ± 0.95^{q}	49.6 ± 0.52^{q}
5 mg/mL	54.8 ± 0.10^{p}	66.3 ± 0.95^{q}	58.2 ± 0.41^{q}	62.3 ± 1.41^{q}
COX-1/COX-2	1.19 ± 0.03^{p}	1.22 ± 0.02^{p}	0.85 ± 0.03^{q}	0.84 ± 0.04^{q}
COX-1/5-LOX	1.28 ± 0.05^{p}	1.09 ± 0.04^{p}	0.76 ± 0.02^{q}	0.71 ± 0.02^{q}

COX-1/COX-2 and COX-1/5-LOX are referred to as selectivity indicators of anti-inflammatory properties of the anti-inflammatory formulations (GME_2 and GME_4) *vis-à-vis* synthetic NSAIDs. p, q: Row wise values with different superscripts of this type indicate significant differences ($P<0.05$) within different samples.

6A.2.6.2 *In vivo* anti-inflammatory activities of the shortlist combinations on carrageenan-induced rat paw edema

GME_2 and GME_4 showed to be potent anti-inflammatory supplements; and, therefore, they were further evaluated for their activities in an *in vivo* system. In the carrageenan administered animals (control) severe swelling was observed at 5^{th} h, and the swelling was maintained until 6^{th} h. Though the aspirin treated groups decreased paw edema significantly ($P<0.05$) with a maximum inhibition (80.87%) after 4^{th} h the edema exhibited an incremental trend during 6^{th} h. The effects of aspirin, ibuprofen and stabilized freeze-dried *P. canaliculus* extract in carageenan-induced inflammation in rodents, which revealed that stabilized mussel extract reduced edema significantly higher (90%) compared with aspirin (40%) and ibuprofen (60%). Notably, the animals challenged with GME_2 and GME_4 significantly mitigated the edema in rats in a time-dependent manner till the end of the 6^{th} h as compared to the control animals throughout the period of study ($P<0.05$) (**Table 6A.10**). GME_2 and GME_4 showed inhibition of the edema to the tune of 52.31 and 50.52%, respectively (250 mg/kg dose) during the 3^{rd} h after the carrageenan injection, which reached to 72.96 and 73.48%, respectively after the 6^{th} h as compared to ~80% for standard synthetic drug (aspirin) (**Fig 6A.6**). However, it was interesting to note that the activity of GME_2 and GME_4 improved with time, indicating their potential to release the active principle throughout an extended period, unlike the standard, which showed a deceleration in activity after 4 h.

Table 6A.10 Effect of the anti-inflammatory treatments GME2 and GME4 compared with the standard drug (aspirin) on carrageenan-induced hind paw edema in BALB/C mice

Samples	Mean paw edema (mm)	Difference in paw edema		%	Difference in paw edema		%	Difference in paw edema		%	Difference in paw edema		%	Difference in paw edema		%
		2h		inhibition	3h		inhibition	4h		inhibition	5h		inhibition	6h		inhibition
	0h	(mm)	%		(mm)	(%)		(mm)	(%)		(mm)	(%)		(mm)	(%)	
N. saline	1.16	2.47	112.93±4.87		2.48	113.79±1.61		2.48	113.79±1.59		2.5	115.52±5.57		2.52	117.24±1.51	
Standard	1.47	1.99	35.37 ±3.33	68.68	1.89	28.57±1.71	74.89	1.79	21.77±2.06	80.87	1.8	22.45±1.36	80.57	1.81	23.13±0.31	80.27
GME_2	1.64	2.62	59.76 ±5.92	47.09	2.53	54.27±1.28	52.31	2.21	34.71±1.93	69.46	2.2	34.15±3.15	70.44	2.16	31.71±3.27	72.96
GME_4	1.19	1.91	60.50 ±2.18	46.42	1.86	56.30±2.86	50.52	1.63	36.97±3.1	67.51	1.6	34.45±4.09	70.17	1.56	31.09±1.72	73.48

Difference in mice paw edema with respect to zeroth hour. Percent difference in mice paw edema was calculated as: $(T_t - T_o)$ X 100/ T_o, where T_t is the average thickness of the edema for each group after treatment in different time intervals and T_o is the average thickness for each group at baseline. Percent inhibition in mice paw edema (in parentheses) was calculated as: $(I_t - I_o)$ X 100/ I_o, where I_t is the percent difference in paw edema of the animals treated with normal saline with respect to the baseline value (zeroth hour) and I_o is the percent difference in paw edema of animals treated with standard, GME_2 and GME_4 at the same time interval.

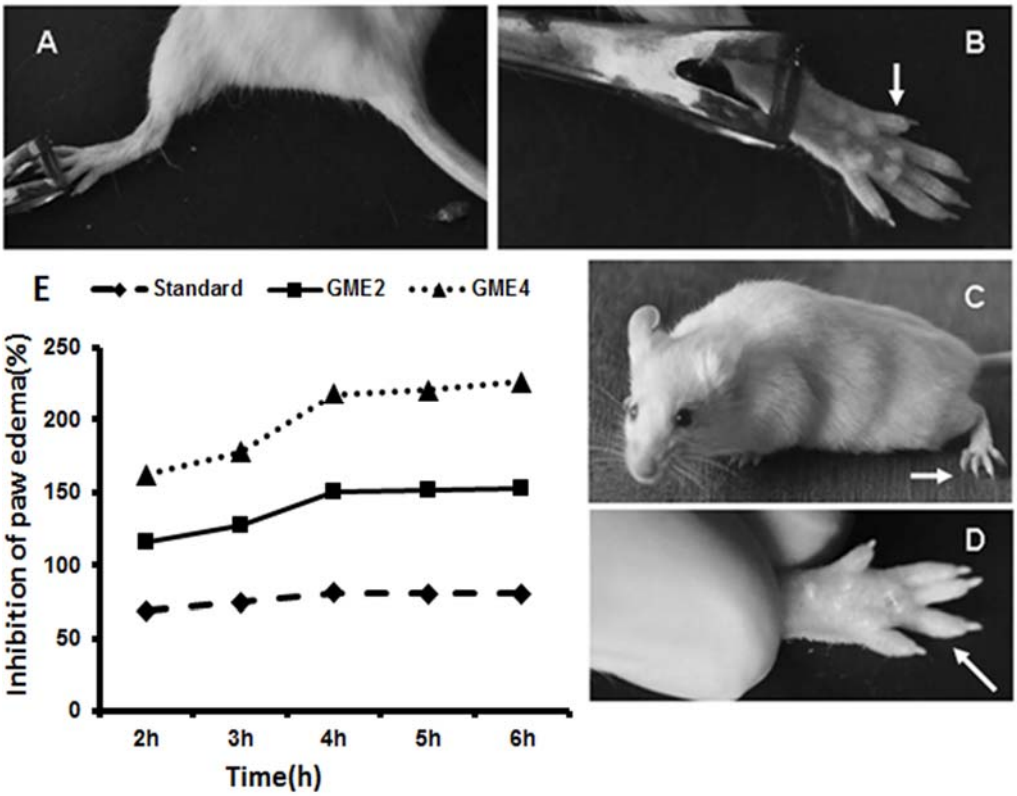


Fig 6A.8 Effect of the anti-inflammatory treatment GME_2 and GME_4 compared with the standard drug (aspirin) on carrageenan-induced hind paw edema in BALB/C mice. (**A**) & (**B**): Reductions in rat paw edema in treated (by GME_2) rats; (**C**) & (**D**): Carrageenan-induced hind paw edema in BALB/C mice without any treatment; (**E**) Rate of change in rat paw edema (%) after administration of anti-inflammatory treatments GME_2 and GME_4 *vis-à-vis* standard drug (aspirin).

6A.3 Conclusions

The dual inhibitory activity of the aqueous extract of Indian green mussel, *P. viridis* against pro-inflammatory COX-2 and 5-LOX enzymes demonstrated its better therapeutic profile and fewer side effects than synthetic NSAIDs. From the present study, it is revealed that different additives from natural origin, such as, *Curcuma longa*, *Rosmarinus officinalis* etc., added in

different combinations can retain the anti-inflammatory activity of *P. viridis* extract by stabilizing its active anti-inflammatory ingredients. The FT-IR spectra at various time intervals showed the possible degradation/retention of the anti-inflammatory principles of *P. viridis vis a vis* different combinations, in a time-dependent manner demonstrating that the combinations GME_2 and GME_4 have the potential to retain the active principles throughout the experimental period under shelf. A lesser COX-1/COX-2 and COX-1/5-LOX, simultaneous inhibition of COX-2 and 5-LOX enzymes and significant *in vivo* activity of GME_2 and GME_4 indicates higher selectivity and lower side-effect profiles. Hence, the anti-inflammatory formulations, GME_2 and GME_4 are effective new generation green alternatives to the synthetic NSAIDs for use against a cascade of mammalian inflammatory diseases.

6.B Enrichment of the potential anti-inflammatory combination by anti-inflammatory principles isolated from *P. viridis*

Background of the study

The green mussel *P. viridis* is a bivalve mussel (family Mytilidae) found in the coastal sea beds along the south west coast of India, and considered to be an economically important bivalve mollusk. Green mussel, abundant in C_{20}-C_{22} *n*-3 PUFAs, particularly eicosapentaenoic acid (20:5*n*-3, EPA) and docosahexaenoic acid (22:6*n*-3, DHA), which are the precursors of anti-inflammatory resolvins (E- and D-series). EPA is more efficient in reducing cellular inflammation preventing depression disorders, and in the reduction of serum triacylglycerols (formerly known as triglycerides). DHA is a component of the phospholipid membrane of brain and retina cells; consequently, it is essential for human health. Phospholipids and phosphatidylcholine applied to either the apical or the basolateral surfaces, was integrated into the cells and significantly inhibited TNF-α-induced inflammatory responses. In animal studies the exogenous phospholipids or phospholipids contained in food have been shown to prevent mucosal damage induced by acids, nonsteroidal anti-inflammatory drugs, or bile salts in the stomach, duodenum, and small intestine. Phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) are the major phospholipid classes found in the mucus, the anti-inflammatory action of choline in guinea pigs.

Polysaccharides and polysaccharide–protein complexes have been found as common polymers in shellfish and received considerable attention in recent years because of their anti-inflammatory, antitumor and immunoregulatory activities. Polysaccharides have been used for decades to stimulate the immune system and to fight against cancer. And several researches have shown that

some polysaccharide possessed an antioxidant activity. *Perna viridis* was reported to yield heparin and heparin-like glycosaminoglycans with fewer impurities (Arumugam and Shanmugam, 2004).

The present work involves the extraction and isolation of different anti-inflammatory principles from *P. viridis*. Specifically the anti-inflammatory lecithin and polysaccharides along with glycolipoprotein and *n*-3 polyunsaturated fatty acids were isolated from this bivalve mollusk in an attempt to enrich the anti-inflammatory activity of the formulations. The isolation of active components from whole organisms requires several purification steps, with each step requiring a screening process to target the active components. These isolated naturally derived anti-inflammatory components were added in various compositions to the said anti-inflammatory combination GME_2 (discussed in section 6A) to prepare different experimental combinations (Q_1 to Q_2).

Another area of concern is that a product of green mussel origin is embedded in unsaturated lipid matrix. This can potentially result in changes in its physicochemical characteristics (formation of high risk decomposition substances *viz.*, free radicals, small molecular weight aldehydes, and ketones) during storage thereby changing its anti-inflammatory properties. The present work explored the role of various natural additives with antiradical active principles to retain the anti-inflammatory potential of the anti-inflammatory concentrate. Naturally derived antioxidants were added to the anti-inflammatory combination, and were subjected to accelerated shelf life study (50°C) for a period of 90 days (d=90) to evaluate the anti-inflammatory activities of different combinations, supplemented with natural additives during the study period.

6B.1 Isolation of anti-inflammatory principles from *P. viridis*

The anti-inflammatory activity of *P. viridis* extract might be due to the presence of one or more constituents present in the extract. These components might act synergistically to reduce inflammation, limit further cartilage degeneration, and potentially support the regeneration of damaged joint cartilage and synovial fluid. The exact natures of the anti-inflammatory compounds can be elucidated by the purification of *P. viridis* sample by different methods. The identification of major compounds in it may be helpful in delineating the pharmacological activity and the underlying mechanisms.

6B.1.1 Isolation of polysaccharides from *P. viridis*

The ground fresh mussel sample (100 g) was extracted thrice with water (60ºC, 1:1 ratio, 2 h) and centrifuged (model superspinR-V/Fm, Plastocrafts) at 9500 rpm for 20 min to separate the water insoluble impurities from the extracts. The combined extracts were concentrated (50°C) *in vacuo* to one-third volume and precipitated with ice-cold 95% ethanol under rigorous stirring. The polysaccharide pellets were obtained by centrifugation and freeze dried to obtain crude polysaccharides **11** (MPS) (30.01 g, yield based on raw mussel, 3.01%). The crude polysaccharide obtained from *P. viridis* was purified by ion exchange chromatography DEAE (Bio-Rad, USA) column (Cl^- form, 2.5 x 9 cm) equilibrated with 0.1 M NaCl. Briefly, the solution of ethanol precipitate in NaCl (0.1 M) was charged to the anion exchanger, and the column was successively eluted with a NaCl linear gradient (0.1 to 1.5 M) until the disappearance in the eluent of positive reaction for carbohydrates. The fractions containing polysaccharide were vacuum concentrated, dialyzed against distilled water for 36 h, and lyophilized. The major peak was collected and further purified on a Sephacryl S-300 HR gel-permeation chromatography to afford (→4)-2, 4 di-(N-acetyl-β-D-mannosamine) -(1→4)-2-N-acetyl- β-D-

galactosamine-(1→) - (4-N-acetyl *p*-phenoxyl) motifs. The purified fraction was monitored by HPLC on a SUPELCOSIL LC-NH_2 column (Supelco, 5 µm, 25 cm × 4.6 mm) using acetonitrile: water (75:25; % v/v, 1 mL/min) as mobile phase at 35ºC using a RI detector for the presence of oligosaccharide units (**12**).

6B.1.2 Isolation of lysolecithin from *P. viridis*

The FDPE was further extracted with MeOH (3 x 650 mL,70 ºC for 2-4 h under N_2), filtered through Whatman No. 1 filter paper, and the pooled filtrate was concentrated (50ºC) in *vacuo* to one-third volume. The methanolic extract was partitioned with $CHCl_3$: MeOH (100 mL x 3**)** and concentrated in *vacuo* to furnish a fraction termed as PME_0. The PME_0 was subjected to a silica gel (60–120 mesh) column (60 g) chromatography, and eluted using a stepwise gradient system from $CHCl_3$ (100%) to MeOH (100%) to obtain eleven column fractions (PME_{1-11}). The purification procedure was monitored by different chromatographic analyses which revealed the purity of PME_{11} fraction, which was characterized as lysolecithin (**12**).

6B.1.3 Extraction and purification of glycolipoprotein

The extraction of glycogen from *P. viridis* samples were carried out following the method of Miller *et al* (Miller *et al.*, 1993) with suitable modifications. The fresh, ground *P. viridis* sample (500g) was extracted with 5 volumes (w/v, 2500mL) of aqueous phenol (w/v 15%) at 15-20ºC for 45-60 min. The mixture was then centrifuged (8000-12000 rpm) for 5-8 min and the upper aqueous layer containing glycogen was aspirated. About 6 volumes (3000 mL) of 95% ethanol was added and centrifuged (8000-12000 rpm) for 5-10 min to obtain the precipitate. This precipitate was washed with distilled water (5 x 200 mL) to remove the phenol content and lyophilized to get the GLP (GLP, 3.35g; yield based on raw material, 0.7%).

The GLP was refluxed with aqueous KOH (20%, 30mL) in a water bath at 100ºC for 2-4 h under N_2 and left to cool at 4ºC overnight. The mixture containing the precipitate was centrifuged and washed with distilled water (3x40 mL) to make the pH neutral. The obtained glycoprotein was lyophilized (GP, 0.1g; yield based on raw mussel, 0.02%). The GP (50mg) was dissolved in phosphate buffer (0.1M, 1mL, pH 7.4) and hydrolysed by proteinase K enzyme (2% in distilled water; 12µL) and kept at 37ºC for 8-24 h preferably for 16 h. The precipitate was centrifuged (8000-12000 rpm) for 20min, washed with distilled water (2x40 mL) and the obtained product was lyophilized to get glycogen (G, 0.05g; yield based on raw mussel, 0.008%).

6B.1.4 Enrichment of polyunsaturated fatty acids by amide complexation

The FDPE (160g) was homogenized with ethyl alcohol (640 mL), sonicated for 10 min, and the homogenate was refluxed (55-60 ºC) for 30 min. The alcoholic solution was vacuum-filtered by using a buchner funnel through Whatman No. 1 filter paper, and concentrated using a rotary evaporator at 40 ºC. The obtained extract was saponified to furnish free fatty acids (FFAs) as follows.

The extract (ALE, 18 g, yield,12.1%) was treated with 0.001 % butylated hydroxytoluene (BHT) and saponified by refluxing for 1 h under a blanket of N_2 using aqueous mixture of KOH (55.5 mL, 1.33N). To the saponified mixture, distilled water (50 mL) was added and the unsaponifable matter was extracted with *n*-hexane (100 mL x 2) and discarded. The aqueous layer containing saponifable matter was acidified (pH 1.0) with 3N HCl (19 mL). The mixture was transferred to a separatory funnel and the liberated fatty acids were extracted with of *n*-hexane (50 mL). The *n*-hexane layer, containing free fatty acids, was then dried over anhydrous Na_2SO_4 and the solvent was removed at 40 ºC to recover the free fatty acids. The polyunsaturated fatty acids (PUFAs) in these free fatty acids were concentrated by urea-fatty acid complexation. Briefly, the free fatty acids (6

g), to which urea (24 g) in ethyl alcohol (95% v/v, 240 mL) was added, and the contents were heated (60–65ºC) until a clear homogeneous solution was obtained. The urea complexes were allowed to crystallize overnight at 4ºC. The formed crystals (urea complexing fraction, UCF) were separated from the liquid (non-urea complexing fraction, NUCF) by filtration. The NUCF was diluted with water (720 mL) and acidified to pH 4-5 with using 6N HCl (5 mL).The mixture was further extracted with *n*-hexane (20 mL), and the combined organic layers were washed with water, and dried over anhydrous Na_2SO_4, and the solvent was removed at 40ºC using a rotary evaporator.

6B.1.5 Preparation of enriched anti-inflammatory compositions, experimental treatments and designing of stability studies

The best anti-inflammatory formulation GME_2 (section 6A) was enriched with anti-inflammatory components (MPS, glycolipoprotein, PME_0, NUCF), isolated from *P. viridis*. For the identification of potent ratios of MPS, two different formulations with MPS were prepared by assigning GME_2 as the base material. The enriched compositions were stabilized by adding 0.1% of *E. officinalis* extract (PEE). *E. officinalis* (100 g) was extracted thrice with distilled water (100 mL), centrifuged and lyophilized to obtain crude aqueous extract. The anti-inflammatory components, such as, MPS, glycolipoprotein, NUCF, PME_0 along with PEE were homogenized in distilled water and added to FDPE in different ratios according to **Table 6B.1**. In order to find the potent anti-inflammatory combination, these combinations were studied for their different biochemical characteristics in order to find their individual and synergistic effects. The samples were kept in glass vials (10 g each) in an incubator at 50°C, were evaluated the 0^{th} day, and at regular time intervals according to the experimental design for a period of 90 days (3 months). A time-dependent accelerated shelf life

studies were conducted in order to find the effects of different compositions of additives in the enriched anti-inflammatory composition.

Table 6B.1: Composition (in percent) of different formulations:

Components added	Q_1	Q_2
GME_2	97.7	97.7
MPS	1.2	0.8
GLP	0.4	0.4
PME_0	0.4	0.8
NUCF	0.2	0.2
PEE	0.1	0.1

FDPE: freeze dried mussel extract without additives (control). MPS (crude polysaccharides isolated *P. viridis*), PME_0 (methanol extract of *P. viridis*), GLP (Glycolipoprotein), NUCF (non-urea complexing fraction), PEE (lyophilized aqueous extracts *of E. officinalis*)

6B.1.6 Antioxidant and anti-inflammatory assays

The capability of the samples to scavenge hydrogen peroxide (H_2O_2) was determined according to the established method (Ruch *et al*., 1989). Lipid compositions of the samples (0.1g each) were determined using established methods (Bligh and Dyer, 1959) and the fatty acid composition of the total lipids was determined as described elsewhere (Chakraborty and Paulraj, 2009). The amount of total phenolics, 2, 2-diphenyl -1- picrylhydrazyl (DPPH) radical scavenging activity and degree of lipid peroxidation were determined as described earlier (Tai *et al*., 2011). Cyclooxygenase (COX-1 and COX-2) inhibition assays were performed using 2, 7-dichlorofluorescein method (Cheung *et al*., 2007). The 5-lipoxygenase (5-LOX) inhibition assay was carried out using the principle of 1-4 diene (linoleic acid) oxidation to 1- 3-diene (Baylac *et al*., 2003). The *in vivo* carrageenan-induced mice paw edema experiment was carried out as previously described (Winter *et al*., 1962).

6B.1.7 Spectroscopic analyses

Fourier Transform Infra Red spectrometer (FTIR) spectra of the compounds under KBr pellets were recorded in a Thermo Nicolet, Avatar 370 explained in 5.1.1.2A. The Gas Chromatography-Mass Spectrometry (GC-MS) analyses were performed in electronic impact (EI) ionization mode in a Varian GC (CP-3800) interfaced with a Varian 1200L single quadruple Mass Spectrometer and the 1H and 13CNMR spectra were recorded on a Bruker AVANCE III 500 MHz (AV 500) DRX 500 NMR spectrometer (Bruker, Karlsruhe, Germany) detailed in 5.1.1.2B and 5.1.1.2C respectively.

6B.1.8 Statistical analyses

One-way analysis of variance (ANOVA) was carried out with the Statistical Program for Social Sciences 13.0 (SPSS, USA, ver. 13.0) to assess for any significant differences between the means. Differences between means at the 5% (P<0.05) level were considered significant.

6B.2 Result and discussion

6B.2.1 Enrichment of polyunsaturated fatty acids by amide complexation

The fatty acid compositions of the two fractions were determined using GC and recorded in **Table 6B.2**. FDPE showed a total PUFA content of 28.4%. The monounsaturated fatty acids (MUFAs) accounted for approximately 20.9%, of which the dominant was found to be 22:1*n*-9. Gordan *et al* reported the fatty acid profiles of mollusks, usually dominated by saturated fatty acids (SFAs), apparently our study reported 42.3% SFA in *P. viridis* (Gordan *et al*., 1982). The most dominant SFA was 16:0 (25.9%). The major long chain PUFAs was found to be 22:6*n*-3 (7.9%) and 20:5*n*-3 (7.0%). The total SFAs (72.2%) in ALE comprised of 8.8, 43.6, 4.5, and 1.8% of C14:0, C16:0, C17:0, C18:0 and C20:0 SFAs, respectively. The MUFA was

recorded to be higher (11.4%) compared to total PUFA (10.9%). PUFA was mainly contributed by the fatty acids 20:5*n*-3 (2.4%) and 22:6*n*-3 (3.1%). The FFA (6 g, yield-3.7%), obtained after saponification were purified by complexation with urea, to obtain non-urea complexed fraction (NUCF, 3 g; yield-1.9%) and urea crystallized fraction (UCF,16g). The interfering SFAs and most of the MUFAs (long-and straight-chain molecules) were removed in the form of urea inclusion complex, while the PUFAs remained in solution. Urea fractionation resulted in significant reduction in SFAs (72.2% to 39.2%TFA). The total PUFA after urea fractionation was recorded to be 34.6%, mainly comprising by EPA (9.8% of TFA), 18:3*n*-3 (2.6% of TFA), and 22:6*n*-3 (10.1% of TFA). The complete removal of SFAs by urea complexation is impossible since some of saturated fatty acids do not form complex with urea during crystallization (Ratnayake *et al*., 2006). A small proportion of PUFAs appeared to be complexed with urea, and was detected in UCF (9.1%).

Table 6B.2: Fatty acid composition of ethanolic extract (AEE), urea complexed fraction (UCF) and non-urea complexed fraction (NUCF).

	Fatty acid (as weight %)			
	FDPE	**AEE**	**NUCF**	**UCF**
		Saturated fatty acids		
12:0	0.14^{a}	0.15^{a}	0.13^{a}	0.25^{a}
13:0	0.07^{a}	0.08^{a}	0.05^{a}	0.13^{a}
14:0	6.09^{a}	8.79^{b}	6.15^{a}	7.63^{c}
15:0	0.13^{a}	1.96^{b}	0.72^{a}	1.85^{b}
iso 15:0	0.06^{a}	0.19^{a}	0.12^{a}	0.19^{a}
16:0	25.9^{a}	43.6^{b}	21.2^{a}	46.8^{b}
iso 16:0	0.99^{a}	0.9^{a}	0.45^{a}	0.95^{a}
17:0	0.33^{a}	4.52^{b}	1.52^{c}	4.85^{b}
18:0	6.09^{a}	9.64^{b}	8.13^{c}	10.1^{b}
20:0	2.14^{a}	1.85^{a}	0.3^{b}	2.39^{a}
22:0	0.16^{a}	0.31^{a}	0.26^{a}	0.45^{a}
24:0	0.21^{a}	0.25^{a}	0.15^{a}	0.3^{a}
∑ **SFA**	42.3^{a}	72.2^{b}	39.2^{a}	76^{b}

Monounsaturated fatty acids				
14:1n7	1.21[a]	1.23[a]	1.81[a]	1.06[a]
16:1n7 trans	0.27[a]	0.07[a]	0.05[a]	0.05[a]
16:1n7 cis	8.8[a]	4.39[b]	8.86[a]	3.14[c]
16:1n9 cis	1.42[a]	1.53[a]	2.41[b]	1.65[a]
17:01	0.22[a]	0.16[a]	0.35[a]	0.27[a]
18:1n7 cis	2.56[a]	0.58[b]	1.41[b]	0.64[b]
18:1n9 trans	0.08[a]	0.05[a]	0.03[a]	0.04[a]
18:1n9 cis	2.63[a]	1.51[b]	3.55[a]	1.62[b]
20:1n11	0.03[a]	1.05[b]	1.11[b]	1.02[b]
22:1n9	2.76[a]	0.52[b]	1.69[c]	0.87[b]
24:01:00	0.93[a]	0.3[a]	0.45[a]	0.26[a]
∑ MUFA	20.9[a]	11.3[b]	21.7[a]	10.6[b]
Polyunsaturated fatty acids				
16:2n4	0.21[a]	0.27[a]	1.51[b]	0.18[a]
16:3n4	0.14[a]	0.18[a]	0.26[a]	0.21[a]
18:2n6 trans	0.11[a]	0.09[a]	0.08[a]	0.11[a]
18:2n6 cis	2.39[a]	0.55[b]	3.28[a]	0.62[b]
18:3n6	2.46[a]	1.53[a]	2.03[a]	1.85[a]
18:4n6	2.28[a]	0.28[b]	0.45[b]	0.15[b]
18:3n3	1.61[a]	0.42[b]	2.61[c]	1.34[a]
18:4n3	1.1[a]	0.12[b]	0.35[b]	0.15[b]
20:2n6	0.31[a]	0.4[a]	0.49[a]	0.39[a]
20:3n6	0.47[a]	0.21[a]	0.41[a]	0.11[a]
20:4n6	0.58[a]	0.45[a]	0.6[a]	0.08[a]
20:3n3	0.28[a]	0.28[a]	0.68[a]	0.3[a]
20:5n3	6.98[a]	2.37[b]	9.78[c]	1.28[b]
22:5n3	1.56[a]	0.69[b]	1.92[a]	0.15[b]
22:6n3	7.92[a]	3.11[b]	10.1[c]	2.21[b]
∑ PUFA	28.4[a]	10.9[b]	34.6[c]	9.13[b]
∑ n3	8.49	6.99	25.5	5.43
∑ n6	9.84	3.51	7.34	3.31
∑ C_{18}PUFA	8.62	2.99	8.8	4.22
∑ C_{20}PUFA	2.29	3.71	11.9	2.16
n3/n6	0.67	1.99	3.47	1.64
∑PUFA/∑SFA	12	0.15	0.88	0.12
EPA/AA	1.13	5.26	16.3	16
DHA/EPA	0.46	1.31	1.03	1.72

Data are expressed as mean ± standard deviation of three replicates. ΣSFA Total saturated fatty acids, ΣMUFA Total monounsaturated fatty acids, ΣPUFA Total polyunsaturated fatty acids. Means with different superscripts (a, b, c, d) in the same row indicates a statistical difference (P<0.05).

6B.2.2: Anti-inflammatory compounds isolated from *P.viridis*

The biopolymers in shellfish received considerable attention in recent years because of their anti-inflammatory, antitumor and immunoregulatory

activities (Dai *et al.*, 2009). The oligosaccharide (compound **11)** was characterized as di-(N-acetyl-β-D-mannosamine) -(1→4)-2-N-acetyl-β-D-galactosamine-(1→) - (4-N-acetyl *p*-phenoxyl) motifs. IR spectrum of (→4)-2, 4 di-(N-acetyl-β-D-mannosamine) -(1→4)-2-N-acetyl- β -D-galactosamine-(1→) - (4-N-acetyl *p*-phenoxyl) unit revealed the broad and intense stretching at 3400 cm^{-1} is characteristic of hydroxyl groups and the weak stretching at 2930 cm^{-1} is attributed to the C–H bond. The fraction also exhibited an obvious characteristic absorption at 925 and 800 cm^{-1} corresponding to the existence of mannose. The characteristic absorptions at 850 cm^{-1} in the IR spectra indicated the presence of α-glycosidic linkages. The ^{1}H NMR spectrum (500 MHz) (**Fig 6B.1A**) showed anomeric proton signals at δ 5.28-5.35 and δ 4.5 in a molar ratio of about 2:1, which were assigned to the two types of sugar units. The structure characteristics of oligosaccharide, especially the chain linkage and conformation of the sugar units, were completed and confirmed by 1D and 2D NMR. The signals around δ 3.5–4.5 were assigned to be as the cross ring protons **Fig 6B.1.**

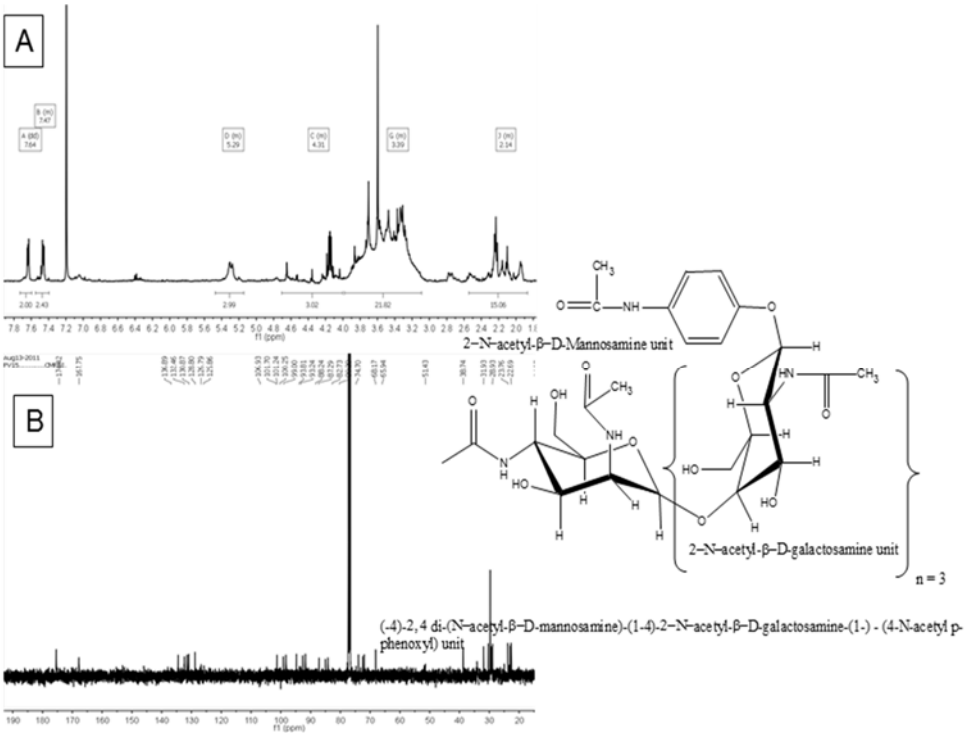


Fig 6B.1 (A) ^{1}H NMR, and (B) ^{13}C NMR spectra of polysaccharide isolated from *P. viridis.*

Similarly, the ^{13}C spectrum (**Fig 6B.1B**) showed four signals δ 102.2, 100.8, 98.2 and δ 94.6 in a molar ratio of nearly 6:1:1:3, which were assigned to be the anomeric carbon of sugar units. The other signals around δ 60–85 were also assigned to be as carbon signals for C2–C5. The complete assignment of the chemicals-shifts of the sugar units were obtained from 2D NMR (**Table 6B.3**), including ^{1}H-^{1}H COSY (Supplementary Figure S1), NOESY (Supplementary Figure S2b), ^{1}H-^{13}C HMBC and HMQC (Figure 4) were acquired, according to the published methodology (Shankaracharya, 1998).

Table 6B.3 NMR spectroscopic data of(⟶4)-2, 4 di-(N-acetyl-β-D-mannosamine) -(1⟶4)-2-N-acetyl- β-D-galactosamine-(1⟶) - (4-N-acetyl *p*-phenoxyl) unit (**11**)

	Carbon Position	^{13}C NMR	δ^{1}H NMR (int., mult., J in Hz)[b]	^{1}H-^{1}H COSY
(4-N-acetyl-p-phenoxyl) unit- A	A-1	132		
	A-2	130	7.42-7.52 (J=9.09 Hz)	A-3
	A-3	126	7.59-7.67 (J=9.01 Hz)	
	A-4	136		
	A-5	125	7.59-7.67 (J=9.01Hz)	A-6
	A-6	128	7.42-7.52 (J=9.09 Hz)	
	A-7	N	3.47-3.50	
	A-8	174		
	A-9	33	1.98-2.30	
2-N-acetyl-β-D-galactosamine unit -B	B-1	106.93	5.28-5.35	B-2
	B-2	99.00	3.20-3.30	
	B-3	87.29	3.75-3.85	B-4
	B-4	100.25	4.20-4.25	
	B-5	93.24	3.60-3.75	B-6
	B-6	68.54	3.80-3.90	
	B-7	N	3.85-3.90	
	B-8	174		
	B-9	33.97	1.98-2.30	
Di-(N-acetyl-β-D-mannosamine)-C	C-1	106.93	5.28-5.35	C-2
	C-2	101.70	3.20-3.30	
	C-3	82.73	4.10-4.20	C-4
	C-4	93.81	3.50-3.70	
	C-5	82.73	3.20-3.30	
	C-6	74.70	3.60-3.75	C-5
	C-7,7'	N	3.29	
	C-8,8'	164		
	C-9,9'	31.93	2.05-2.30	

[a]NMR spectra recorded using Bruker AV III 500 MHz spectrometer. [b]Values in ppm, multiplicity and coupling constants (J¼ Hz) are indicated in parentheses. Assignments were made with the aid of the 1H–1H COSY, HMQC, and HMBC experiments.

The down field-shifts in carbon signals of the sugar units compared to the native glucan might suggest the possible linkage information, e.g., the downfield-shift in the C-3 position of the W indicated it might be 3-linked mannose. Similarly, units U, V and X were deduced to be as 6-linked glucose, terminal glucose and 3,6-linked mannose, respectively. The linkage information of the sugar units were further confirmed by NOESY and HMBC experiments. In the HMBC spectrum, the intra- and inter-residual connectivities of both anomeric protons and carbons of each of the glycosyl residues were summarized in **Table 6B.3**. The cross peaks indicated the correlation signals which were found between C-1 of the residue U (δ 102.2) with H-6 of residue X (U C-1, X H-6), H-1 of residue U (δ 5.33) with C-6 of residue X (U H-1, X C-6), indicating that U was linked to the 6-position of X. Similarly, the correlation signals W H-1–X C-3 indicated the unit W linked to the 3-position of X. Thus, both 3 and 6 positions of X were substituted. The correlation signals V H-1–W C-3 and V C-1–W H-3 indicated unit V linked to the 3-position of the unit W; an intraresidual coupling between H-1 of residue U with its own C-6 (U H-1, U C-6) indicated that the 6-linkage was among different U repeats. In the NOESY spectrum (Supplementary Figure S2b), similar correlations of the protons were observed: U1–X6, V1–W3, W1–X3 and X1–U6, which confirmed the above results from HMBC. Based on all these results from methylation analysis and 2D NMR, the main repeating unit structure of BEPS-IB was deduced and was shown in **Fig 6B.2**.

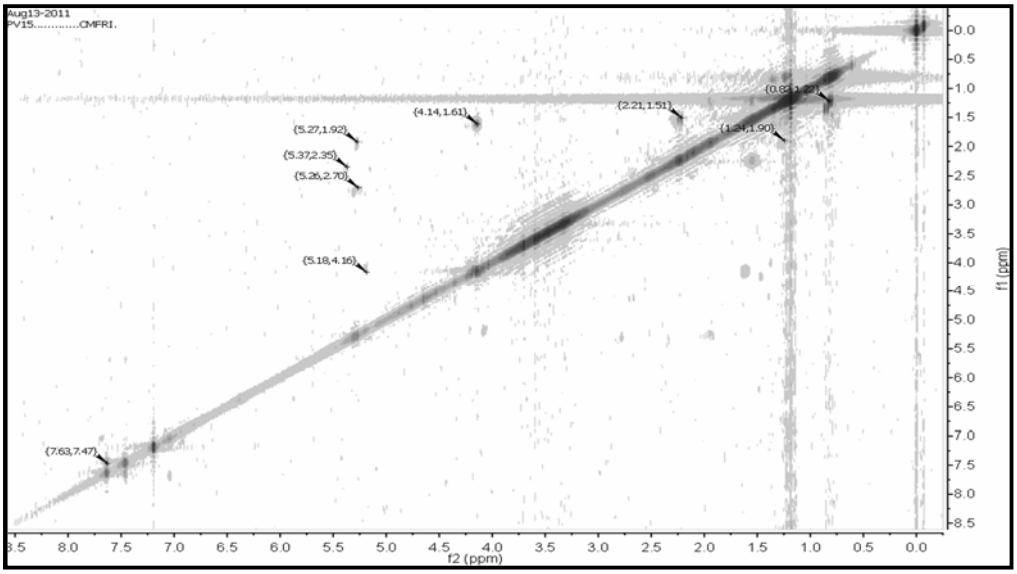


Fig 6B.2A ^{1}H-^{1}H COSY spectra of (⟶4)-2, 4 di-(N-acetyl- β -D-mannosamine) -(1⟶4)-2-N-acetyl-β-D-galactosamine-(1⟶) - (4-N-acetyl p-phenoxyl) unit isolated from *P. viridis.*

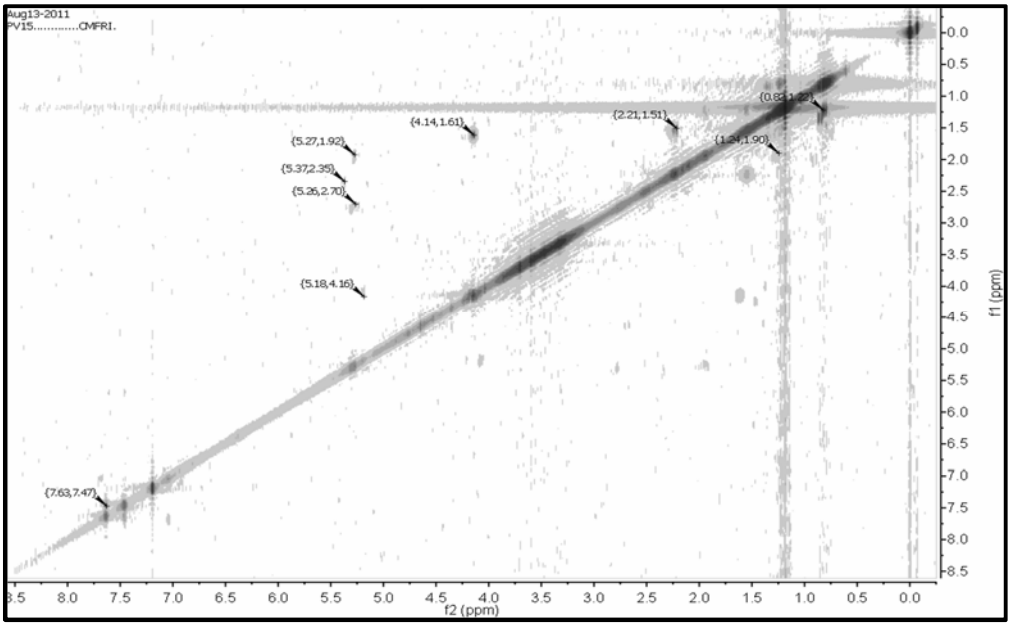


Fig 6B.2B HSQC spectra of (⟶4)-2, 4 di-(N-acetyl-β-D-mannosamine) -(1⟶4)-2-N-acetyl- β-D-galactosamine-(1⟶) - (4-N-acetyl p-phenoxyl) unit isolated from *P. viridis.*

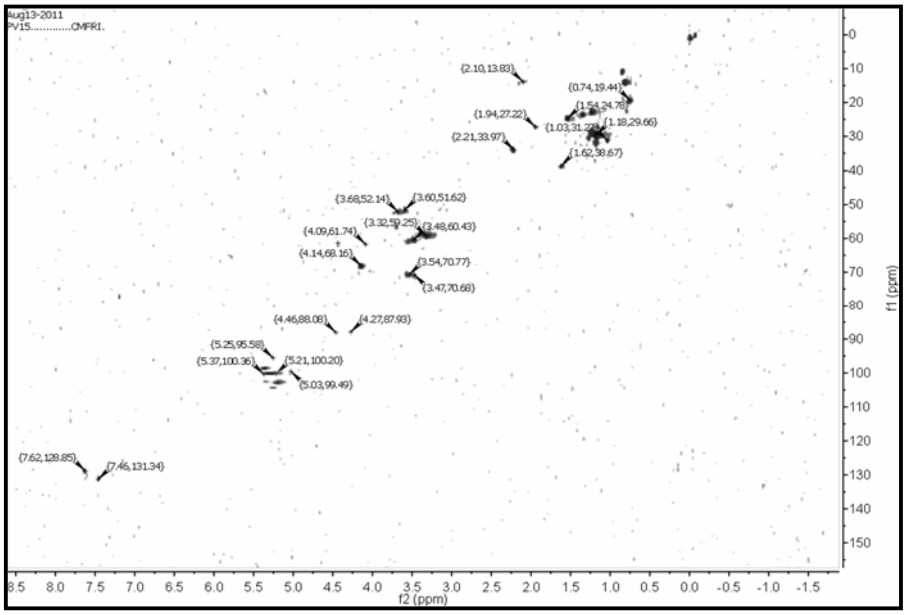


Fig 6B.2C HMBC spectra of (⟶4)-2, 4 di-(N-acetyl- β -D-mannosamine) -(1⟶4)-2-N-acetyl- β-D-galactosamine-(1⟶) - (4-N-acetyl p-phenoxyl) unit isolated from *P. viridis.*

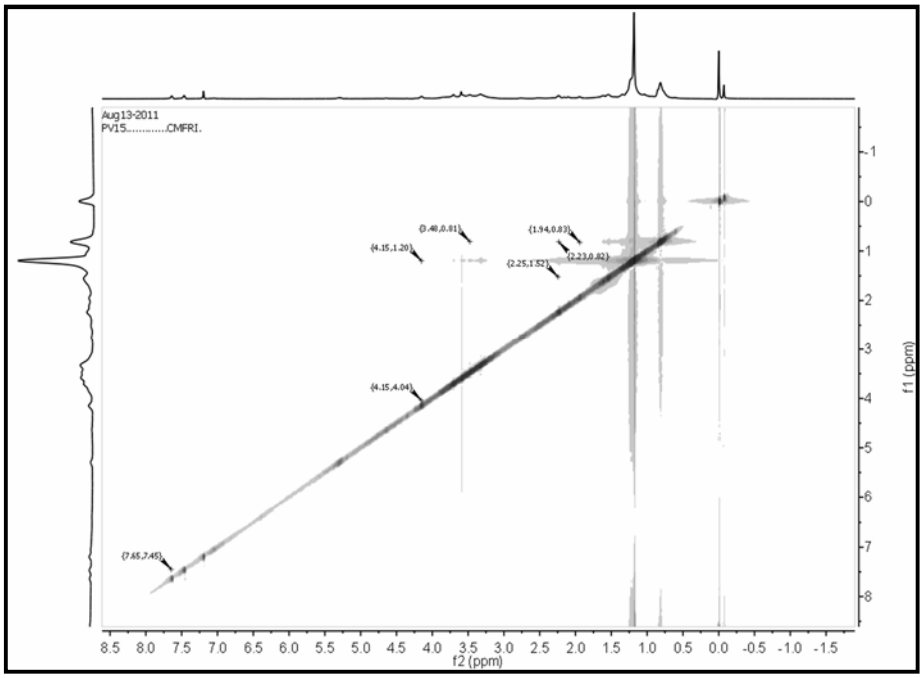
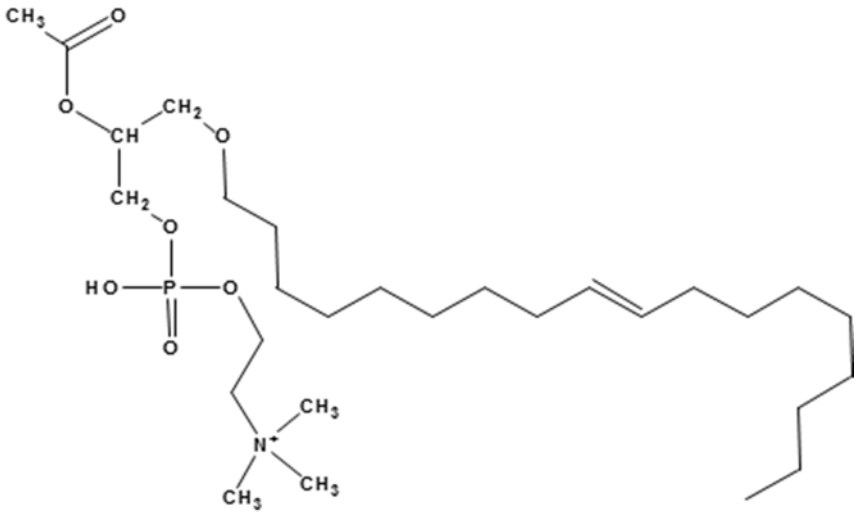


Fig 6B.2D NOE spectra of (⟶4)-2, 4 di-(N-acetyl- β -D-mannosamine) -(1⟶4)-2-N-acetyl- β-D-galactosamine-(1⟶) - (4-N-acetyl p-phenoxyl) unit isolated from *P. viridis.*

The compound **12** contain C-28 carbon lysolecithin isolated from the green mussel, whereas the ultraviolet absorbance at λ_{max} (log ε) 244 (3.11) nm (in MeOH) has been assigned to the choline and glycerol systems. Its mass spectrum exhibited a molecular ion peak at m/z 550 ($C_{28}H_{57}NO_7P$). ^1H NMR in conjugation with ^{13}C NMR recorded the signature peaks of choline, glycerol and fatty acid portion of the lysolecithin. ^{13}C NMR spectra displayed three close peaks at δ 54.31(C-1), δ 54.21(C-2) and δ 53.56(C-3) that were attached to the nitrogen represented singlet at δ 3.19 (s) with an integral value of 9 (**Table 6B.4)**.

Table 6B.4 NMR spectroscopic data 2-[(2-(Acetyloxy)-3-[(E)-9-octadecenyloxy] propoxy hydroxyphosphoryl) oxy] ethyl (trimethyl)ammonium (12)



	Carbon Position	^{13}C NMR ppm	H -type	δ^{1}H NMR(int., mult., J in Hz)[b]	^1H-^1H COSY	HMBC $^1H \rightarrow ^{13}C$
	1	54.31	1-H	3.19(s)		
	2	54.21	2-H	3.19(s)		C-4
Choline	3	53.56	3-H	3.19(s)		
	4	66.17	4-H	3.62(dd,2H)	5-H	C-5
	5	59.49	5-H	4.22(t,2H)		

Glycerol	6	63.30	6-H	3.69(d,2H)	7-H	C-7,9
	7	69.48	7-H	3.83(m,1H)	8-H	
	8	59.61	8-H	4.03(d,2H)		
	9	176.48				
Ether/Fatty Acid part	10	34.12	10-H	2.26(m,3H)		
	11	71.79	11-H	3.32(m,2H)	12-H	C-12
	12	31.96	12-H	1.46	13-H	
	13-17	29.90-25.63		2.01-1.21(m)		
	18	27.08	18-H	1.99(m,2H)	19-H	
	19	129.87	19-H	5.27(dd,1H J=9.4Hz)	18-H	C-18
	20	129.54	20-H	5.30(dd,1H J=9.2Hz)	21-H	
	21	25.62	21-H	2.78(m,2H)		
	21-28	29.61-14.12		1.48-0.81(m)		

[a] NMR spectra recorded using Bruker AV III 500 MHz spectrometer.[b]Values in ppm, multiplicity and coupling constants (J¼ Hz) are indicated in parentheses. Assignments were made with the aid of the 1H–1H COSY, HMQC, and HMBC experiments

The carbon atoms at δ 66.17 (C-4) and δ 59.49 (C-5) as shown HSQC correlated with δ 3.62 (H-4) and δ 4.22 (H-5), respectively, supports the presence of the choline moiety. The downfield shift of δ 3.69 (H-6), δ 3.83 (H-7) and δ 4.03 (H-8) attached to δ 63.60 (C-6), δ 69.48 (C-7) and δ 59.61 (C-8), respectively revealed the presence of glycerol moiety with acetyl group attached to the C-7 position. The proton integral values and ^{13}C NMR peaks appeared at upfield region in combination with COSY and HMBC spectra revealed the presence of 18 carbon fatty acid chain with double bond at C-19. The proton and carbon connectivities deduced from the HSQC and HMBC experiments confirmed the side chain framework, and was presented in **Fig 6B.3**.

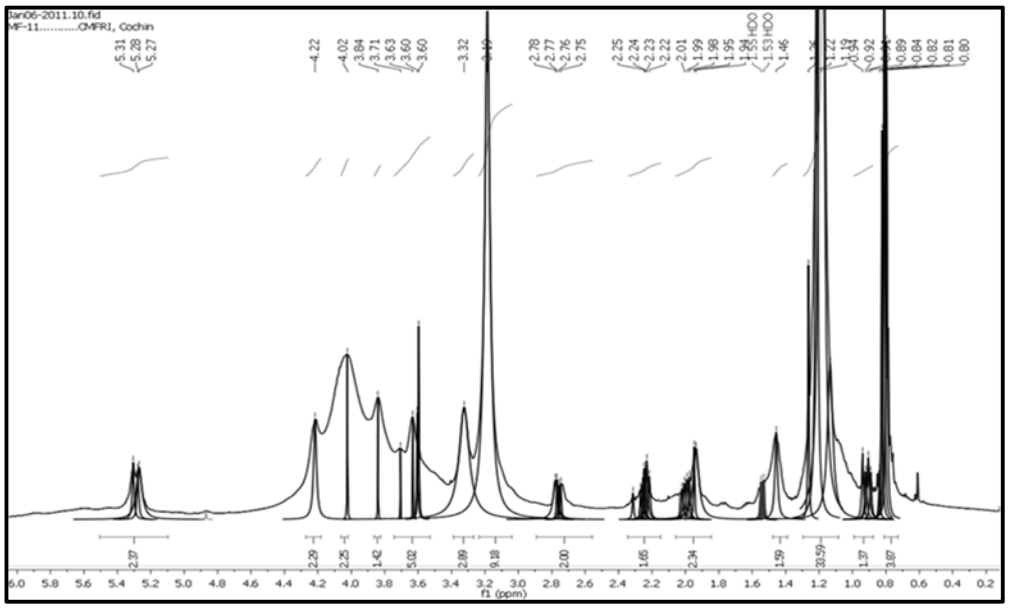


Fig 6B.3A ^{1}H NMR spectra of 2-[(2-(acetyloxy)-3-[(E)-9-octadecenyloxy] propoxy hydroxyphosphoryl) oxy] ethyl (trimethyl)ammonium (12)

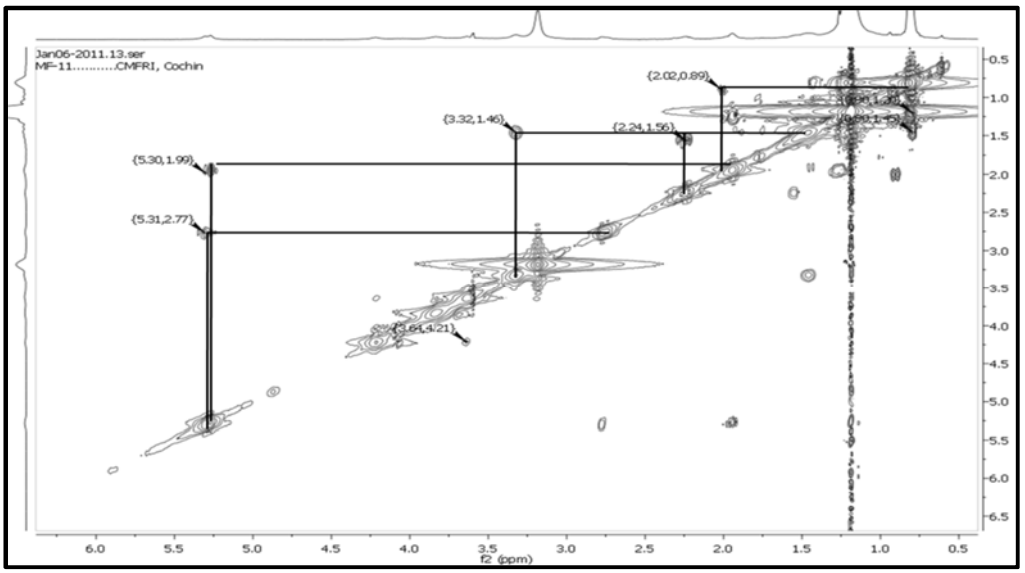


Fig 6B.3B ^{1}H-^{1}H COSY spectra of 2-[(2-(acetyloxy)-3-[(E)-9-octadecenyloxy] propoxy hydroxyphosphoryl) oxy] ethyl (trimethyl)ammonium (12)

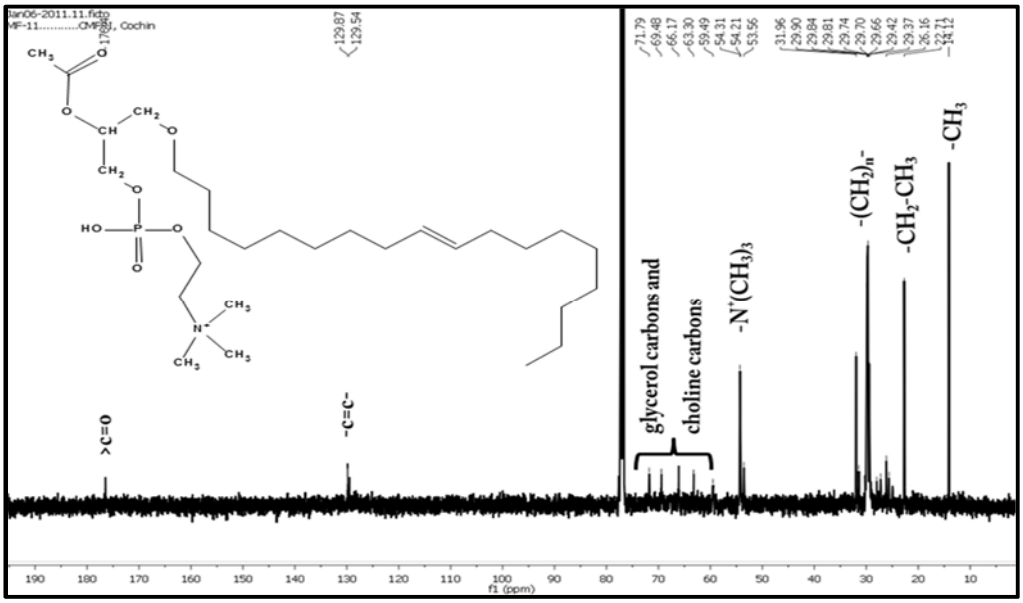


Fig 6B.3C ^{13}C NMR spectra of 2-[(2-(acetyloxy)-3-[(E)-9-octadecenyloxy] propoxy hydroxyphosphoryl) oxy] ethyl (trimethyl)ammonium (12)

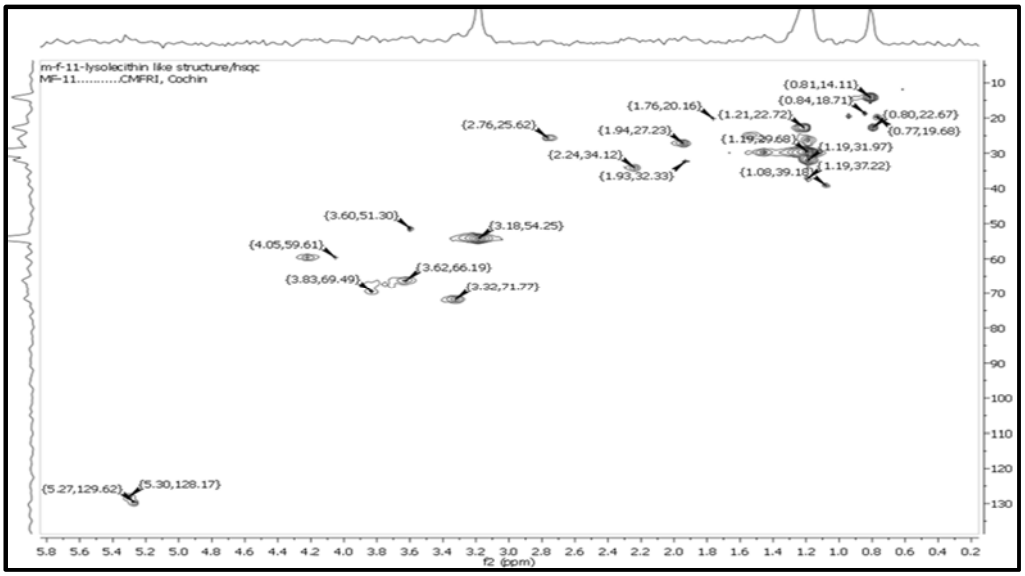


Fig 6B.3D HSQC spectra of 2-[(2-(acetyloxy)-3-[(E)-9-octadecenyloxy] propoxy hydroxyphosphoryl) oxy] ethyl (trimethyl) ammonium (12)

6B.2.3: *In vitro* anti-inflammatory activities of the isolated components from *P. viridis*.

The *in vitro* anti-inflammatory activities were studied by using indomethacin and aspirin as positive controls (**Table 6B.5)**. MPS registered significantly greater COX-2 inhibition (74.8%) than aspirin and indomethacin (58.6% and 59%, respectively, 5mg/mL). No significant differences were apparent between MPS (32.5%) and indomethacin (34.6 %) at lower concentration (0.5 mg/mL). MPS showed significantly lesser (56.4%) inhibition of COX-1 than aspirin (70.0 %) and indomethacin (72.1%). Earlier studies indicated the anti-inflammatory properties of several glucan-type polysaccharides (Czarnecki and Grzybek, 1995). PME_{11} (68.9 %) and PME_0 (64.5%) exhibited strong, concentration dependent inhibition of COX-2, and was more inhibitory than the traditional NSAID COX inhibitor, indomethacin, at comparable concentrations. PME_{11} (68.9%, 54.6%) showed significantly greater inhibition towards COX-2 than that of PME_0 (64.5%, 44.6%) (at 5 mg/mL and 1 mg/mL, respectively). PME_{11} (55.68%) and PME_0 (51.7%) showed significantly lesser inhibition of COX-1, when compared with aspirin (78.8%) and indomethacin (72.1%). PME_0 (27.4%) exhibited significantly lesser inhibition of COX-1 than FDPE (33.2%) at lower concentration (0.5 mg/mL). GP showed significantly greater inhibition of COX-1 and COX-2, and did not appear to exhibit selectivity in their inhibition pattern towards these inflammatory enzymes. Glycogen (59.7% and 69.6%) recorded significantly greater COX-1 and COX-2 inhibition than that of glycoprotein and glycolipoprotein (54.5% and 64.5%, & 57.2% and 67.8 %, respectively, 5 mg/mL). Glycolipoprotein and glycogen exhibited a similar level of inhibition

at the same concentration, suggesting that they contain related bioactive compounds. NUCF (68.2%) recorded significantly greater COX-2 inhibition than aspirin and indomethacin, in higher concentration (5 mg/mL). NUCF (68.2%) was found to be more efficient than aspirin (58.6 %) to inhibit COX-2. It is likely that the PUFA is biologically active component explained by the greater bioactivity NUCF fraction. Studies have shown that the phospholipids (polar lipid) fraction of Lyprinol® exhibited moderate inhibition towards COX-2, whilst COX-1 inhibition was not significant (McPhee *et al*., 2007). Upon examination of the IC_{50} inhibitory concentration values, it was found that NUCF exhibited approximately 2 times more inhibitory activity against COX-2 than that of FDPE. Among different isolated compounds, MPS (1.21 mg/mL) was found to have the lowest IC_{50} value to inhibit COX-2 than NUCF (1.90 mg/mL), PME_{11} (1.89 mg/mL) and glycolipoprotein (1.83 mg/mL).in other words, the polysaccharide fraction (MPS) was found to be significantly active against COX-2 followed by glycolipoprotein, lysolecithin (PME_{11}), and polyunsaturated fatty acid complex (NUCF) in descending order.

LOXs are sensitive to antioxidants, and the most common way of their action is inhibition of lipid hydroperoxide formation appeared to be due to the scavenging of lipidoxy- or 9-lipidperoxy-radicals formed during course of enzymic peroxidation. This can limit the availability of lipid hydroperoxide substrate necessary for the catalytic cycle of LOX. Since COX isozymes and 5-LOX share the same substrate arachidonic acid, inhibition of COX pathways could lead to a shift towards the production of cysteinyl-LT and LTB4 by 5-LOX due to increased substrate availability, thereby leading to development of gastrointestinal ulcers (Hudson *et al*., 1993). MPS showed significantly greater

inhibition towards 5-LOX (IC_{50} 0.88 mg/mL) than aspirin (IC_{50} 3.88 mg/mL) and indomethacin (IC_{50} 2.35 mg/mL). However lysolecithin derivative showed comparable inhibition towards 5-LOX (IC_{50} 2.63 mg/mL) as recorded with aspirin (IC_{50} 2.67 mg/mL) and indomethacin (IC_{50} 2.07 mg/mL). Glycoprotein (5 mg/mL) showed significantly greater 5-LOX inhibition than glycolipoprotein (70.2%) and glycogen (70.20%). At lower concentration (1 mg/mL & 0.5 mg/mL) the inhibition of glycoprotein towards 5-LOX was significantly higher (53.20% and 32.50%, respectively) than glycogen (48.7%, 33.1%, respectively) and glycolipoprotein (50.5%, 31.40%, respectively). The studies implicated that oxygen free radicals in the process of inflammation and phenolic compounds that might block the cascade process of arachidonic acid metabolism by inhibiting lipoxygenase activity, and might serve as a scavenger of reactive free radicals, which are produced during the arachidonic acid metabolism. The NUCF (56.30%) showed significantly greater 5-LOX inhibition than aspirin (at 5 mg/mL) but lower than indomethacin. Since COX isozymes and 5-LOX share the similar substrate (arachidonic acid), inhibition of COX pathways could lead to a shift towards the production of cysteinyl-LT and LTB_4 by 5-LOX due to the increased substrate availability, thereby leading to development of gastrointestinal ulcers (Hudson *et al*., 1993).

Table.6B.5 Comparison of *in vitro* anti-inflammatory activity of isolated compounds, with FDPE and standard anti-inflammatory drugs

Samples	Percentage inhibition of COX 1				Percentage inhibition of COX 2				Percentage inhibition of 5-LOX			
	0.5	1	5	IC50	0.5	1	5	IC50	0.5	1	5	IC50
Aspirin	44.6±0.55	52.0±1.83	70.0±0.10	1.1	38.5±0.25	52.2±0.72	58.6±0.67	2.23	31.3±0.23	40.2±0.74	54.8±1.12	3.88
Indomethacin	45.6±0.80	54.0±1.05	72.1±0.92	0.82	34.6±0.36	46.3±0.69	59±0.81	2.9	32.9±0.56	47.2±0.35	66.3±0.64	2.35
FDPE	33.2±0.71	40.8±2.59	48.9±0.80	5.25	26.1±0.94	36.4±1.02	48.4±0.84	5.27	35.6±0.34	45.8±0.18	51.9±0.19	4.14
MPS	30.1±0.32	45.5±0.94	56.4±1.12	3.44	32.5±0.58	62.6±0.55	74.8±0.92	1.21	32.8±0.61	67.6±0.54	71.6±0.45	0.88
GLP	30.3±0.21	42.9±0.94	57.2±1.16	3.46	35.6±0.14	52.5±0.81	67.8±0.11	1.83	31.4±1.29	50.5±0.11	70.2±0.71	2.07
GP	29.2±1.14	45.7±0.87	54.5±0.25	3.76	31.2±0.62	50.6±0.54	64.5±1.08	2.38	32.5±0.52	53.2±0.84	74.5±0.94	1.73
G	31.9±0.67	44.6±1.07	59.7±0.73	3.04	32.5±0.84	48.7±1.24	69.6±0.99	2.13	33.1±0.67	48.7±0.42	70.1±0.89	2.08
NUCF	35.7±0.14	51.4±0.16	75.7±0.78	1.61	31.2±0.35	55.6±0.86	68.2±0.38	1.9	29.6±1.13	51.6±0.94	56.3±1.24	3.18
PME_{11}	27.4±0.36	41.3±0.89	51.7±1.09	4.45	29.6±0.88	44.6±0.16	64.5±0.63	2.73	24.6±0.19	40.4±0.88	55.6±1.05	3.88
PME_0	24.78±0.44	39.35±0.65	55.6±0.86	3.9	31.8±0.62	54.6±0.49	68.9±0.57	1.89	22.6±0.34	47.2±1.02	53.2±0.94	4.05

FDPE: freeze dried mussel extract without additives (control). MPS (crude polysaccharides isolated *P. viridis*) PME_0 (methanol extract of *P. viridis*), PME_{11} (Purified fraction of PME_0), GLP (Glycolipoprotein), GP (Glycoprotein), G (Glycogen), NUCF (non-urea complexing fraction)

6B.2.4 *In vivo* anti-inflammatory activity of the isolated compounds.

Carrageenan induced paw edema is a standard and most commonly used technique to screen the anti-inflammatory activity (Winter, 1962). Carrageenan is a sulfated polysaccharide that promotes acute inflammation by activating the proinflammatory cells. Inflammation induced by carrageenan, is acute, nonimmune, well-researched, and highly reproducible. It is expected that after tissue injury, an animal will display spontaneous pain behavior. This peripheral hypersensitivity or pain perception can be explained on the basis of local release of various inflammatory mediators *i.e*. bradykinin, prostaglandins or cytokines, which can activate and sensitize the peripheral nerve endings (Baccaglini, 1983). Inflammation is characterized by increased tissue water and plasma metabolism of arachidonic acid by COX and LOX enzyme pathway (Gamache *et al*., 1986). There are biphasic effects in carrageenan induced edema. The first phase begins immediately after injection and diminishes in 1 hour and the second phase begins after 1 hour. It has been suggested that the early hyperemia of carrageenan induced edema results from the release of histamine and serotonin. On the other hand, the delayed phase of carrageenan induced edema results mainly from the potentiating effect of prostaglandins on mediator release, especially of bradykinin. Different extracts demonstrated a time dependent anti-inflammatory effect in rats with carrageenan induced footpad edema. Comparison of purified compounds with the reference drug (aspirin) on carrageenan-induced hind paw edema in mice were recorded in **Table.6B.6**. MPS registered significantly greater edema inhibition (64.4%, h) than FDPE (34.3%, 2 h). Maximum inhibition of edema by MPS was achieved in 4 h (77.2%). The study showed that MPS effectively

suppressed the edema produced by the histamine, which indicated that the polysaccharide exhibited its anti-inflammatory action by means of either inhibiting the synthesis, release or action of inflammatory mediators, such as, histamine, serotonin and prostaglandin's. Polysaccharide might be the potential therapeutic agent involved in inflammatory disorders, controlling the initial phase of inflammation and provoking an inhibition of edema formation similar to the reference drug (aspirin, 68.67% in 2h) compounds. MPS and aspirin exhibited highest activity in 4 h (77.2% and 82.1%, respectively) and started losing activity around 5 h (72.2% and 65.7%, respectively) which mightbe related to a lower level of these compounds in the blood circulation. The result was in accordance with the previous reports that several glucan-type polysaccharides were shown to possess anti-inflammatory activities (Czarnecki & Grzybek, 1995). The administration of glycolipoprotein limited the edema to 51.1% which was 34.3% for FDPE after 2h. Glycolipoprotein registered significantly greater inhibition (61.2 %, 3 h) than glycogen and glycoprotein (57.1% and 58.7%, 3 h respectively). Glycoprotein (65.7 %, 5 h) recorded significantly lesser inhibition than glycolipoprotein (71.9%, 5 h). These results indicated that the activity of glycoprotein was lost following the KOH formulation of glycolipoprotein. Essentially, the same result pattern as that GLP (65.8 %, 4h) was obtained when proteinase-K-treated glycogen (64.1%, 4 h) was administered to the subjects challenged with carrageenan. The glycolipoprotein (78.7%, 6 h) showed significantly greater inhibition than glycogen (74.1%, 6 h).The results revealed that the active component of mussel resided within a protein moiety associated with glycogen (Miller *et al*., 1993). Maximum suppression of inflammation by glycogen and

glycolipoprotein administration was achieved during 6th h (78.4% and 75.4%, respectively). These results demonstrated that the glycogen and glycolipoprotein, exerts an anti-edematous effect during the second phase of paw edema due to the reduction of prostaglandins, which are second phase inflammatory mediators. Therefore, the present results suggest that the mechanism of the anti-inflammatory effect of glycogen might involve the inhibition of the COX pathway. NUCF showed a gradual increase in inhibition from 2nd to 6th h (49.23 to 70.33%). The PME_0 extract (6 h) exhibited significant anti-inflammatory activity (72.4%) at 100 mg/kg whilst aspirin showed 80.56% inhibition of edema (200 mg/kg, 6 h). It was observed that the increase in the paw thickness was inhibited to about 82.06% (3 h) by aspirin, whereas the fraction PME_{11} inhibited the paw thickness to 65.36%. At the end of 6 h, PME_{11} fraction (lysolecithin) (79.45%, 50 mg/kg) was found to show greater activity towards the inhibition of paw edema than aspirin (80.27%, 200 mg/kg). In the present study, the edema inhibition by PME_{11} was more pronounced at the second phase, which suggests that the inhibition of prostaglandin production could be one of the ways by which PME_{11} exerted the anti-inflammatory effect.

Table.6B.6 Comparison of purified compounds with FDPE and reference drug (aspirin) on carrageenan induced hind paw edema in mice.

Samples	Mean paw edema(mm)	Difference in paw edema 2h		% inhibition	Difference in paw edema 3h		% inhibition	Difference in paw edema 4h		% inhibition	Difference in paw edema 5h		% inhibition	Difference in paw edema 6h		% inhibition
	0h	(mm)	(%)		(mm)	(%)		(mm)	(%)		(mm)	(%)		(mm)	(%)	
N.Saline	1.16	2.47	112.76±4.87		2.48	113.7±1.61		2.48	113.7±1.59		2.5	115.5±5.57		2.52	117.2±1.51	
Std	1.47	1.99	35.32±3.33	68.67	1.89	28.67±1.71	74.8	1.82	20.41±2.06	82.06	1.8	22.45±1.36	80.56	1.77	23.13±0.31	80.27
FDPE	1.56	2.72	74.04±2.89	34.33	2.53	62.05±2.45	45.46	2.19	44.87±3.14	60.56	2.16	44.23±2.61	61.71	2.15	42.95±2.56	63.36
MPS	1.62	2.27	40.12±1.64	64.41	2.12	30.86±2.86	72.87	2.04	25.92±2.56	77.21	2.14	32.09±2.49	72.21	2.18	34.56±2.18	70.51
G	1.25	2.01	60.8±3.45	46.08	1.86	48.8±3.14	57.11	1.76	40.8±2.96	64.14	1.7	36±1.65	68.83	1.61	28.8±2.93	75.43
GP	1.49	2.24	50.33±2.76	55.36	2.19	46.97±1.53	58.71	2.11	41.61±1.98	63.43	2.08	39.59±1.14	65.72	2.05	37.58±1.58	67.94
GLP	1.54	2.39	55.19±1.95	51.05	2.22	44.15±2.57	61.19	2.14	38.96±1.84	65.76	2.04	32.46±1.85	71.89	1.93	25.32±1.63	78.39
NUCF	1.38	2.17	57.24±1.45	49.23	2.05	48.55±2.01	57.33	1.91	38.40±4.25	66.25	1.89	36.95±3.69	68.01	1.86	34.78±2.99	70.33
PME_0	1.45	2.21	52.41±1.64	53.51	2.16	48.96±2.69	56.96	2.05	41.37±1.52	63.63	2.02	39.31±3.56	65.97	1.92	32.41±1.36	72.35
PME_{11}	1.37	2.05	49.63±2.16	55.98	2.01	46.71±3.54	58.94	1.91	39.41±3.25	65.36	1.81	32.11±2.69	72.19	1.7	24.08±1.99	79.45

FDPE: freeze dried mussel extract without additives (control). MPS (crude polysaccharides isolated *P. viridis*) PME_0(methanol extract of *P. viridis*), PME_{11} (Purified fraction of PME_0).Percent inhibition in mice paw edema (in parentheses) was calculated as: $(I_t - I_o) \times 100 / I_o$, where I_t is the percent difference in paw edema of the animals treated with normal saline with respect to the baseline value (zeroth hour) and I_o is the percent difference in paw edema of animals treated with standard and FDPE at the same time interval

6B.2.5 Identification of potent formulations by *in vitro* anti-inflammatory and antioxidant assays.

The *in vitro* anti-inflammatory and antioxidant activities of the various treatments have been compared with control mussel (FDPE) and were recorded in **Table 6B.7**. Various inflammatory parameters have been determined in order to understand whether prolonged storage had any negative effect on the target bioactivity. FDPE recorded 48.9% inhibition towards COX-1 in 0^{th}day (baseline) whereas Q_1 and Q_2 showed 51.2 and 49.5% inhibition, respectively. During the 60 days of shelf life study, Q_1 and Q_2 recorded 49.6 and 47. 6% inhibition, respectively, while the FDPE showed only 44.9%. The formulations Q_1 and Q_2 showed 62.2 and 61.5% inhibition respectively in 0^{th} day while the FDPE showed only 48.6% inhibition for COX-2 enzyme. During 60 days shelf life study Q_1 and Q_2 showed 59.2% and 59% inhibition, respectively, while FDPE showed only 43.02%. The 5-LOX inhibition study of Q_1 showed 56. 9 % inhibition at the 0^{th}day, while FDPE showed only 51.9% inhibition for 5-LOX. The LOX inhibition of Q_1 during 30 days shelf-life study was 42.2% although for Q2 and FDPE the inhibition was found to be as 41.6 and 32. 9%, respectively. During the 60 days shelf-life study, the decrease in LOX inhibition of Q_1 (40.7%) was lower, compared with Q_2 (39.64 %) and FDPE (29.54%).

The DPPH inhibition assay measured the ability of compounds to transfer the labile hydrogen atoms to radical. The DPPH activity of FDPE was 13% at baseline (t=0) and decreased to 2.07% at 60^{th} day during storage at 50°C, whereas Q_1 showed a lower reduction in DPPH radical scavenging capacity (34.5%-26.8%) than that of FDPE. During 60 days of accelerated shelf life study, the reduction in total antioxidant capacity (TARSC) of Q_1 (34.5%-26.8%) was lower than Q_2 (33.895-24.11%) and FDPE (13.0%-2.07%). The malondialdehyde formation in FDPE was increased from 3.94 – 8.20 mM MDAequivalent/kg sample during the 60 days of accelerated shelf life study. The TBARS value of Q_1 and Q_2 was increased from 2.02-4.37 and 2.11 - 4.7 mM MDA equivalent /kg

sample, respectively. The rate of increase of TBARS in Q_1 lower when compared to Q_2, which meant that lipid peroxidation was more for Q_2 than in Q_1. The anti-inflammatory composition containing greater concentration of MPS in (1.2%) (ie.Q_1) showed greater antioxidant activity and lesser lipid peroxidation, which are the preferred indices to assess the stability of the anti-inflammatory neutraceutical.

Table.6B.7 Anti-inflammatory and antioxidant activities of FDPE and different formulations (5 mg/mL) during accelerated shelf life study.

	0 day	30 day	60 day	90 day
H_2O_2 inhibition activity (%)				
FDPE	48.64±1.07^a	45.96±1.68^a	33.86±1.56^b	26.21±0.53^c
Q_1	55.08±1.03^a	51.83±0.73^a	50.65±1.26^a	46.35±0.60^b
Q_2	53.11±1.12^a	51.07±1.83^a	48.14±1.98^a	45.48±1.04^a
COX-1 inhibition activity (%)				
FDPE	48.9±0.13^a	46.11±0.41^a	44.85±0.43^b	43.15±0.43^c
Q_1	51.2±0.22^a	50.36±0.16^a	49.61±0.26^b	47.88±0.11^b
Q_2	49.5±0.76^a	48.77±0.29^a	47.56±0.32^b	46.91±0.24^b
COX-2 inhibition activity (%)				
FDPE	48.4±0.19^a	45.01±0.05^a	43.02±0.70^a	35.21±1.61^b
Q_1	62.23±0.43^a	61.05±0.06^a	59.21±0.26^a	55.24±1.47^a
Q_2	61.49±0.50^a	59.87±0.27^a	58.96±0.42^a	55.01±1.42^a
5-LOX inhibition activity (%)				
FDPE	51.9 ±0.50^a	49.3 ±0.01^a	48.65 ±0.41^a	42.25 ±0.63^b
Q_1	56.89 ±0.79^a	55.23 ±0.08^a	53.76 ±0.25^a	51.11 ±0.82^a
Q_2	53.42 +0.33^a	51.97 ±0.03^a	50.35 ±0.03^a	50.99 ±0.81^a
DPPH scavenging activity (%)				
FDPE	13.01+0.33^a	6.6+0.21^b	2.07+0.37^c	1.03±0.46^d
Q_1	34.53+0.29^a	30.78+0.44^a	26.80+0.47^b	20.15±0.44^c
Q_2	33.89+0.07^a	29.80+0.52^b	24.11+0.85^c	19.84±0.5^d
TBARS (mM MDA/kg)				
FDPE	3.94+0.04^a	5.18+0.03^b	8.20+0.06^c	10.4±0.46^d
Q_1	2.02+0.03^a	3.98+0.05^a	4.37+0.09^a	5.69±0.6^b
Q_2	2.11+0.06^a	4.02+0.13^b	4.7+0.07^b	6.02±0.65^c

Data are expressed as mean ± standard deviation of three replicates. FDPE: freeze dried mussel extract without additives (control). Q1 and Q2 were the enriched formulations prepared from the extract of *P. viridis*)

6B.2.6 Identification of potent formulation with high nutritional value *viz.* fatty acid compositions

The essential characteristic of the nutritional and functional quality of marine food product is its fatty acid composition. Depending on the time, temperature and other conditions of storage, highly lipidic material like *P. viridis* can undergo various changes in its fatty acid profile owing to the activity of various enzymes. Fatty acids are very important biochemical indicators contributing to the nutritional quality of the samples. Unfavourable composition of nutritional fatty acids is a major risk factor for cardiovascular and inflammatory diseases. The total SFAs in Q_1 andQ_2 were found to be higher than in FDPE. In particular, Q_1 effectively arrested the increase of the SFA at various scheduled intervals of accelerated shelf-life study than did Q_2 and FDPE, which realized a significant increase from 0^{th} day to 90^{th} day (**Table 6B.8**). Q_1 and Q_2 realized a decelerated increase of palmitic acid ($C_{16:0}$), the predominant among the SFAs than in FDPE.

The PUFAs *viz.*, arachidonic acid (20:4*n*-6), 20:5*n*-3 and 22:6*n*-3 in *P. viridis* concentrate is important biochemical indicators contributing to the nutritional quality in addition to the reported anti-inflammatory properties (Calder, 2009). The addition of natural additives with polyphenolic moieties in Q_1 and Q_2 apparently shielded the mussel PUFAs against oxidation as evident by higher PUFA content in Q_1 and Q_2 as compared to FDPE during 90 days of accelerated shelf life study. The FDPE showed a total PUFA content of 29.03 % in 0^{th} day while Q_1 and Q_2 showed 32.48% and 32.58% respectively. The total *n*-3 PUFA content of FDPE was found to be lesser than Q_1 and Q_2 at 0^{th} day, thereby indicating the added *n*-3 fatty acid pool to FDPE by the natural

additives. The *n*-3 fatty acid, 22:6*n*-3 registered a significant reduction in FDPE, at 90th day. The combinations Q_1 and Q_2 effectively prevented 22:6*n*-3 from oxidative degradation at 90th day than did FDPE. The fatty acid 20:5*n*-3, which competes with AA for COX-2 and 5-LOX active sites to synthesize anti-inflammatory PG metabolites (Calder, 2009) were found to be higher in Q_1 and Q_2 than in FDPE. It is interesting to note that a major reduction of EPA content were apparent in Q_1 at 90th day as compared to that in FDPE and Q_2, which realized a significant reduction in EPA content at 90th day. Earlier studies (Whitehouse *et al*., 1997) reported that the anti-inflammatory activity in a lipid extract from green lipped mussel is associated with PUFAs with multiple (4-6) double bonds. In the present study, the PUFAs with 4-6 double bonds in FDPE were found to be significantly reduced at 90th day. However, no significant reduction of this group of fatty acids was noticeable for Q_1 and Q_2. Apparently, the antioxidant additives serve as oxidation "chain breakers" for Q_1 and Q_2 by intercepting the free radicals generated from PUFAs during accelerated storage

Table.6B.8: Fatty acid profile of FDPE and other formulations for accelerated storage studies for a period of 30 days

Fatty acid (% total fatty acids, TFA)												
	FDPE				Q_1				Q_2			
	0 days	30 days	60 days	90 days	0 days	30 days	60 days	90 days	0 days	30 days	60 days	90 days
Saturated fatty acids												
C12:0	0.22±0.01^a	0.45±0.01^a	0.59±0.06^a	0.66±1.1^a	0.22±0.01^a	0.13±0.21^a	0.14±0.04^a	0.16±0.02^a	0.12±0.01^a	0.15±0.01^a	0.17±0.03^a	0.19±0.02^a
C13:0	0.11±0.02^a	0.03±0.02^a	0.02±0.01^a	0.01±1.01^a	0.10±0.02^a	0.10±0.03^a	0.09±0.01^a	0.08±0.01^a	0.11±0.02^a	0.12±0.02^a	0.13±0.01^a	0.15±0.01^a
C14:0	5.99±1.2^a	6.61±0.31^b	6.75±0.04^b	6.82±1.05^b	6.26±0.11^b	6.01±0.01^b	6.09±0.06^b	6.19±0.05^b	6.11±0.11^b	6.53±0.12^b	6.68±0.06^b	6.74±0.05^b
C15:0	0.62±0.02^a	0.98±0.04^a	1.13±0.02^a	1.24±1.03^a	1.67±0.05^a	1.64±0.02^a	1.61±0.02^a	1.60±0.01^a	1.64±0.12^a	1.72±0.02^a	1.79±0.01^a	1.82±0.03^a
iso 15:0	0.06±1.87^a	0.05±0.01^a	0.08±0.01^a	0.09±1.01^a	0.22±0.02^a	0.15±0.01^a	0.13±0.01^a	0.11±0.01^a	0.12±0.01^a	0.37±0.07^a	0.42±0.01^a	0.49±0.02^a
C16:0	26.1±0.99^a	32.62±0.3^b	35.1±2.23^b	36.9±1.45^b	23.19±1.31^a	22.54±0.04^a	22.9±1.23^a	23.3±0.87^a	22.79±0.9^a	23.9±0.99^a	24.5±0.15^a	25.1±0.12^a
iso 16:0	0.87±0.85^a	0.03±0.01^a	0.24±0.01^a	0.37±1.01^a	0.91±0.02^a	0.87±0.03^a	0.88±0.01^a	0.93±0.04^a	0.87±0.01^a	0.88±0.85^a	0.91±0.1^a	0.94±0.07^a
C17:0	0.88±0.53^a	1.06±0.03^a	1.19±0.02^a	1.31±1.13^a	2.19±0.03^b	3.54±0.05^c	4.02±0.03^c	4.03±0.05^c	1.64±0.15^a	3.37±0.53^c	3.54±0.02^c	3.86±0.03^c
C18:0	6.14±1.23^a	6.05±0.02^a	5.94±1.23^a	5.86±1.11^a	5.44±0.11^a	5.30±0.01^a	5.35±0.07^a	5.39±0.11^a	5.34±0.19^a	5.47±1.23^a	5.52±0.01^a	5.59±0.06^a
C20:0	2.04±0.64^a	2.09±0.11^a	2.13±0.05^a	2.16±1.04^a	0.31±0.01^a	0.24±0.02^a	0.27±0.09^a	0.29±0.07^a	0.23±0.02^a	0.25±0.64^a	0.26±0.02^a	0.09±0.03^a
C22:0	0.21±0.11^a	0.22±0.01^a	0.19±0.01^a	0.17±1.01^a	0.05±0.01^a	0.40±0.04^a	0.42±0.04^a	0.45±0.06^a	0.31±0.01^a	0.49±0.11^a	0.54±0.01^a	0.61±0.01^a
C24:0	0.16±0.24^a	0.25±0.03^a	0.29±0.01^a	0.34±1.01^a	0.03±0.03^a	0.04±0.03^a	0.05±0.01^a	0.06±0.01^a	0.03±0.13^a	0.05±0.24^a	0.06±0.01^a	0.08±0.01^a
∑ **SFA**	43.4^a	50.43^b	53.65^b	55.93^b	40.59^a	40.94^a	41.95^a	42.59^a	39.3^a	43.3^a	44.52^a	45.66^a
Monounsaturated fatty acids												
14:1n7	1.21±0.01^a	0.22±0.12^b	0.19±0.01^b	0.09±1.02^b	0.35±0.03^b	0.43±0.01^b	0.49±0.02^b	0.52±0.04^b	0.29±0.02^b	0.49±0.01^b	0.65±0.06^b	0.72±0.09^b
16:1n7 trans	0.16±0.01^a	0.48±0.02^a	0.61±0.03^a	0.65±1.03^a	0.12±0.08^a	0.05±0.01^a	0.03±0.01^a	0.02±0.01^a	0.12±0.01^a	0.03±0.01^a	0.02±0.01^a	0.01±0.01^a
16:1n7 cis	8.30±0.02^a	8.85±0.6^a	8.42±0.13^a	8.39±1.11^a	7.35±0.02^b	7.21±0.01^b	7.15±0.05^b	7.09±0.06^b	7.18±0.11^b	7.58±0.02^b	7.75±0.06^b	7.84±0.08^b
16:1n9 cis	1.67±0.01^a	1.71±0.21^a	1.75±0.07^a	1.81±0.1^a	1.71±0.01^a	1.71±0.01^a	1.69±0.04^a	1.65±0.05^a	1.69±0.06^a	1.77±0.01^a	1.61±0.04^a	1.48±0.03^a
17:01	0.39±0.01^a	0.52±0.02^a	0.57±0.02^a	0.59±1.03^a	0.41±0.02^a	0.35±0.03^a	0.25±0.02^a	0.18±0.03^a	0.39±0.00^a	0.34±0.01^a	0.28±0.01^a	0.21±0.02^a
18:1n7 cis	1.59±0.35^a	0.49±0.08^b	0.39±0.02^b	0.25±1.04^b	1.95±0.01^a	1.56±0.03^a	1.45±0.05^a	1.33±0.04^a	2.02±0.13^a	1.54±0.35^a	1.18±0.02^a	1.01±0.01^a
18:1n9 trans	0.08±0.01^a	0.19±0.01^a	0.24±0.01^a	0.31±1.01^a	0.06±1.02^a	0.05±0.00^a	0.04±0.01^a	0.03±0.01^a	0.11±0.00^a	0.02±0.01^a	0.01±0.00^a	0.01±0.00^a
18:1n9 cis	2.91±0.01^a	2.88±0.01^a	2.62±0.05^a	2.39±1.04^a	2.59±0.12^a	2.92±0.02^a	3.01±0.05^a	3.09±0.08^a	2.78±0.02^a	2.86±0.01^a	3.03±0.03^a	3.09±0.06^a
20:1n11	1.09±0.07^a	1.21±0.01^a	1.24±0.03^a	1.09±1.01^a	0.12±0.01^a	0.34±0.05^a	0.41±0.06^a	0.49±0.01^a	0.17±0.04^a	0.28±0.07^a	0.35±0.02^a	0.41±0.01^a
22:1n9	3.74±0.03^a	3.72±0.04^a	3.69±0.03^a	3.51±1.06^a	3.87±0.02^a	3.51±0.01^a	3.25±0.08^a	3.09±0.06^a	3.79±0.02^a	3.57±0.03^a	3.46±0.03^a	3.38±0.04^a
C24:1	0.35±0.01^a	0.11±0.01^a	0.09±0.01^a	0.05±1.01^a	0.08±0.02^a	0.38±0.04^a	0.55±0.01^a	0.64±0.01^a	0.33±0.02^a	0.03±0.01^a	0.02±0.00^a	0.02±0.01^a
∑ **MUFA**	21.48^a	20.37^a	19.81^a	19.13^a	18.61^a	18.29^a	18.32^a	18.13^a	18.87^a	18.5^a	18.36^a	18.18^a

	Polyunsaturated fatty acids											
16:2n4	0.17±0.03^{a}	0.15±0.03^{a}	0.13±0.02^{a}	0.12±0.04^{a}	0.19±0.02^{a}	0.21±0.02^{a}	0.25±0.01^{a}	0.28±0.02^{a}	0.16±0.01^{a}	0.15±0.03^{a}	0.08±0.01^{a}	0.05±0.01^{a}
16:3n4	0.21±0.01^{a}	0.11±0.05^{a}	0.09±0.01^{a}	0.05±0.01^{a}	0.21±0.01^{a}	0.19±0.07^{a}	0.14±0.01^{a}	0.11±0.01^{a}	0.12±0.01^{a}	0.11±0.01^{a}	0.1±0.02^{a}	0.08±0.01^{a}
18:2n6 trans	0.11±0.09^{a}	0.18±0.04^{a}	0.21±0.03^{a}	0.25±0.05^{a}	0.09±0.00^{a}	0.03±0.16^{a}	0.02±0.01^{a}	0.02±0.01^{a}	0.10±0.00^{a}	0.09 ±0.09^{a}	0.08±0.01^{a}	0.07±0.01^{a}
18:2n6 cis	3.07±0.01^{a}	3.19±0.03^{a}	3.23±0.02^{a}	3.27±0.1^{a}	2.19±0.01^{a}	2.05±0.06^{a}	2.01±0.02^{a}	1.98±0.03^{a}	2.17±0.16^{a}	2.11±0.01^{a}	2.09±0.03^{a}	2.06±0.01^{a}
18:3n6	1.87±0.01^{a}	2.39±0.21^{a}	2.48±0.11^{a}	2.59±0.07^{a}	2.09±0.02^{a}	1.69±0.08^{a}	1.46±0.02^{a}	1.35±0.01^{a}	1.94±0.28^{a}	2.04±0.01^{a}	2.11±0.04^{a}	2.25±0.05^{a}
18:4n6	2.16±0.03^{a}	2.37±0.02^{a}	2.52±0.06^{a}	2.68±0.09^{a}	0.21±0.01^{b}	0.13±0.00^{b}	0.09±0.01^{b}	0.05±0.01^{b}	0.08±0.11^{b}	0.92±0.03^{c}	1.18±0.06^{c}	1.26±0.05^{c}
18:3n3	1.70±0.01^{a}	0.73±0.03^{a}	0.41±0.03^{b}	0.35±0.02^{b}	1.05±0.04^{a}	0.90±0.03^{a}	0.84±0.02^{a}	0.75±0.01^{a}	1.04±0.04^{a}	0.14±0.01^{b}	0.11±0.03^{b}	0.09±0.01^{b}
18:4n3	1.05±0.11^{a}	0.52±0.05^{b}	0.44±0.01^{b}	0.32±0.02^{b}	0.16±0.02^{b}	0.13±0.01^{b}	0.09±0.01^{b}	0.06±0.01^{b}	0.16±0.01^{b}	0.03±0.11^{b}	0.02±0.01^{b}	0.01±0.01^{b}
20:2n6	0.41±0.03^{a}	0.71±0.01^{a}	0.82±0.04^{a}	0.99±0.01^{a}	0.81±0.03^{a}	1.62±0.05^{b}	1.79±0.03^{b}	2.01±0.04^{b}	0.77±0.05^{a}	1.66±0.03^{a}	2.12±0.03^{a}	2.35±0.04^{a}
20:3n6	0.39±0.06^{a}	0.28±0.02^{a}	0.21±0.02^{a}	0.18±0.04^{a}	0.43±0.06^{a}	0.38±0.02^{a}	0.27±0.02^{a}	0.21±0.03^{a}	0.42±0.02^{a}	0.21±0.06^{a}	0.19±0.02^{a}	0.15±0.03^{a}
20:4n6	0.69±0.01^{a}	1.32±0.03^{a}	1.44±0.06^{a}	1.55±0.08^{a}	0.29±0.01^{b}	1.65±0.06^{a}	1.97±0.04^{a}	2.16±0.05^{a}	0.29±0.01^{b}	0.30±0.01^{b}	0.48±0.04^{b}	0.65±0.02^{b}
20:3n3	0.31±0.11^{a}	0.16±0.01^{a}	0.11±0.01^{a}	0.08±0.01^{a}	2.54±0.02^{b}	0.89±0.05^{b}	0.75±0.02^{b}	0.64±0.01^{b}	2.85±0.15^{b}	0.86±0.11^{b}	0.79±0.03^{b}	0.65±0.04^{b}
20:5n3	7.05±0.09^{a}	5.54±0.41^{a}	4.73±0.06^{a}	3.82±0.04^{a}	10.15±0.02^{b}	9.49±0.62^{b}	9.42±0.09^{b}	9.39±0.13^{b}	10.39±1.62^{b}	9.42±0.09^{b}	9.36±0.12^{b}	9.32±0.14^{b}
22:5n3	0.73±0.01^{a}	0.52±0.01^{a}	0.45±0.01^{a}	0.39±0.01^{a}	1.46±0.03^{b}	1.31±0.11^{b}	1.27±0.06^{b}	1.25±0.04^{b}	1.44±0.09^{b}	1.29±0.01^{b}	1.23±0.03^{b}	1.08±0.06^{b}
22:6n3	9.12±0.01^{a}	7.78±0.83^{b}	6.14±0.02^{b}	4.95±3.03^{c}	10.61±0.01^{d}	10.15±0.44^{d}	9.89±0.07^{d}	9.81±0.09^{d}	10.75±0.84^{d}	10.3±0.01^{d}	9.98±0.13^{d}	9.95±0.14^{d}
∑PUFA	29.03	25.92	23.41	21.59	32.48	30.74	30.26	30.07	32.58	30.73	29.92	30.02
∑n3	19.95	15.25	12.28	9.91	25.97	22.87	22.26	21.9	26.63	22.04	21.49	21.1
∑n6	8.7	10.42	10.91	11.51	6.11	7.55	7.61	7.78	5.67	8.42	8.25	8.79
∑C_{18}PUFA	9.96	9.38	9.29	9.46	5.79	4.93	4.51	4.21	5.39	5.24	5.59	5.74
∑C_{20}PUFA	8.84	7.99	7.31	6.62	14.22	14.03	14.2	14.41	14.72	13.63	12.94	13.12
∑n3/n6	2.29	1.46	1.13	0.87	4.25	3.03	2.93	2.81	4.7	2.62	2.6	2.4
∑PUFA/∑SFA	0.67	0.58	0.44	0.39	0.8	0.75	0.72	0.71	0.83	0.71	0.67	0.66
∑DHA/EPA	1.29	1.4	1.3	1.3	1.05	1.07	1.05	1.04	1.03	1.29	1.07	1.07
∑trans	0.35	0.85	1.06	1.21	0.27	0.13	0.09	0.07	0.33	0.14	0.11	0.09

Data are expressed as mean ± standard deviation of three replicates. ΣSFA total saturated fatty acids, ΣMUFA total monounsaturated fatty acids, ΣPUFA total polyunsaturated fatty acids. Means with different superscripts (a, b, c, d) in the same row indicates a statistical difference (P<0.05). ND: not detected.

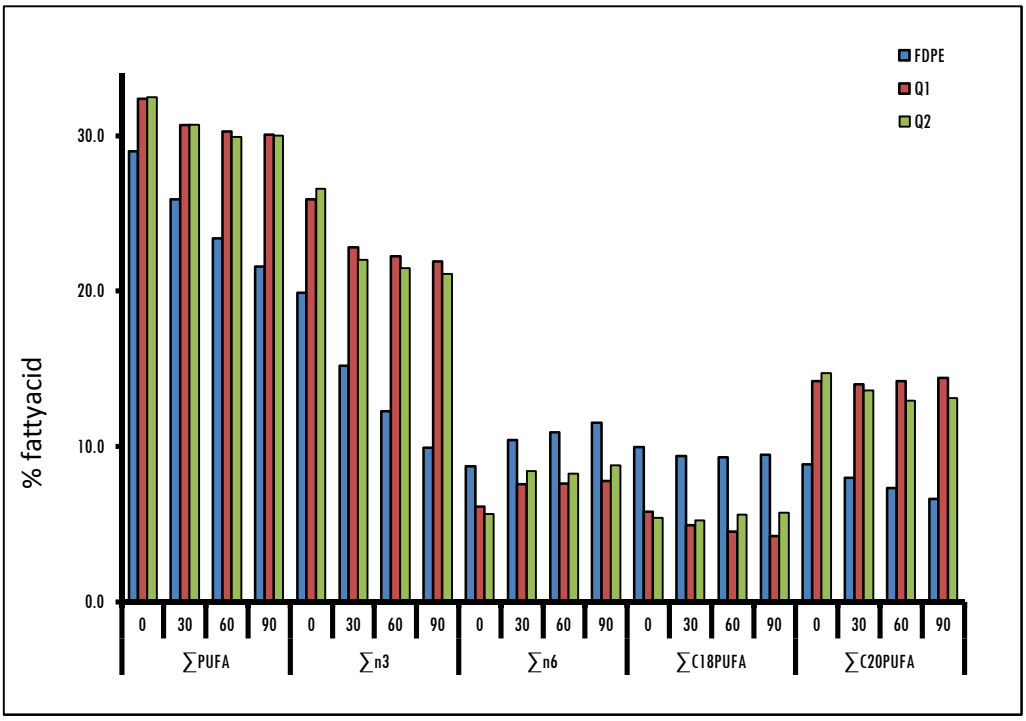


Fig.6B.4: Fatty acid content of control (FDPE) and different formulations (Q1 and Q2) during 90 days shelf life study.

FDPE was found to be susceptible towards peroxidation by free radicals, and therefore, could not retain the *n*-3/*n*-6 ratio for a longer time period under shelf, and the additives (as in Q_1 and Q_2) were used to shield the oxidation of FDPE. Initially, the total content of deleterious *trans* fatty acids were found to be 0.35%, 0.27%, and 0.33%, respectively for FDPE, Q_1 and Q_2 in that order. The combinations Q_1 and Q_2 effectively arrested the isomeric conversion of monoenoic and dienoic *cis*-fatty acid isomers (7 *cis* 18:1*n*-9 and 9 *cis*, 12 *cis* 18:2*n*-6) to their *trans* isomers (7 *trans*18:1*n*-9 and 9 *trans*, 12 *trans* 18:2*n*-6) at different time intervals of incubation during accelerated shelf-life study for 90 days (**Fig 6B.5**). *Trans* 16:1*n*-7 exhibited a significant increase in FDPE, than in Q_1 and Q_2 that realized a moderate increase (upto a maximum of 0.05%) throughout the period on shelf. This increase of *trans*

isomers of fatty acids is related to the initiation of lipid peroxidation, which is initiated by a homogeneous split of a C-H bonds adjacent to *cis* double bonds in the unsaturated fatty acids.

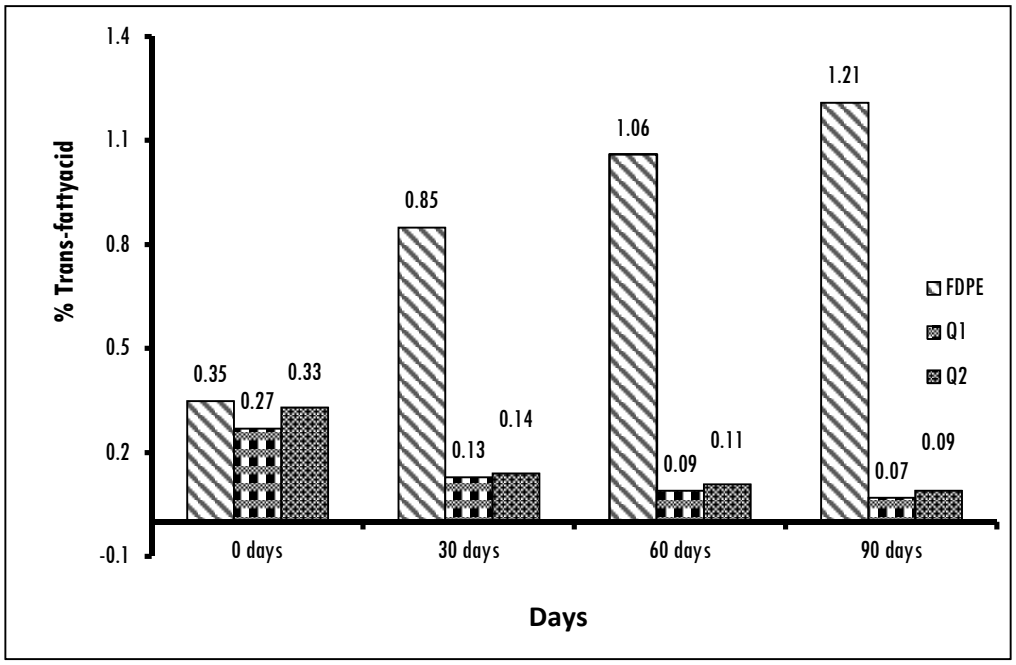


Fig.6B.5 Trans-fatty acid (%) of control (CM) and different formulations (Q1 and Q2) during 90 days shelf life study.

6B.2.7 *In vivo* anti-inflammatory studies of potential formulation

Carrageenan-induced paw edema is a standard and most commonly used technique to screen the anti-inflammatory activity (Winter 1962). It is the most commonly used method in experimental pharmacology causing inflammation by producing inflammatory histamines, and prostaglandins. Therefore, in the present study, time dependent anti-inflammatory properties in mice with carrageenan–induced foot paw edema were investigated and the effect of Q_1 was compared with standard drug, aspirin. The arrows in **Fig 6B.7** indicated the swelling of dorsal surface of the paw. The control group animals

showed maximum swelling at the fifth hour, which increased further until the sixth hour **(Table 6B.9)**. The anti-inflammatory formulation Q_1 showed significant inhibition of the edema (> 50%) during the third hour after the carrageenan injection, which reached ≥ 72.0% after the sixth hour as compared to ~80% noted for the standard (aspirin). It was interesting to note that the activity of Q_1 improved with time, indicating their potential to release the active principle throughout an extended period, unlike the standard, which showed a deceleration in activity after the fourth hour. Comparison of Q_1 with reference drug (aspirin) on carrageenan-induced hind paw edema in mice were given in **Table 6B.9**. The aspirin treated groups significantly decreased the paw edema with maximum inhibition of 82.07% at 4^{th} h. Q_1 (34.34%, 2 h) recorded significantly higher edema inhibition than FDPE (55.98%, 2h).Q_1 showed considerable inhibition on swelling as compared to aspirin (80.27 and 78.37%, respectively) in the 6^{th}h, illustrating that Q_1 is potent but relatively slow-acting anti-inflammatory agent. The development of carrageenan induced edema is biphasic; the first phase is attributed to the release of histamine and kinins, while the second phase is related to the release of prostaglandins (Vane and Booting, 1987). Hence, the present investigation demonstrated that Q_1 showed significant reduction in the biphasic response of carrageenan induced edema.

Table.6B.9 Comparison of Q_1 with reference drug (aspirin) and FDPE on carrageenan-induced hind paw edema in mice.

Samples	Mean paw edema (mm)	Difference in paw edema 2h		% inhibition	Difference in paw edema 3h		% inhibition	Difference in paw edema 4h		% inhibition	Difference in paw edema 5h		% inhibition	Difference in paw edema 6h		% inhibition
	0h	(mm)	(%)		(mm)	(%)		(mm)	(%)		(mm)	(%)		(mm)	(%)	
N.Saline	1.16	2.47	112.76±4.87		2.48	113.79±1.61		2.48	113.79±1.59		2.50	115.52±5.57		2.52	117.24±1.51	
Std	1.47	1.99	35.32±3.33	68.68	1.89	28.67±1.71	74.81	1.82	20.41±2.06	82.07	1.80	22.45±1.36	80.57	1.77	23.13±0.31	80.27
FDPE	1.56	2.72	74.04±3.09	34.34	2.53	62.05±0.84	45.47	2.19	44.87±1.87	60.57	2.16	44.23±1.41	60.57	2.15	42.95±1.49	63.37
Q_1	1.42	2.14	50.7±2.34	55.98	2.06	45.07±2.89	60.39	1.89	33.09±1.56	70.91	1.81	27.46±1.95	76.22	1.78	25.35±2.31	78.37

N.Saline: nomal saline; Std: reference drug (aspirin); FDPE: freeze dried mussel extract without additives (control); Q1:enriched formulations prepared from the extract of *P. viridis*. Percent inhibition in mice paw edema (in parentheses) was calculated as: $(I_t - I_o)$ X 100/ I_o, where I_t is the percent difference in paw edema of the animals treated with normal saline with respect to the baseline value (zeroth hour) and I_o is the percent difference in paw edema of animals treated with standard and Q_1 at the same time interval

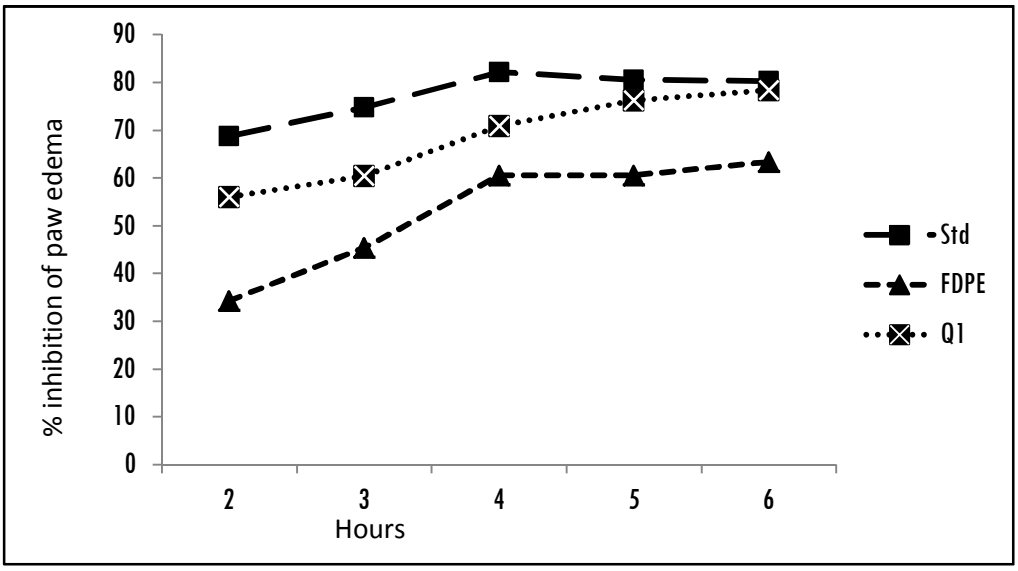


Fig.6B.6 Inhibition of paw edema (%) on Q_1 treated mice

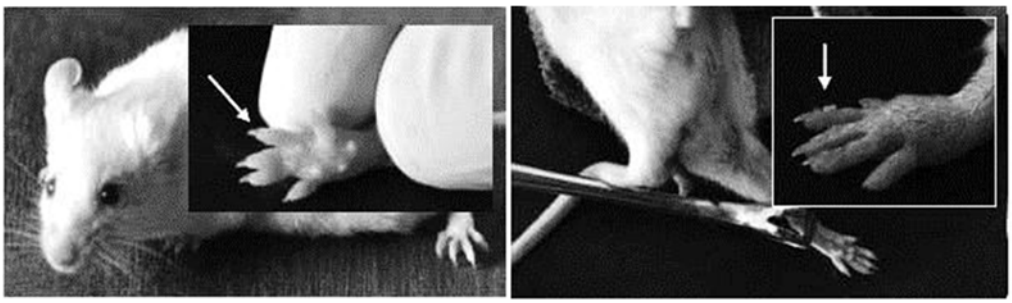


Fig.6B.7 *In vivo* anti-inflammatory activities of anti-inflammatory formulation Q_1 on carrageenan-induced mice paw edema. (**A**) Carrageenan-induced hind paw edema in BALB/C mice. The arrow indicates the swelling of dorsal surface of the paw; (**B**) Reductions in mice paw edema in treated (by Q_1) mice. The reduction in paw swelling is indicated by an arrow.

6B.2.8 Conclusion

Two combinations (Q_1 and Q_2) of the freeze dried *P. viridis* extract (FDPE) supplemented with the antioxidative oleoresins and aqueous extracts of natural additives were prepared based on their antiradical properties. Among the different treatments of enriched green mussel extracts Q_1 exhibited

significant inhibition of pro-inflammatory COX-1, COX-2 and 5-LOX enzymes, indicating their potential for use as effective green alternatives to the synthetic drugs with multiple several adverse effects on human health. Significant *in vivo* activities also indicated better therapeutic profile and fewer side effects of the anti-inflammatory formulation Q_1 as compared to the synthetic non-steroidal anti-inflammatory drugs. The different anti-inflammatory combinations, demonstrated that the combinations Q_1 have the potential to retain the active principles throughout the experimental period under shelf. Therefore, Q_1 proved to be the effective new generation green alternatives to the synthetic non-steroidal anti-inflammatory drugs for use against a cascade of mammalian inflammatory diseases.

6.C Response of pro-inflammatory prostaglandin contents in anti-inflammatory supplements from green mussel *Perna viridis* L.

Background of the study

Bioactive lipids like prostaglandins (PGs) are potent lipid signaling mediators of inflammation, and are derived from arachidonic acid (AA; C20:4*n*-6) and related 20 carbon polyunsaturated fatty acids (PUFAs) by cyclooxygenase (COX) enzymes. COX is a bi-functional enzyme containing a site that converts AA to prostaglandin G_2 (PGG_2) and another site that reduces PGG_2 to prostaglandin H_2 (PGH_2), which is metabolized to prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Fernandes *et al*., 2004). Production of inflammatory PGE_2 is mediated by three enzymatic reactions involving phospholipase A_2 (PLA_2), COX, and PGE_2 synthase (PGES). In this biosynthetic pathway, AA releases from membrane phospholipids by cytosolic or secretory PLA_2 and is converted to PGH_2 by COX. PGH_2 is then isomerized to PGE_2 by terminal PGES enzymes. Studies have shown elevated COX activity and resulting increased levels of PGE_2 in various tissues, including human colon and prostate tumor (Hidalgo *et al*., 2002). PGE_2 and $PGF_{2\alpha}$ have been found to stimulate the proliferation of various cancer cells and promote tumor development (Koehne *et al*., 2004).

Considerable attention has been devoted to study the role of PGE_2 in inflammation and tumor progression, the role of which is supported by high concentrations of the major urinary metabolite of PGE_2, tetranor PGE-M (**Fig.6C.1**), through a series of reaction intermediates in patients suffering from inflammatory responses (Neale and Dean, 2008). The enzyme COX-2 is expressed and up-regulated in the rheumatoid synovium (Claria and Romano, 2005), and inhibition of this enzyme is proposed as a safe and effective treatment

modality in rheumatoid arthritis (Charlier and Michaux, 2003). Current symptomatic treatment of inflammation is mainly by non steroidal anti-inflammatory drugs (NSAIDs) that exert anti-inflammatory actions by inhibiting COX, which exits as two isoforms: COX-1, constitutively expressed in most cells under physiological conditions and COX-2, which induced by pro-inflammatory agents such as TNF-α, LPS and tumor-promoting factors (Jaggi *et al*., 2004). However, NSAIDs inhibit not only COX-2, which produces PGs associated with pain induction, but also COX-1, which produces PGs involved in maintaining the integrity of gastrointestinal mucosa, and, therefore, are noted for undesirable gastrointestinal side effects related to their use (Parente and Perretti, 2003). For this reason, selective COX-2 inhibitors have come to attention in recent years (Chen *et al*., 2005). It is desirable to develop more potent and selective inhibitors of COX-2 for development of the next-generation therapeutics.

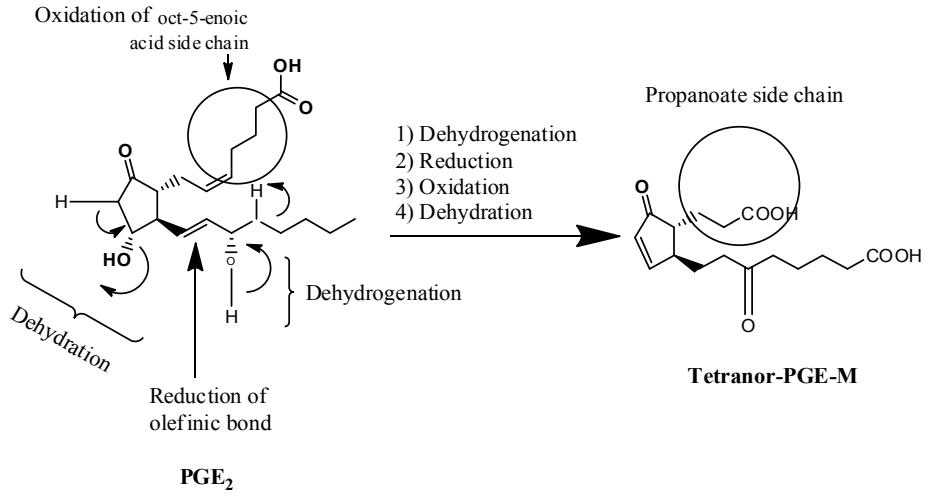


Fig.6C.1 Metabolic conversion of pro-inflammatory PGE_2 to tetranor PGE-M, which is a major causative agent in inducing a series of reactions in inflammation and tumor progression.

Earlier studies reported that commercially prepared freeze-dried extract of New Zealand green-lipped mussel *P. canaliculus* shows a capacity to inhibit experimentally induced inflammation and can act as dual inhibitor of

membrane arachidonic acid oxygenation by both COX and lipoxygenase (LOX) pathways, thus decreasing PG and leukotriene synthesis and down regulating the inflammatory sequence (Miller *et al.*,1993; Tiffany and Bui, 2002). Nutritional supplements Seatone® and Lyprinol containing freeze-dried tissues and mussel oil, respectively, from *P. canaliculus*, are used as anti-inflammatory medication (Cobb and Ernst, 2006).

Marine bivalves are virtually untapped resource for the discovery of novel bioactivities and were reported to contain abundance of bioactive principles, and many of these can influence human health (Chellaram *et al.*, 2004). Marine originated bioactive lipids have been reported to be linked in alleviating the symptoms of inflammatory conditions (Calder, 2004). Green mussel, like *Perna viridis*, is abundant in C_{20}-C_{22} *n*-3 PUFAs, particularly eicosapentaenoic acid (C20:5*n*-3, EPA) and docosahexaenoic acid (C22:6*n*-3, DHA), which are the precursors of anti-inflammatory resolvins (E- and D-series) (King *et al.*, 1990). The coastline of South Indian subcontinent is bestowed with large assemblage of *P. viridis*; and they have been found to possess anti-inflammatory principles. According to recent studies, reactive oxygen species (ROS) are important mediators that initiate and propagate inflammatory responses by stimulating release of pro-inflammatory cytokines such as interleukin-1b (IL-1b) and tumor necrosis factor (TNF-a) (Geronikaki and Gavalas, 2006). This indicates that free radical scavengers might be a useful means of attenuating inflammatory effects. Accordingly, anti-inflammatory and antioxidative agents interfering with eicosanoid synthesis require a well-balanced pharmacological profile to minimize these on-target side effects. In this context, it needs to be mentioned that the harmful ROS attack the multiple olefinic bonds in the lipids, which are abundantly present in any marine fauna like green mussel, leading to the formation of lipid

hydroperoxides, small molecular weight aldehyde and ketones, among various degradation products. *P. viridis* are also rich in long chain PUFAs with 20 carbon atoms, mainly AA, which are precursors of inflammatory PGs. It has been demonstrated that marine organisms are capable of metabolizing C20 *n*-6 PUFA to oxidized fatty acid derivatives, PGs, via oxidative pathways. There is tendency of these multiple inflammatory PGs particularly PGE_2 and $PGF_{2\alpha}$ metabolized from C20 *n*-6 PUFA to increase in mussel meat or extract unless otherwise any attempt is made to arrest the oxidative pathways towards this direction by addition of antioxidative principles (Droge, 2002). It is, therefore, necessary to arrest the formation of inflammatory PGs in the mussel extract from C20 *n*-6 PUFAs (like AA) by different antioxidants, and to retain its anti-inflammatory property for a longer time period in shelf.

The present study explored various natural additives, which are able to reduce the free radicals that cause oxidation reactions, and simultaneously hinder the formation of inflammatory PGs. Antioxidant principles play an important role to deter the formation of PGE_2 and $PGF_{2\alpha}$, and, therefore, have added advantage other than maintaining high shelf-life of the active ingredient. These led us to incorporate natural antioxidants in 'green' and 'environmentally safe' preparations of mussel in different combinations to arrest the formation of inflammatory PGE_2 and $PGF_{2\alpha}$ from C_{20} *n*-6 PUFAs. The different compositions of oleoresins (*Rosmarinus officinalis* and *Curcuma longa*) and other natural antioxidant additives in different proportions were blended with freeze dried green mussel extract (GME_0) to find their individual and synergistic effects to arrest the formation of inflammatory PGE_2 and $PGF_{2\alpha}$. The purpose of the stability study is to determine the levels of PGE_2 and $PGF_{2\alpha}$ in GME_0 and that supplemented with natural antioxidant additives (GME_1, GME_2, GME_3 and GME_4) during the course of an accelerated shelf-life study of 90 days to

understand the differential pattern of PGE_2 and $PGF_{2\alpha}$ in different combinations thereby establishing its shelf life, the time period of its storage.

Because of broad involvement of PGs in normal and pathophysiological processes, their quantification is critical in understanding their role in anti-inflammatory activities. Common methods used to quantify PGE_2, $PGF_{2\alpha}$ and their metabolites include immunological assays, gas chromatography, high performance liquid chromatography (HPLC), and mass spectrometry. Although immunoassays are sensitive and can accommodate high-throughput applications, specificity of the antibody is a concern regarding selectivity for the desired antigen, which can lead to variable results and an overestimation of analyte levels. As compared to other methods, HPLC and HPLC with tandem mass spectrometer detection (LC/MS/MS) has several advantages because of its high selectivity, its high sensitivity, and its potential for simultaneous quantification for several PGs and isoprostanes without any derivatization with relatively low cost of analysis (Yue *et al*., 2007). ^{1}H NMR has been used to further validate the results obtained by HPLC to understand and compare the signal pattern and intensity of GME_0 *vis-à-vis* GME_{1-4} after 90 days of accelerated shelf-life study. Simultaneous ^{1}H NMR and mass spectroscopic monitoring of the PG formation in GME_0 and GME_{1-4} gives a fair description of anti-inflammatory status. The present study therefore also focusses to understand the differential indicative information regarding the presence of characteristic signature peaks of PGE_2 and $PGF_{2\alpha}$.

6C.1 Materials and methods

6C.1.1 Preparation of *P. viridis* extract, experimental combinations and designing of stability studies.

The *P. viridis* meat (3.0 kg) acquired manually from the shellon samples was freeze dried to furnish *P. viridis* extract (GME; 213.6 g; yield - 7.1%). By assigning FDPE as the base material, four different combinations GME_1, GME_2,

GME_3 and GME_4 with anti-oxidative oleoresins (ROO and CLO) and aqueous extracts of the additives were prepared as discussed in chapter 6A.The samples (GME_0, GME_1, GME_2, GME_3, and GME_4) were kept in glass vials (10 g each) and were evaluated at 0^{th} day and after a period of 90 days (3 months) for the changes in inflammatory PGE_2 and $PGF_{2\alpha}$ and the fatty acid C20:*n*-6 and C22:*n*-3. All the samples were withdrawn in triplicate at same intervals. Cyclooxygenase (COX) inhibition assay was performed using established 2, 7-dichlorofluorescein method (Larsen *et al.*, 1996) as described in the section 4.1.2.2.1.

6C.1.2 C_{20} and C_{22} polyunstaurated fatty acid compositions during an accelerated shelf life study of 90 days

Lipids of GME_1, GME_2, GME_3 and GME_4 *vis-à-vis* GME_0 collected for baseline (d =0) and at the end of the study (d=90) were extracted according to Bligh and dyer (Bligh and Dyer, 1959). The fatty acid composition of the total lipids was determined as described elsewhere with suitable modification (Metcalf et al., 1966; Chakraborty and Paulraj, 2007). Identification of FAMEs was confirmed by chromatographic comparison of retention times with known FAME standards. Results were expressed as percent weight of total fatty acids. The fatty acid methyl esters (FAMEs) were also resolved by TLC (5 cm X 20 cm), pre-coated with silica gel and impregnated with $AgNO_3$ following reported method (Chakraborty and Paulraj, 2007).

6C.1.3 Isolation and identification of prostaglandins PGE_2 and $PGF_{2\alpha}$ in the mussel extract (FDPE) and different treatments during an accelerated shelf life study for a period of 90 days

GME_0 (5g) was extracted thrice with EtOAc (3 X 20mL) following the modified method of Jurenka et al. (Jurenka et al., 1999), and filtered under vacuum to yield 55 mL of total PG extract. The combined EtOAc extracts were evaporated under N_2 to dryness (150 mg), which was redissolved in EtOAc (2 mL), before being charged to a small silicic acid column (2 mm i.d.

90 mm long, 60-120 mesh silicic acid). The PGs were eluted with the sequential addition of 30 mL of increasingly polar solvents starting with 100% *n*-hexane, followed by *n*-hexane:EtOAc (1:1, v/v) EtOAc: acetonitrile (1:1, v/v), 100% acetonitrile, acetonitrile: MeOH (1:1, v/v), and 100% MeOH. PGs were eluted in the acetonitrile: MeOH fraction. For the quantitative analysis of PGE_2 and $PGF_{2\alpha}$ analytical HPLC was performed on a Phenomenex ODS column (C_{18} 25 cm X 4.6 mm i.d.) (Phenomenex, Torrance, CA) with UV detector (196 nm) in an isocratic mobile system of MeOH–0.1% formic acid in H_2O (70:30) at a flow rate of 0.8 mL/min. The run time was 30 min.

6C.1.4 Identification of prostaglandins in the mussel extract (GME_0) and different treatments by positive mode ESI-MS and ^1H NMR experiments

The NMR spectra of PGs were recorded in $CDCl_3$ on a Bruker DRX NMR spectrometer operating at 400 MHz for ^1H NMR. Chemical shift (δ) values are expressed in parts per million (ppm) and are referenced to the residual solvent signals of $CDCl_3$. The identification of the products was confirmed by comparing NMR spectra with earlier published data. Mass spectrometric detection was performed using a FT-ESI-MS with positive mode electrospray ionization and on a Bruker Daltonic (GmbH, Germany) micro-TOF series with electrospray ionization (ESI-MS). Positive ion ESI ($M+H^+$) was calculated. HPLC-MS has been carried out with an Agilent G1312 binary gradient pump with the solvent program for elution: 30% B (30% water and 0.1% HCOOH) and A 70% A (70% MeOH and 0.1% HCOOH) upto 100% MeOH (and 0% water) with a total run time is 25 min with slice width of 1.0 points/sec. The flow rate was 0.6 mL/min, and the injection volume was 5µL. Equilibration time between runs was 5 min. The separation was carried out using a Luna C_{18} column (3 µm, 100 Å pore diameter, 150 x 2.0 mm, Phenomenex, Torrance, CA, USA) with a stainless steel frit filter (0.5 µm) and security guard cartridge system (C_{18}) (Phenomenex, Torrance, CA, USA). The HPLC system consisted of an Agilent 1100 series LC pump equipped with a

wellplate autosampler (Agilent Technologies, Santa Clara, CA). A 5 µL out of 30 µL sample was injected onto a chromatographic column. Detector used in this study was Agilent DAD G131B and scanned through 190-600 nm wavelength; the spectra has been started at 190 min and completed at 1200 min. The maximum pressure was 380 bar. The HPLC column was kept at column oven under 25°C. Total ion chromatogram (TIC) in MS was used to identify the PGs. Zero-grade air was used as the nebulizing and curtain gases at a temperature of 700°C. The ionization energy was set to 4000 V, and the declustering potential was 30.0 V. The scan rate was 4000 amu/s with a range from 100 to 500 amu. Complete system control, data acquisition, and processing were performed using Analyst 1.4.2 software.

6C.2 Results and discussion

6C.2.1 Time series studies of *in vitro* anti-inflammatory activity of mussel extract (GME_0) and different treatments during an accelerated shelf life study of 90 days

The anti-inflammatory properties of GME_1, GME_2, GME_3 and GME_4 were evaluated *in vitro* during accelerated shelf life study of 90 days to determine their COX-2 inhibitory potencies, and were compared to GME_0 (**Table 6A.1**).

Table 6C.1 *In vitro* anti-inflammatory activities of different combinations (GME_1- GME_4) compared with control (GME_0) (5 mg/mL) during accelerated shelf life study

Days (d)	COX-2 Inhibition activity (%)				
	GME_0	GME_1	GME_2	GME_3	GME_4
0	$48.4^{apq} \pm 0.03$	$50.02^{bp} \pm 1.02$	$52.1^{cp} \pm 0.59$	$51.6^{dp} \pm 0.23$	$52.9^{ep} \pm 0.56$
15	$48.4^{aq} \pm 1.56$	$50.23^{bq} \pm 2.05$	$52.0^{cp} \pm 2.0$	$51.4^{dq} \pm 1.2$	$52.9^{eq} \pm 0.9$
30	$47.69^{ap} \pm 1.25$	$49.89^{br} \pm 1.65$	$51.89^{cp} \pm 1.35$	$51.0^{dr} \pm 0.59$	$52.01^{cr} \pm 0.45$
60	$43.02^{ar} \pm 0.25$	$47.06^{bs} \pm 0.38$	$49.56^{cq} \pm 1.97$	$49.56^{ct} \pm 0.89$	$51.02^{ds} \pm 1.4$
90	$37.23^{as} \pm 2.36$	$46.56^{bt} \pm 0.56$	$49.02^{cr} \pm 0.56$	$47.56^{du} \pm 0.74$	$51.0^{es} \pm 1.45$
x	23.1^{a}	6.9^{b}	5.9^{c}	7.8^{b}	3.6^{d}

*Results are expressed as percent activity with respect to the samples, and as mean ± SD (n = 3). 'x' represents percent reduction in activity on 90^{th} day (d=90) with respect to 0^{th} day (d=0).

a-e: Row wise values with different superscripts of this type indicate significant differences ($P < 0.05$) within different samples; p-t: Column wise values with different superscripts of this type indicate significant differences ($P < 0.05$) within different days of shelf life study

GME_2 and GME_4 were more effective in inhibiting COX-2 enzymes compared to GME_1, GME_3 and GME_0. The principle anti-inflammatory ingredients used in GME_2 and GME_4 are rosemary oleoresin (with carnosic and rosmarinic acid), and curcuminoids, which share a close physicochemical status with AA, the natural COX-2 substrate, thereby indicating the potential of GME_2 and GME_4 active principles to mimic the target enzyme (COX-2) (**Fig.6C.2**). Also to note that *n*-3 PUFAs (present in GME_0) compete with AA for COX-2 active site to synthesize anti-inflammatory lipid mediators thereby inhibiting this enzyme. The treatment GME_4 was more effective in inhibiting COX-2 than other treatments at day 0. The COX-2 inhibition rate of GME_0 was significantly reduced on d=15, and a significant percent reduction in COX-2. inhibitory activity was apparent after 90 days with respect to the baseline activity (d=0). In general, the reduction in inflammatory response is directly proportional to the active ingredients in GME_0 as apparent from the potential of the same to inhibit COX-2. However, it could not retain the activity for a longer time period, and therefore, various additives have been tried to retain the activity of the active ingredient of GME_0. The functional advantages of the additives are two-fold, the first to arrest the oxidation of GME_0, and second to increase the anti-inflammatory potential. Apparently, among the treatments, GME_4 and GME_2 showed marginal percent reduction in COX-2 inhibition activity with respect to initial activity as compared to GME_1 and GME_3. The ability of *Z. officinale* to reduce inflammation is due to its neutralizing action upon free radicals by phenolic compounds *viz*., gingerol, zingerone, and shogaol (with 2-methoxy-4-propylphenol moiety) (Onyenekwe and Hashimoto, 1999). The active ingredients in *Z. officinale* (gingerol) and Indian gooseberry (*E. officinalis*) contributed towards mimicking the substrate (AA, C20*n*-6 PUFA) to competitively inhibit the production of pro-inflammatory PGE_2 and $PGF_{2\alpha}$.

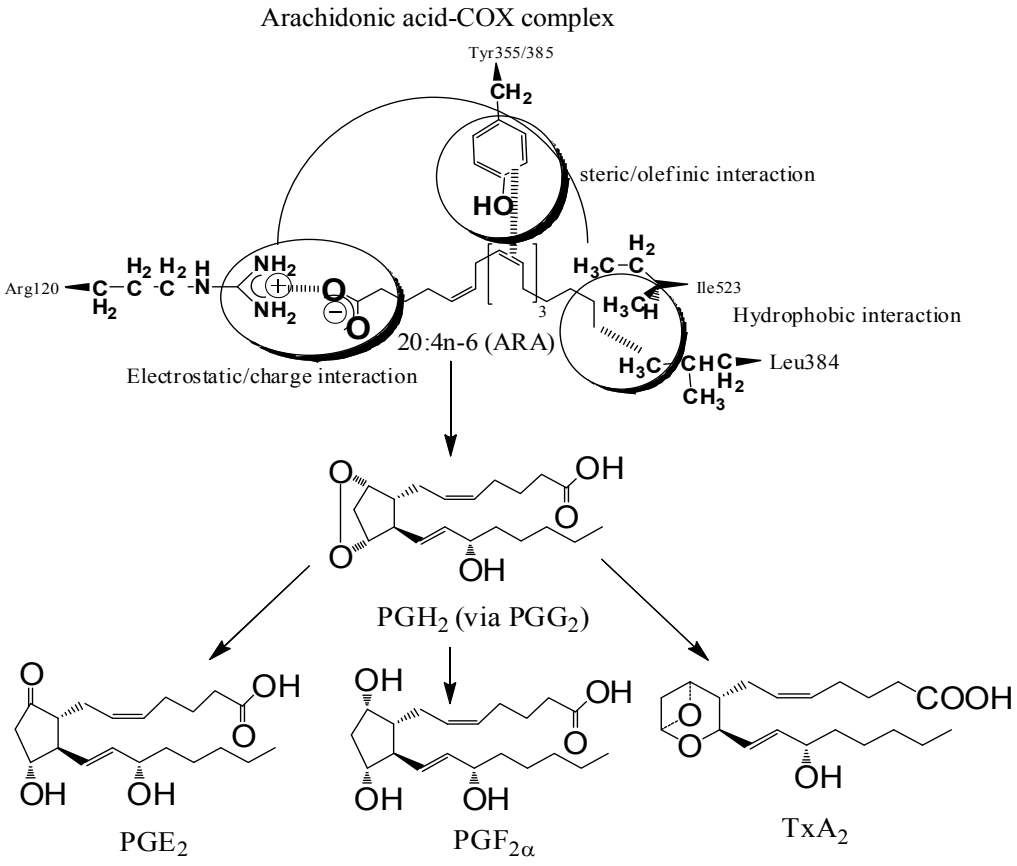


Fig.6C.2 Schematic representation of the COX-2 active site amino acyl residues with the substrate (AA) forming the inflammatory PGH_2, PGE_2, $PGF_{2\alpha}$, and TxA_2. The Δ11-double bond of AA interacts with $Tyr^{355/385}$ in an olefinic interaction with COX-2. PGE_2 is metabolized to PGH_2 by peroxidase activity intrinsic to COX-2/ COX-1 to give PGH_2 that serve as the precursor to inflammatory prostanoids.

6C.2.2 Effect of various combinations of natural additives on C20-22$_{n-3}$ and C20$_{n-6}$ PUFAs during an accelerated shelf life study for a period of 90 days

C20*n-6* and C22*n*-6 fatty acid profiling of GME_0 *vis-à-vis* different combinations supplemented with antioxidative ingredients (GME_{1-4}) used in this study as a factor of time are detailed in **Table 6A.2.** PUFAs with more

than 20 carbon atoms were found to represent more than 17% of the total fatty acids for GME_0 and different combinations (GME_{1-4}). However, the C20*n*-6 PUFAs in GME_0 experienced an increase in terms of their total content from the initial composition after accelerated shelf life study of 90 days as compared to the treatments GME_2, GME_3 and GME_4, which point towards the beneficial effect of natural additives added to it. In the present study, total C20-22*n*-3 PUFA content in GME_0 was found to be lesser than recorded in GME_1 - GME_4 at d=0, thereby indicating the added *n*-3 fatty acid pool to GME_0 by the additives. The content C22:6*n*-3 PUFA, registered a significant reduction in GME_0, after 90 days. GME_2 and GME_4 were able to effectively protect C22:6*n*-3 from oxidative degradation even after 90 days of accelerated storage than did GME_1 and GME_3. The comparatively poor shelf-life of GME_3 than GME_2 in terms of containing degradation of C22:6*n*-3 to a minimum in spite of higher concentration of antioxidant principles can be explained by the fact that a threshold level of CLO: ROO of 0.8%:0.4% is sufficient for optimum antioxidant activity, and further addition of endogenous antioxidants (ROO to 0.8% as in GME_3) does have negative impact on stability of the fatty acid. This observation was supported in an earlier study Lampi *et al*. (Lampi *et al*. 1999) on fish oil, which implies that above a certain concentration, added antioxidants lose efficiency in stabilizing PUFA. The fatty acid C20:5*n*-3, which compete with AA for COX-2 active site to synthesize EPA-derived anti-inflammatory PG metabolites including series-E resolvins were found to be higher in GME_2 and GME_4 than in GME_1 and GME_0 **(Fig.6C.2)**.

Table 6C.2 C20*n*-6 and C22*n*-3 fatty acid profile of freeze-dried green mussel extract (GME_0), different combinations GME_{1-4} during accelerated shelf life study

Fatty acid descriptors	Fatty acids (% total fatty acids TFA)				
	DAY 0 (d =0)				
	GME_0	GME_1	GME_2	GME_3	GME_4
20:2*n*-6	0.31±0.01	0.28±0.01	0.14±0.01	0.24±0.02	0.36±2.51
20:3*n*-6	0.47±0.01	0.45±0.01	0.27±0.01	0.34±0.01	0.31±2.25
20:4*n*-6	0.58±0.01	1.29±0.01	1.17±0.01	1.18±0.16	1.19±0.02
20:5*n*-3	6.98±0.23	6.79±0.6	7.85±0.34	7.89±0.02	7.74±0.01
22:5*n*-3	1.56±0.09	1.73±0.02	1.89±0.01	1.86±0.02	1.74±0.01
22:6*n*-3	7.92±0.18	7.96±0.01	8.7±0.22	7.58±0.24	7.43±0.02
Σ C20*n*-6 PUFA	1.36	2.02	1.58	1.76	1.86
Σ C20-22*n*-3 PUFA	16.46	16.48	18.44	17.33	16.91
Σ C20*n*-6/ C22*n*-3 PUFA	0.08	0.12	0.09	0.1	0.11
	DAY 90 (d =90)				
	GME_0	GME_1	GME_2	GME_3	GME_4
20:2*n*-6	1.13*±0.09	0.76±0.31	0.21±0.05	0.28±2.77	0.28±0.06
20:3*n*-6	0.07*±0.001	0.06±0.01	0.05±0	0.05±0.02	0.07±2.31
20:4*n*-6	1.96*±0.05	1.86±0.05	1.23±0.03	1.34±0.01	1.29±0.06
20:5*n*-3	4.53*±0.15	5.12*±0.31	7.61±0.2	7.63±0.06	6.74±0.06
22:5*n*-3	0.73*±0.01	0.86*±1.25	1.42±0.11	1.46±0.01	0.97±0.01
22:6*n*-3	5.11*±0.26	5.36*±0.11	7.84±0.31	5.24*±0.02	7±0.03
Σ C20*n*-6 PUFA	3.16*	2.68*	1.49	1.67	1.64
Σ C20-22*n*-3 PUFA	10.37*	11.34*	16.87	14.33*	14.71
Σ C20*n*-6/ C22*n*-3 PUFA	0.3	0.24	0.09	0.12	0.11

All samples were analyzed in triplicate (n=3) from a pooled samples. The values are expressed as mean ± standard deviation. ΣPUFA: total polyunsaturated fatty acids.
* shows significant difference ($P < 0.05$) at d=90 compared to d=0

It is interesting to note that insignificant reduction of C20:5*n*-3 were apparent in GME_2 and GME_4 after 90 days as compared to that in GME_0 and GME_1, which realized a significant reduction in the content of this fatty acid. In the present study, the PUFAs with C20-22$_{n-3}$ PUFAs in GME_0, were found to be significantly reduced after 90 days (37% with respect to initial value at d=0) of accelerated storage. However, no significant reduction of this group of fatty acids was noticeable for GME_2 and GME_4 (% reduction = 8.5 & 13.0%, respectively). Apparently, the antioxidant additives serve as oxidation "chain breakers" for the treatments (GME_{1-4}) by intercepting the free radicals generated from unsaturated fatty acids during accelerated storage (**Fig.6C.3**).

Fig.6C.3 Reaction mechanisms to PGG_2 synthesis and role of curcuminoid and carnosic acid functional groups to contain the free radicals responsible to metabolize arachidonate to PGG_2. PGG_2 is a precursor of pro-inflammatory prostaglandins PGE, and $PGF_{2\alpha}$.

The PUFAs with C20*n*-6 PUFA configuration (e.g., arachidonate) may potentially be oxidized to pro-inflammatory PGE_2 and $PGF_{2\alpha}$ (**Fig.6C.3**). In recent times, considerable attention has been devoted to study the role of inflammatory PGs in inflammation. There is tendency of these multiple inflammatory PGs metabolized from C20*n*-6 PUFA to increase in the product based on green mussel meat or extract unless otherwise any attempt is made to arrest the oxidative pathways towards this direction by addition of antioxidative principles. A schematic diagram illustrating lipid peroxidation inhibitory and antioxidant mechanism of rosemary active principles carnosic acid with 1-(1, 1-dimethylperhydro-4-naphthalenyl)-1-ethanone) and carnosol (with perhydro-1-isochromenone and 3-isopropyl-1, 2-benzenediol moieties has been depicted under **Fig.6C.3**. These phenolic active principles together are responsible for ~90% of antioxidant activity of rosemary. Hydrogen atom transfer (HAT or H-donation) and chain breaking mechanism, as in phenolic antioxidants, is the mode of action of carnosic acid and carnosol. It was found to form radical species due to HAT, undergoing rearrangement in multiple steps to satisfy the vacant electron shell to exhibit its antioxidant activity. The effective inhibition of lipid peroxidation in GME_4 and GME_2 may be due to the presence of polyphenolic antioxidants that were reported to disrupt free-radical chain reaction by donating proton to fatty acid radicals to terminate chain reactions. Though carnosic acid is the primary antioxidant in rosemary, and it metabolized to other compounds like carnosol, rosamanol and rosmariquinone, which too were reported to possess antioxidant and lipid peroxidation inhibitory properties. Another mechanism by that these polyphenolic compounds exhibit lipid peroxidation is by metal (Fe^{2+}) ion chelation by phenoxyl radical and free hydrogen on -OH group. The polyphenols with *ortho*-dihydroxy groups provide improved stability to polyphenol radical by electron delocalization through the

system accompanying radical formation. The lipid peroxidation inhibitory and antioxidant mechanism of curcumin-I has also been illustrated under **Fig.6C.3**, whereas the *ortho*-methoxyl group of curcumin-I can form intramolecular hydrogen bond with the phenolic hydrogen, making the H-atom abstraction from the *ortho*-methoxyphenols easy. The antioxidant efficiency of phenolics is enhanced by the introduction of a second OH group and is increased by one or two methoxy substitutions in positions *ortho* to the OH group as in curcuminoids. Antioxidant action of curcuminoids was also found to be associated with the β-dicarbonylic system in the heptadienone link of curcumin-I, which has conjugated double bonds (dienes). H-atom abstraction from the -CH_2 group in the heptadienone link of curcumin-I also may be involved in antioxidant activity (**Fig.6C.3**).

The C20*n*-6/C20-22*n*-3 fatty acid ratio is directly proportional to the anti-inflammatory activities due to the fact that C20:5*n*-3 is a precursor to anti-inflammatory lipid mediators (E-series of resolvins, RvE1), whereas docosanoids C22:6*n*-3 to D-series of resolvins (RvD1) and protectins (neuroprotectin D1, NPD1). The combinations GME_1 and GME_4 were found to exhibit high C20*n*-6/C20-22*n*-3 ratio than recorded in GME_2, GME_3, and GME_0. It is interesting to note that the combinations, GME_2 and GME_4 did not exhibit any change in this ratio after a period of 90 days accelerated shelf-life study whereas in GME_0 an increase in this ratio was observed. It is apparent that ROO and CLO contain phenolic compounds with multiple centers of unsaturation capable of deactivating free radicals to initiate chain breaking peroxidation of olefinic bonds in C20-22*n*-3 PUFAs (**Fig 6C.3**). GME_0 is susceptible towards peroxidation by free radicals, and therefore could not retain the C20*n*-6/C20-22*n*-3 ratio for a longer time period under shelf, and the additives (as in GME_{1-4}) were used to shield the oxidation of GME_0. The

combination GME_1 realized an increase of C20*n*-6/C20-22*n*-3 as compared to the initial value, apparently due to comparatively less titre of curcuminoids (T_1: CLO and ROO of 0.4 and 0.8%, respectively), which were reported to possess potential to arrest oxidation of long chain PUFAs.

The long chain C20-22*n*-3 PUFAs have been demonstrated to modulate the inflammatory reaction especially by a competition mechanism at the level of COX-2, resulting in a reduced release of pro-inflammatory eicosanoid metabolites PGE_2 and $PGF_{2\alpha}$. The C20-22*n*-3-PUFAs viz., EPA and DHA modulate the inflammatory reaction especially by a competition mechanism at the level of COX-2, resulting in a reduced release of pro-inflammatory AA metabolites PGE_2, and $PGF_{2\alpha}$. The long chain-C20-22*n*-3-PUFAs have been demonstrated to combat chronic inflammatory diseases by inhibiting the formation of inflammatory PGs derived from AA by replacing the fatty acid as an eicosanoid substrate and inhibiting AA metabolism.

6C.2.3 Effect of potential combinations of natural additives on pro-inflammatory prostaglandins PGE_2, $PGF_{2\alpha}$ in a time course accelerated shelf-life study for a period of 90 days

PGE_2 is one of the major PGs produced in substantial amounts during inflammation by AA metabolism, and a positive correlation between PGE_2 and $PGF_{2\alpha}$ concentration during the course of inflammatory response was reported in earlier studies (Sanchez-Moreno *et al*., 2006). An isocratic reversed-phase HPLC separation of the PG derivatives in GME_0 and $GME_{1\text{-}4}$ in a time course accelerated shelf-life study for a period of 90 days was achieved within 30 min in a single run. The peaks of esters were completely resolved when a 25-cm column was used. With a 15-cm column, the resolution of PGE_2 and $PGF_{2\alpha}$ was poorer, but the analysis time was reduced to 16 min. As a rule, we

determined the sum of PGE_2 and $PGF_{2\alpha}$ because many tests on a 25-cm column showed that the amount of PGE_2 related to that of $PGF_{2\alpha}$ as 25:10 and this value were quite stable in all experiments. HPLC chromatogram of authentic standards, PGE_2 and $PGF_{2\alpha}$ along with GME_0, GME_2 and GME_4 after 90 days of accelerated stability study is shown in **Fig.6C.4**.

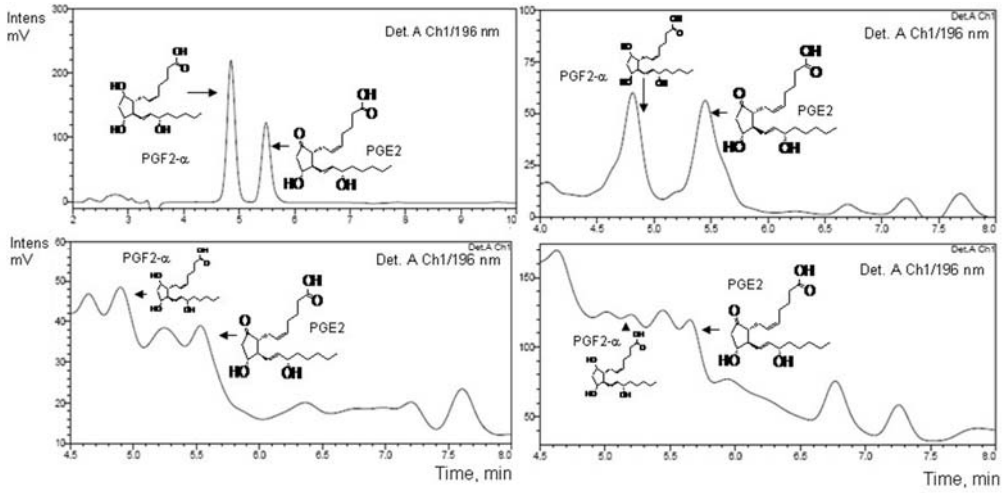


Fig.6C.4 HPLC Chromatograms of (A1) standard prostaglandins, PGE_2 and $PGF_{2\alpha}$; (A2) Control mussel extract (MSL) after 90 days; (A3) concentration of PGE_2 and $PGF_{2\alpha}$ in $GMET_2$ after 90 days of accelerated shelf life study; (A4) concentration of PGE_2 and $PGF_{2\alpha}$ in $GMET_4$ after 90 days of accelerated shelf life study.

The concentrations of PGE_2 and $PGF_{2\alpha}$ after an accelerated shelf-life study of 90 days (d=90) were significantly higher ($P < 0.05$) in GME_0 on day 90 of the intervention than among GME_2 and GME_4 (**Table 6A.3**). An inverse correlation between antioxidative effects of the additives (ROO and CLO: 0.4 and 0.8%, respectively) and PGE_2 concentrations in both GME_2 and GME_4 at baseline (day 0, d=0) and at the end of the study (day 90, d=90) was apparent. The relative increases and time-courses of formation of AA and PGE_2/ $PGF_{2\alpha}$ were highly correlated. Of note again, however, is that the levels of PGE_2 registered an approximately 24.35 times increase in GME_0 at the end of the

study (day 90, d=90) than at baseline (day 0, d=0) compared with GME_2 and GME_4 realizing a meager increase of 2.49 and 2.56 times (at d=90). The decelerated increases of inflammatory PGs of both GME_2 and GME_4 during later times of incubation were due to the presence of antoxidative components present in the additives. This was confirmed by demonstrating that an increase in levels of PGE_2 and $PGF_{2\alpha}$ in GME_0 was mirrored by a decline in levels of antioxidant status (**Table 6A.3**) with an increase in COX-2 titre. The fold-increase in levels of $PGF_{2\alpha}$ in GME_2 and GME_4 treated with additives are significantly less (6.40 and 6.52) as compared to levels measured in GME_0 greatly exceeded (18.30 fold increase) that of GME_2 and GME_4. The results of this study highlight the greater utility of measuring PGE_2 and $PGF_{2\alpha}$ as an index of antioxidant status and lipid peroxidation. The principle anti-inflammatory ingredients used in GME_2 and GME_4 are rosemary oleoresin (with carnosic and rosmarinic acid), and curcuminoids (curcumin-I and II), which share a close physicochemical status with arachidonate (as detailed under section 3.1), the natural COX-2 substrate, thereby indicating the potential of the former to mimic the enzyme (COX-2) inhibiting the biosyntheses of inflammatory PGE_2/ $PGF_{2\alpha}$.

Table 6C.3 Changes in inflammatory prostaglandin contents (PGE_2 and $PGF_{2\alpha}$ ppm) in different treatments of *P. viridis* (GME_1, GME_2, GME_3 and GME_4) compared with freeze-dried green mussel extract (GME_0) during before (day 0, d=0) and after accelerated shelf life study of 90 days (d= 90).

	PGE_2 (ppm)		$PGF_{2\alpha}$ (ppm)		$PGE_2 + PGF_{2\alpha}$ (ppm)	
Samples	**0th day**	**90 th day**	**0th day**	**90 th day**	**0th day**	**90 th day**
GME_0	74.82	1822.2*	44.3	810.6*	119.12	2632.8*
GME_1	75.21	241.32*	46.21	354.21*	121.42	595.53*
GME_2	76.89	189.72*	45.68	292.21*	122.57	481.93*
GME_3	76.89	206.35*	47.21	326.35*	124.1	532.7*
GME_4	75.23	192.35*	46.23	301.37*	121.46	493.72*

All samples were analyzed in triplicate (n=3) from a pooled samples. The values are expressed as mean standard deviation. * shows significant difference ($P < 0.05$) at d=90 compared to d=0

A higher titre of $PGF_{2\alpha}$ represents oxidative stress status in the system, and its role in the pathogenesis of diseases (Roberts and Morrow, 2000). Evidence from prospective studies suggests that an overexpression of PGE_2 associated with increased susceptibility to inflammatory response, including cancer, aging and cystic fibrosis (Stables and Gilroy, 2011).

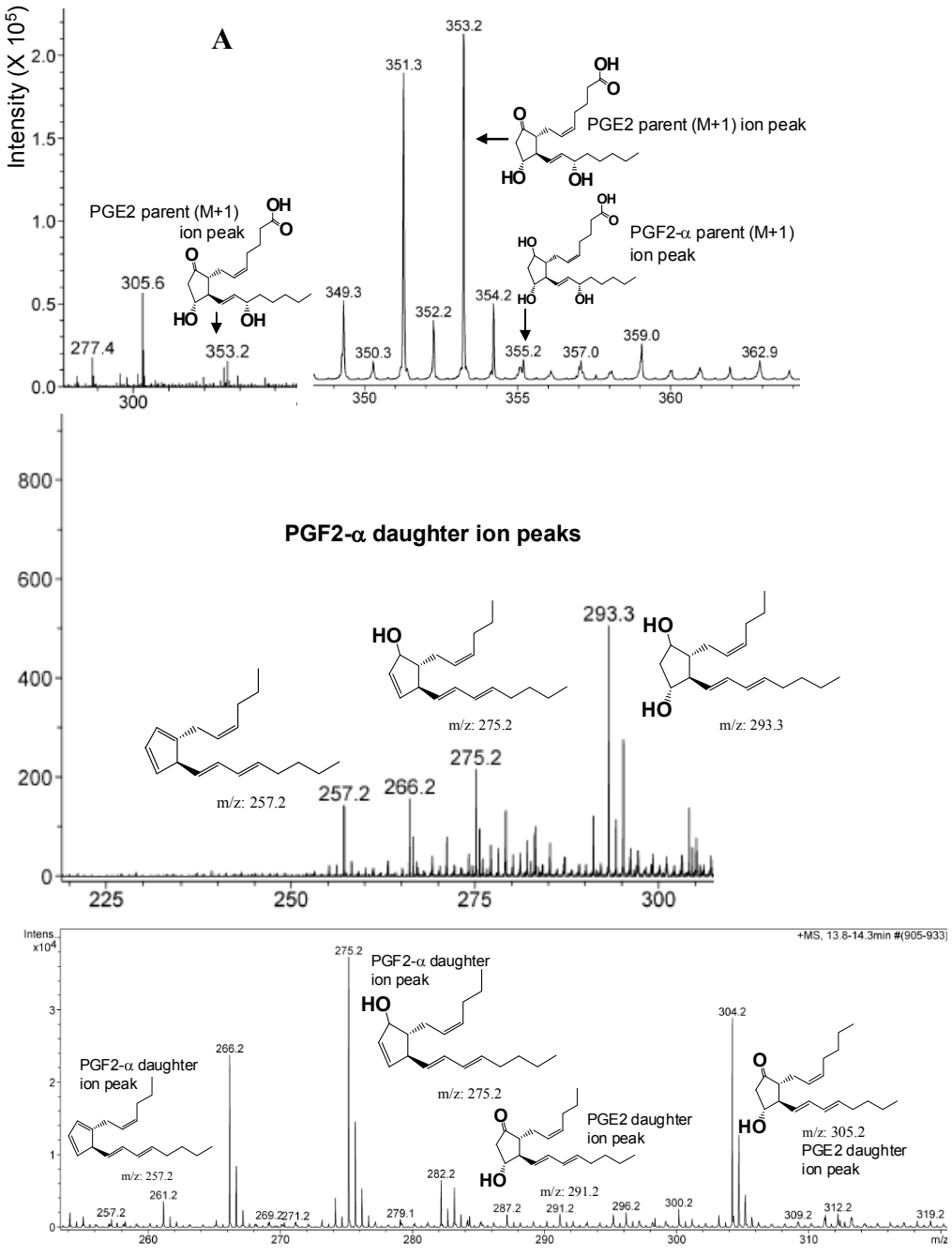
We have identified PGE_2 and $PGF_{2\alpha}$, as major eicosanoids in GME_0. The presence of these compounds was confirmed by co-chromatography with the authentic PG standards using TLC and HPLC. The structure of PGE_2 and $PGF_{2\alpha}$was unequivocally proved by analysis of their ^1H NMR spectra which were identical to those of authentic PG and the spectra reported previously (Fusetani and Hashimoto, 1984).

6C.2.4 Electrospray ionization (ESI) liquid chromatographic tandem mass spectrometric (LC/MS/MS) analyses of prostaglandins PGE_2 and $PGF_{2\alpha}$

Tandem LC/MS/MS methods allow a highly selective, sensitive, simultaneous analysis for analysis for PGs without derivatization. In the application of LCMS of biological samples, ESI has been proven to desolvate and ionize fragile chemical species (Sajiki and Kakimi, 1998). A systematic approach was used to evaluate the ESI mass spectral (ESI-LCMS) analysis of PGE_2 and $PGF_{2\alpha}$. The LC/MS/MS data shows parent peak of PGE_2 and $PGF_{2\alpha}$ at 5.5 and 4.8 minutes exhibiting parent $[M+H]^+$ ions at m/z 353.2 and 355.2, respectively.

The parent ion (M+1, m/z 353.2) of PGE_2 undergoes the addition of a Na^+ ion to yield an additive peak at m/z 375.2. Ions at m/z 291.2 (2-(Z)-hex-2-enyl)-4-hydroxy-3-((1E,3E)-octa-1,3-dienyl)cyclopentanone) would indicate the loss of –COOH group from ion (7-((1R,2R,3R)-3-hydroxy-2-((1E,3E)-octa-1,3-dienyl)-5-oxocyclopentyl) hept-5-enoic acid (M+1, m/z 335.2). The

mass spectral pattern agreed well with that of authentic standard of PGE_2. The daughter ions of PGE_2 at m/z 291.2 and 305.2 as shown in the **Fig. 6C.5A** are due to (M+1) signals of (2R,3R,4R)-2-((Z)-hex-2-enyl)-4-hydroxy-3-((1E,3E)-octa-1,3-dienyl) cyclopentanone and (2R,3R,4R)-2-((Z)-hept-2-enyl)-4-hydroxy-3-((1E,3E)-octa-1,3-dienyl) cyclopentanone, respectively. Other PGE_2 daughter ions viz.,(5Z)-7-((1R,2R,3R)-3-hydroxy-2-((1E,3E)-octa-1,3-dienyl)-5-oxocyclopentyl)hept-5-enoic acid (M+1, m/z 335.2) and (4S,5R)-5-((Z)-hex-2-enyl)-4-((1E,3E)-octa-1,3-dienyl)cyclopent-2-enone (M+1, m/z 317.2) are also apparent due to the ESI cleavage of neutral loss of uncharged molecule of water. Likewise the daughter ions of $PGF_{2\alpha}$ at m/z 337.2 and 319.2 resulted from the loss of water molecules are due to (M+1) signals of (5Z)-7-((1R,2R,3R)-3,5-dihydroxy-2-((1E,3E)-octa-1,3-dienyl)cyclopentyl) hept-5-enoicacid and(5Z)-7-((1R,5S)-2-hydroxy-5-((1E,3E)-octa-1,3-dienyl) cyclopent-3-enyl)hept-5-enoic acid, respectively resulted from ESI cleavage of parent ion. The PGE_2 daughter ion at m/z 293.3 identified as(1R,4R,5R)-4-((Z)-hex-2-enyl)-5-((1E,3E)-octa-1,3- dienyl)cyclopentane-1,3-diol (M+1, m/z 293.3) as shown in the **Fig. 6C.5A** resulted from the ESI cleavage of CO_2 from (5Z)-7-((1R,2R,3R)-3,5-dihydroxy-2-((1E,3E)-octa-1,3-dienyl) cyclopentyl) hept-5-enoic acid (M+1, m/z 337.2). Further loss of a water molecule from (1R,4R,5R)-4-((Z)-hex-2-enyl)-5-((1E,3E)-octa-1,3-dienyl) cyclopentane-1,3-diol (M+1, m/z 293.3) resulted in (4S,5R)-5-((Z)-hex-2-enyl)-4-((1E,3E)-octa-1,3-dienyl)cyclopent-2-enol (M+1, m/z 275.5), which eliminates another molecule of water to yield (S)-1-((Z)-hex-2-enyl)-5-((1E,3E)-octa-1,3-dienyl) cyclopenta-1,3-diene (M+1, m/z 257.2), which is a characteristic fragment of $PGF_{2\alpha}$, where the proposed fragmentation is shown in **Fig. 6C.5B**. The data have been compared with authentic PG standards.


A
Intensity (X 10^5)
PGE2 parent (M+1) ion peak
PGF2-α parent (M+1) ion peak
PGF2-α daughter ion peaks
m/z: 257.2
m/z: 275.2
m/z: 293.3
PGF2-α daughter ion peak
PGE2 daughter ion peak
m/z: 291.2
m/z: 305.2
PGE2 daughter ion peak
+MS, 13.8-14.3min #(905-933)

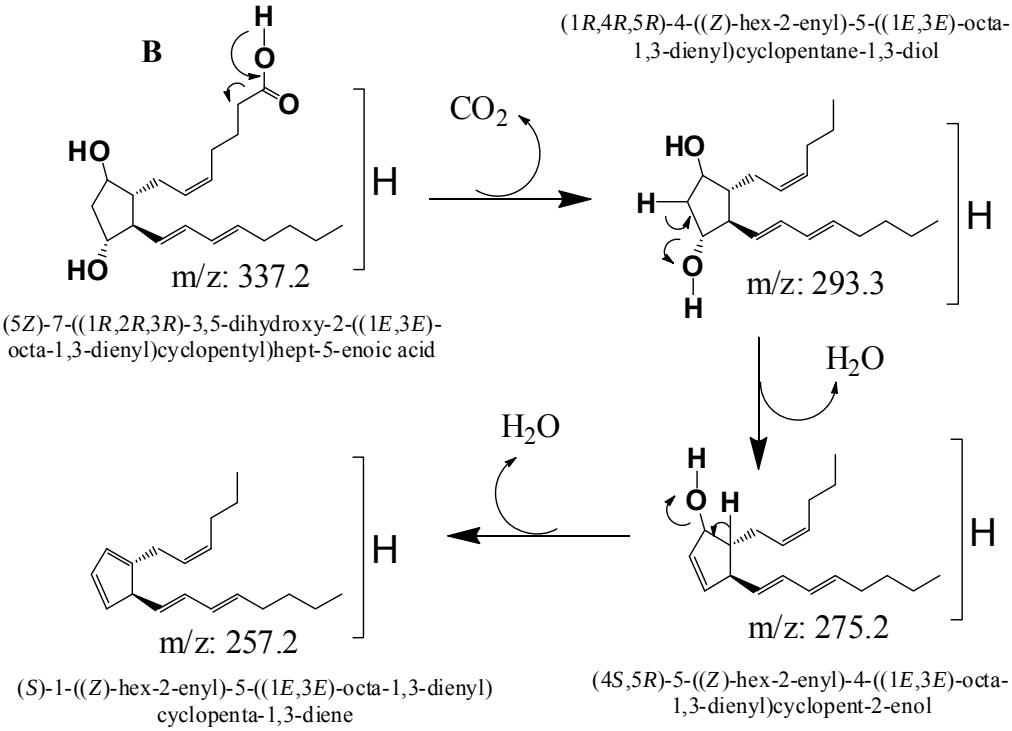


Fig. 6C.5 **(A)** Positive mode electrospray ionization mass (ESI-MS, $M+H^+$) spectra of PGE_2 and $PGF_{2\alpha}$ parent and daughter ions as recorded on a Bruker Daltonic (GmbH, Germany) micro-TOF series with ESI-MS and an Agilent HPLC fitted with G1312 binary gradient pump. **(B)** Schematic representation of the signature LC-MS fragmentation pattern of parent and daughter ions of PGE_2 and $PGF_{2\alpha}$.

6C.2.5 NMR analyses of prostaglandins PGE_2 and $PGF_{2\alpha}$

Proton NMR (^{1}H NMR) has been carried out for the purified PG mix to further validate the results obtained by understand the component intensities and ratios in GME_0 (not fortified with antioxidant additives) and GME_{1-4} (fortified with antioxidant additives) after 90 days of accelerated shelf-life study **(Fig.6A.6)**. Since the spectra is a mixture of different PGs, we have found it difficult to analyse some multiplets due to their complexity to

interpret (due to the strong coupling) and peak overlap as a result of natural line width limitation imposed by the T2 (spin-spin relaxation), which prevents valuable information to be detected in the multiplets. Large number of transitions in ^{1}H NMR spectra in the mixture of PGs was made simplified by the peak analysis algorithm, Global Spectral Deconvolution (GSD), which applies a deconvolution of the whole spectrum thereby making us to work out the signature peaks in an effective manner. ^{1}H NMR has been used to further validate the results obtained by HPLC to understand and compare the signal pattern and intensity of the control samples of *P. viridis* (GME_0) vis-à-vis the samples fortified with antioxidant additives (GME_2) after a 90 days of accelerated shelf-life study. The NMR spectrum (Fig.6C.6A) has been obtained from the purified PGs of GME_2, whereas (Fig.6C.6B) has been derived from the purified PGs of GME_0. The signal at δ4.6 ppm, which is the signature peak of –OH groups, was integrated to result number of protons as 4.92 in the sample GME_0 as compared to 1.56 proton integer in GME_2. This result apparently indicates the lower concentration of PGs (4 times) in GME_2 than GME_0. HPLC data validate the observation as $PGF_{2\alpha}$ recorded to be present at 810 ppm in GME_0 and 292 ppm in GME_2 (about 4 times less than in control) after 90 days of accelerated shelf-life study. In particular $PGF_{2\alpha}$ possess three –OH groups as compared to PGE_2 (with two –OH groups), and a higher peak intensity at δ 4.6 ppm with respect to GME_0 apparently indicates the presence of the former ($PGF_{2\alpha}$) PGs in a higher quantitities in GME_0 than recorded in GME_2. The ^{1}H NMR data presented here give indicative information regarding the presence of characteristic signature peaks of PGE_2 and $PGF_{2\alpha}$.

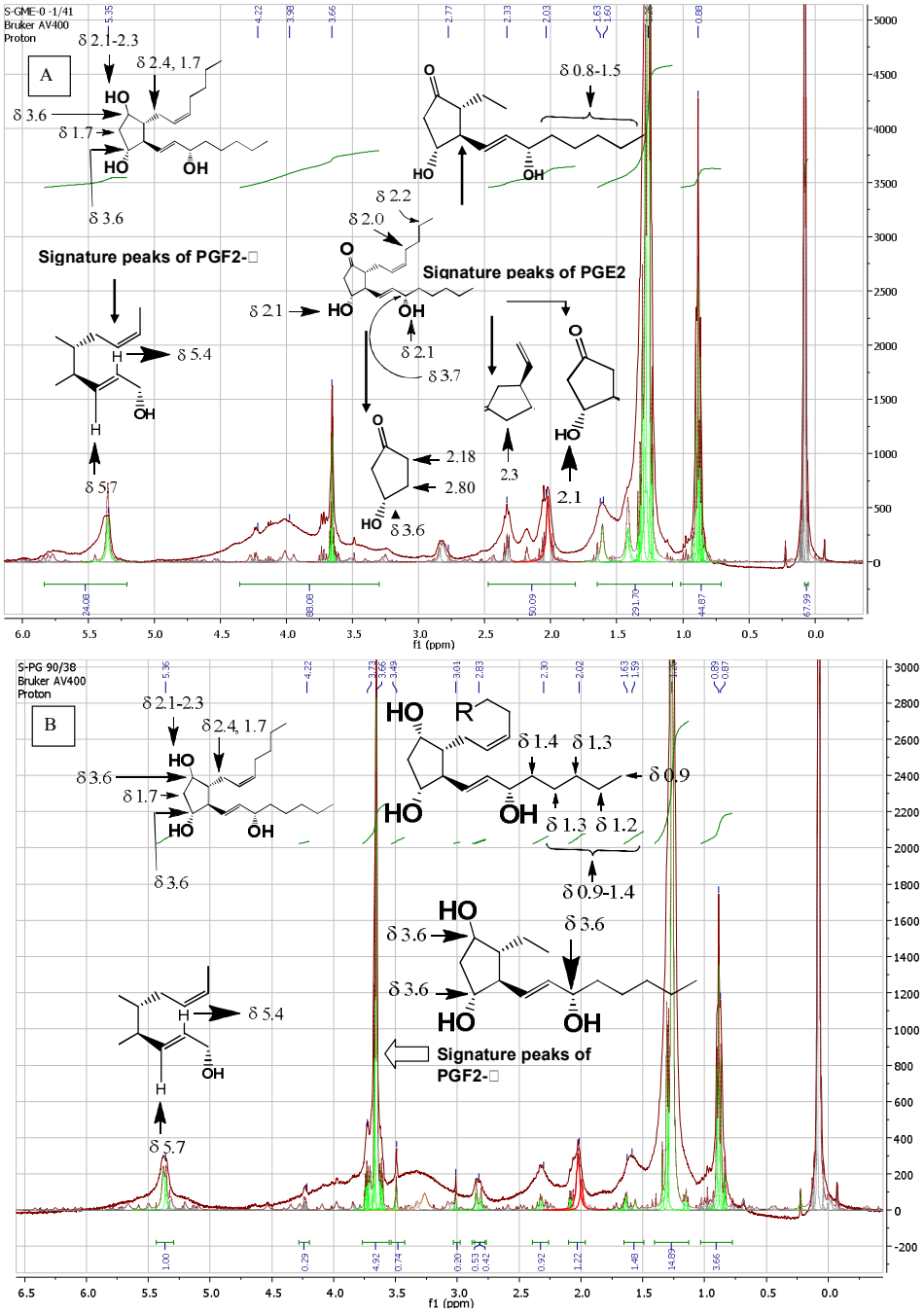


Fig.6C.6 H NMR spectra of (A) PGE_2 and (B) $PGF_{2\alpha}$ as recorded in $CDCl_3$ on a Bruker DRX NMR spectrometer operating at 400 MHz for ^1H NMR. Chemical shift (δ) values are expressed in parts per million (ppm) and are referenced to the residual solvent signals of $CDCl_3$.

The methine signal (CH) at δ 2.7 ppm belongs to the cyclopentanone moiety of PGE_2 and the downfield shift of the CH proton was found to be due to the proximity of 1 alpha -C=C from CH group under consideration. The other CH group of cyclopentanone appeared at δ 2.12 ppm. One of the protons of the methylene group (CH_2) of the cyclopentanone moiety appeared at δ 2.34. The downfield shift of the proton is due to the proximity of >C=O group. The methine signals at δ CH 3.9 ppm and 3.8 ppm appeared downfield. The CH signal at δ 3.8 ppm is due to propan-2-ol moiety of the side chain (non-2-en-4-ol) of PGE_2. The downfileld shift appeared to be due to 1α-O from -CH and 1β-C=C from -CH group. Likewise the downfield shift of the methine (CH, δ 3.8) proton of cyclopentanone moiety is due to 1α-C=C and 1α-O. The olefinic protons appeared in the range between δ 5.4-5.7 ppm and apparently reside at the side chains of PGE_2, *viz*., oct-5-enoic acid and non-2-en-4-ol. The downfield shift of the olefinic protons was found to be due to 1-C and 1-C-O gem gem groups of PGE_2. The methylene signals at δ 3.3 and 2.1 ppm of the 5-octenoate moiety of PGE_2 were shifted downfield due to the proximity of the electron withdrawing effects of the groups 1α-C=C and 1β-C (=O)-C. The CH_2 groups of 5-octenoate moiety flanked between carboxylate and olefinic groups of PGE_2 appeared downfield at δ 1.7-2.2 ppm. Also the signals at δ1-1.4 ppm are due to the – CH_2 and –CH_3 signals of non-2-en-4-ol moiety.

The ^1H NMR spectrum of PGs showed a terminal methyl signal at δ 0.89 ppm (t), one carbinol methine at δ 4.09 ppm (m), three sets of double bound at δ 5.40, 5.43, 5.61, 5.70 ppm, and a methylene adjacent to a carboxyl group at δ 2.32 ppm. The values of 5, 6, and 16 Hz coupling constants are typical of the δ 5(Z) and δ 13(E) olefin functionalities. A set of double bound at δ 6.18, 7.50

ppm suggested the presence of α, β-unsaturated cyclopentenone. These features were quite similar to those of PGE_2 ester. The spectrum also showed signals of four coumarin protons at δ 6.33, 6.84, 6.99, 7.42 ppm and a methoxy group at δ 3.66 ppm (s). The ^1H NMR spectra of other compounds contained the described signals of the methyl methoxy coumarin group and signals similar to ^1H NMR shifts of PG esters published elsewhere (Mikolajczyk *et al*., 2000).

The characteristic ^1H NMR signals of $PGF_{2\alpha}$ resemble to a large extent to that of PGE_2. However, the peak intensities (integer values) of the signature peaks of PGE_2 and $PGF_{2\alpha}$ are higher than expected apparently due to the addition of these two PG components. The only difference of PGE_2 and $PGF_{2\alpha}$ are with an extra –OH group in the cyclopentane moiety (as cyclopentane-1, 3-diol) of the former ($PGF_{2\alpha}$), which result in the additional signals of a –CH group attached with a –OH. The CH and CH_2 signals of the cyclopentane-1, 3-diol moiety in $PGF_{2\alpha}$ appeared slight upfield than in PGE_2 apparently due to the substitution of one >C=O with –OH group in $PGF_{2\alpha}$. For example, the –CH peaks appeared at δ 1.7 and 2.3 ppm, which are not the part of isopropanol part of cyclopentane-1, 3-diol ring system unlike δ2.1 and 2.8 ppm with respect to 3-hydroxycyclopentanone ring system of PGE_2. Likewise, the –CH groups, which are part of propan-2-ol system of 3-hydroxycyclopentanone ring system appeared upfield (than in PGE_2) at δ 3.3 ppm due to the reason stated earlier.

6C.3 Conclusion

The present study provides the effects of various proportions of natural antioxidant additives *viz*., *Rosmarinus officinalis* (ROO) and *Curcuma longa*

oleoresins (CLO) in four different proportions as GME_1, GME_2, GME_3 and GME_4 on pro-inflammatory PGE_2 and $PGF_{2\alpha}$ in an accelerated shelf-life study of 90 days. Among the four combinations, GME_2 and GME_4 were found to effectively arrest the activity of pro-inflammatory COX-2 for a longer time period on shelf as compared to GME_1, GME_3 and GME_0. A threshold level of CLO:ROO of 0.8%:0.4% in GME_3 was found to be effective for optimum antioxidant activity. GME_2 and GME_4 were able to effectively protect C20:5*n*-3 and C22:6*n*-3 from oxidative degradation even after 90 days (d=90) of accelerated storage as compared to that in GME_0, which realized a significant reduction in the content of these fatty acids. The phenolic compounds with multiple centers of unsaturation in the antioxidant additives used to prepare the formulation intercepted the free radical chain reaction on unsaturated fatty acids during accelerated storage resulting in the storage stability of the long chain C20-22*n*-3 PUFAs. The concentrations of pro-inflammatory PGE_2 and $PGF_{2\alpha}$ after an accelerated shelf-life study of 90 days were significantly higher ($P < 0.05$) in GME_0 than among GME_2 and GME_4 due to the presence of phenolic antoxidative components present in the additives. The study also highlights the utility of measuring PGE_2 and $PGF_{2\alpha}$ as an index of antioxidant status and lipid peroxidation. The results obtained by HPLC were further validated using tandem LC/MS/MS and ^{1}H NMR analyses to understand and compare the signal pattern and intensity of the PG in GME_0 *vis-à-vis* the samples fortified with antioxidant additives after 90 days of accelerated shelf-life study.

6.D Toxicity profile of a nutraceutical formulation derived from green mussel *Perna viridis*

Background of the study

Bivalves are considered vital, next to fish and prawns from the nutritive point of view. Bivalve mollusks were reported to contain bioactive lipids, which include fatty acids; sphingolipids, phytosterols, diacylglycerols, etc. and many of these can influence human health and disease linked to alleviating the symptoms of inflammatory conditions (Li and Sinclair, 2007). The green mussel *Perna viridis* (family: Mytilidae) is a bivalve mollusk native of the Indian coast and throughout the Indo-Pacific and Asia-Pacific (Benson *et al*., 2001). It forms a significant fishery and contributes nearly 50% to the total bivalve production of the area (Laxmilatha *et al*., 2011)

There are several drugs like NSAIDs (aceclophenac, diclophenac etc.), steroids (glucocorticoid) and DMARDs (methotrexate, cyclosporin A), coxibs (celecoxib, rofecoxib) for managing moderate to severe cases of arthritic pain, stiffness and inflammation (Quan *et a*l., 2008). However, the side effects of these drugs are often deleterious, which includes gastrointestinal ulcers, cardiovascular diseases, and reported toxic effects on the vital organs in the body (Schnitzer *et al*., 1999).

The *in vitro* and *in vivo* anti-inflammatory studies of the green mussel derived nutraceutical formulation showed that the green mussel *Perna viridis* contains anti-inflammatory ingredients, which can be useful against inflammatory pain. With the interesting pharmacological properties of the said formulation, it has become imperative that the anti-inflammatory preparation be evaluated for its toxicity profile. As a part of the safety evaluation of this nutraceutical formulation, the present study was carried out to determine the

changes in body weight, food and water consumption, hematological parameters, serum biochemistry and histopathological changes as indices of toxicosis with the aim to provide guidance for selecting a safe dose of its use. The acute oral toxicity study in 14-days were carried out at a very high dose, whereas the repeated dose 90-day oral toxicity study was performed to establish the no-observed-adverse-effect level (NOAEL) of the formulation as parts of a safety assessment according to the internationally accepted guidelines.

6D.1. Materials and methods

6D.1.1 Animals

The short-term and sub-chronic toxicity studies were conducted in adult Wistar rats (both males and females) which purchased from Sri Venkateshwara Enterprises, Bangalore. They were housed in well ventilated polypropylene cages under controlled temperature (22-25^{o}C), pressure, relative humidity (60-80%), light-dark cycle of 12 h and provided with rat feed (Sai Durga Feeds and Foods, Bangalore, India) and water *ad libitum*. The age of the animal was approximately 7 - 8 weeks with body weights (BW) in the range of 200-250 g. All animal experiments were conducted after getting prior permission from the Institutional Animal Ethics Committee and as per the instructions prescribed by the Committee for the Purpose of Control of Supervision of Experiments on Animal (CPCSEA), Ministry of Environment and Forest, Government of India.

6D.1.2 Test article and evaluation of anti-inflammatory activities

The test article (GME_2) is a nutraceutical formulation prepared from the green mussels (*Perna viridis*) and the detailed collection of the raw material, processing, method(s) used to assure stability under storage

conditions, chemical analysis demonstrating the composition of the material have been described section 6A.1.

6D.1.3 Lethal dose 50 (LD_{50}) of green mussel formulation

Fifteen animals were divided randomly into three groups containing 5 numbers each. After being fasted for 16 h, the animals were administered with different doses of green mussel formulation suspended in distilled water (5000, 2500 and 1500 mg/kg BW), and administered as a single dose through oral gavage to be monitored for 14 days for mortality, clinical and behavioral symptoms, and any adverse reaction.

6D.1.4 Acute oral toxicity study of green mussel formulation

Forty animals (20 males and 20 females) were divided into 4 groups, each consisting of 5 male and 5 female rats, and three doses (2.5, 1.0 & 0.5 g/kg) of the green mussel formulation were administered orally (once daily) for 14 days. The control received 1 mL of water as vehicle every day. The animals were monitored for mortality, clinical and any adverse reaction of the test material. The body weight and food consumption were determined on every third day. After 14 days, the animals were sacrificed under mild ether anesthesia and the blood was collected by direct heart puncture method. Necropsy was performed and observations were recorded. Selected organs such as the liver, kidney, brain and spleen were dissected out and weights were recorded and histopathological analyses were performed.

6D.1.5 Sub-chronic oral toxicity study of green mussel formulation

Forty animals (20 males and 20 females) were divided into 4 groups, each consisting of 5 male and 5 female rats, and three doses (2.0, 1.0 & 0.5 g/kg) of the green mussel formulation (1 g suspended in 6 mL double distilled water) were administered orally (once daily) for 90 days (Ezeja *et al.*, 2014).

The control received 1 mL of water as vehicle every day. The test animals were monitored, during this period for any type of clinical symptoms, mortality, and adverse reaction. The body weight and food consumption were determined every seven days. On the 91st day, the animals were sacrificed under mild ether anesthesia. Blood was collected by direct heart puncture method. Necropsy was performed and observations were recorded. Selected organs such as the brain, kidney, liver and spleen were dissected out, weights were recorded and histopathological analyses were performed.

6D.1.6. Hematology and clinical chemistry parameters

Blood collected in EDTA tubes were analyzed for hematological parameters (Chiranthanut *et al*., 2013). Red blood cell (RBC), total white blood cell count (WBC), platelet count and hemoglobin (HGB) using a hematology analyzer (Model-Diatron, Wein, Austria). Total white blood cells were measured after diluting the blood in Turk's fluid and counting them using a hemocytometer (Nelson *et al*., 1984). For differential counts (lymphocytes, eosinophills, neutrophills) blood was spread on a clean slide, treated with Leishman's stain before being counted manually with a microscope (100 X) (Pal and Parvati, 2003).

A part of the blood was collected in non-heparinized tubes and serum was separated after centrifugation at 5000 rpm for 10 min which was used for the following investigations: serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) were assayed according to the method described by Bergmeyer *et al*. (Bergmeyer *et al*., 1976). Alkaline phosphatase (ALP) was estimated by p-nitrophenyl phosphate (PNPP) hydrolysis (McComb and Bowers, 1979). Total bilirubin was determined by Jendrassik-Diazotized sulphanilic acid method (Jendrassik and Grof, 1938). The total protein concentration was determined by biuret method (Nelson and

Morrie, 1984). Albumin was determined based on its reaction with bromocresol green. Markers of kidney function such as creatinine and blood urea nitrogen were estimated by Jaffe-Kinetic and urease method respectively (Pearlman and Lee, 1974). Serum sodium, potassium and bicarbonate were estimated using flame photometer 129 ion selective electrolyte analyzer. Chloride was estimated by mercurous thiocyanate method using a kit from Raichem Lifesciences Pvt Ltd, India. Total cholesterol was estimated by CHOD–PAP (cholesterol oxidase–phenol + aminophenazone) enzymatic method (Jaffe, 2003), triglyceride was estimated by GPO–PAP (glycerol-3-phosphate oxidase–phenol + aminophenazone) method (Cole *et al*., 1997), and high-density lipoprotein (HDL) was determined after precipitation with phosphotungistic acid. Very low-density lipoprotein (VLDL) was estimated by the Friedewald equation (VLDL = triglyceride/5) and low-density lipoprotein (LDL) by calculation: LDL = total cholesterol–(HDL + VLDL) (Ezeja *et al*., 2014).

6D.1.7 Histopathological analysis

A portion of the selected organs (brain, kidney, liver and spleen) of control and treated group (high dose groups) were fixed in 10% neutral buffered formalin. Embedded organs tissue samples were cut into slices of 2–4 µm and stained with hematoxylin-eosin and the sections were observed under light microscope (40X).

6D.1.8 Statistical analysis

Statistical evaluation was carried out with the Statistical Program for Social Sciences 13.0 (SPSS Inc, Chicago, USA, ver. 13.0). Analyses were carried out in triplicate, and the means of all parameters were examined for significance by analysis of variance (ANOVA). The values were compared with that of untreated control animals.

6D.2 Results and discussion

The active principles in the formulation derived from green mussel *P. viridis* were competitively inhibited inflammatory cycloxygenases (COX-1/COX-2) and lipoxygenase (5-LOX) in an inflammation and oxidative stress reaction, resulting in decreased production of pro-inflammatory prostaglandins and leukotrienes. Many of the allopathic prescriptions include NSAIDs and cyclooxygenase inhibitors used in controlling arthritic conditions have known side effects, especially with long term usage. About 25% of the users experience some kind of side effect and 5% develop serious health consequences such as stomach bleeding, stroke, and acute renal failure. The green mussel formulation proved to be a safer and effective alternative to these synthetic NSAIDs and other products available in the market.

As it is has been observed to be slow acting, long-term use of the green mussel formulation may be required in treatments of arthritis related diseases. In this aspect, long term toxicity studies of the green mussel formulation are of vital importance for the assessment of its safety in mammalian systems. In order to provide safety evidence for the green mussel formulation as a prospective nutraceutical medication for joint pain and arthritis, short-term and sub-chronic toxicity studies were conducted on the rats to evaluate the possible toxicity.

6D.2.1 LD_{50} of green mussel formulation

The single dose administration of the green mussel formulation up to a concentration of 5000 mg/kg BW did not produced any mortality after 14 days of observation, which indicates that the mean lethal dose (LD_{50}) of the formulation is greater than 5000 mg/kg BW. The oral toxicity of this formulation can be classified in category 5 (the lethal acute toxicity is greater than 5000 mg/kg) according to the Globally Harmonized Classification

System of OECD (Betti *et al*., 2012). According to the classification of Loomis and Hayes (Hayes, 1996), *viz.*, a chemical substance with an LD_{50} within the range of 5000–15,000 mg/kg is considered as practically non-toxic. The calculated LD_{50} for the green mussel formulation found in this range, and therefore, this nutraceutical formulation should be regarded as practically non-toxic in acute ingestion. The green mussel formulation too did not cause any toxic symptoms, behavioral changes or mortality when acutely administered at 5000 mg/kg to rats, and therefore, this formulation can be included under category 5 (low or no toxicity) in accordance with OECD guidelines.

6D.2.2 Acute toxicity study of green mussel formulation

Under various regulatory guidelines, changes in body weight have been used as an integral part of the conventional safety evaluation of test materials, drugs and chemicals (Schilter *et al*., 2003; Hilaly *et al*., 2004). No treatment related signs of mortality were observed in the animals over short-term administration (maximum dose of 2500 mg/kg BW). In addition, the administration of the green mussel formulation at different doses did not produce any treatment-related changes in the body weight of the animals and any differences in the food consumption of male and female rats when compared to controls. This indicates that the food conversion rate was not affected and growth of rats in treatment groups was comparable to that of control. Since, no significant changes were observed in the general behavior, body weight, food and water intake of rats in the treated groups as compared to the untreated control after the administration of the green mussel formulation during the short-term toxicity study spanning over 90-day period; it could be concluded that oral administration of this test material had no effect on the normal growth of rats on the concentration studied.

No significant changes were noticed during necropsy and there was no change in the organ weight. Organ weights are widely accepted in the evaluation of toxicity related studies (Wooley, 2008). No significant differences were recorded in the relative weights of kidney, brain, spleen and liver indicating that the acute oral administration of the green mussel formulation did not detrimentally affect the wet weight, organ-to-body weight ratio and the color of the organs.

The haematopoietic system is one of the most sensitive targets of toxic compounds and is an important index to assess the toxicity of the test material on the physiological and pathological status in human and animals (Mukinda *et al*., 2007). No treatment-related biologically significant effects of the green mussel formulation treatment at dose levels of 0.5-2.5g/kg in hematology parameters such as haemoglobin, RBC count, platelet count, total and differential leukocytes counts were apparent in both genders of rats when compared to untreated animals. After 90 days, there were no treatment-related changes in hematological parameters between the untreated and the green mussel formulation treated groups indicating that the test material had no effects on the circulating blood cells**,** nor it interfered with their production. Some statistically insignificant differences were noted in WBC and differential counts when the control and treatment groups were compared. However, these changes did not appear to be related to the test article treatment since they were still within the limits of normal biological variation. The changes in WBC counts were probably due to the normal responses to foreign bodies or stress associated with the toxicity studies. Decreased hemoglobin and differential counts were found previously in the rats fed with polyunsaturated fatty acids, such as *n*-6 fatty acid arachidonic acid and *n*-3 fatty acid docosahexaenoic acid containing oils (Lina *et al*., 2006). However, in the

present study, no significant changes in these parameters were apparent. Taken together, the normal range of hematological indicators indicated the absence of hematotoxic potential of the green mussel formulation

Biochemical determinations of blood parameters in serum serve as an indicator of toxicity of a test material (Schilter *et al*., 2003). The enzymes *viz.,* serum aspartate transaminase (AST) or serum glutamic oxaloacetic transaminase (SGOT), serum alanine transaminase (ALT) or serum glutamic pyruvic transaminase (SGPT), and alkaline phosphatase (ALP) are well-known enzymes used as good indicators of liver function and as major markers of hepatic injury (Hilaly *et al*., 2004). In general, aminotransferases AST and ALT are normally contained within liver cells, and their activity in the blood is generally low. If the liver is damaged, these enzymes diffuse across the damaged cell membrane due to altered plasma membrane permeability (Upur *et al*., 2009), before being entered into the circulation, raising the enzyme levels in the blood and signaling liver disease.The green mussel formulation up to a concentration of 2.5g/kg did not produce any change in the hepatic function parameters in serum such as SGOT, SGPT and ALP, These results suggest that the test material did not alter the hepatic function and prevent hepatocyte enzyme go into the blood. No significant alterations in ALP in the animal subjects treated with the green mussel formulation are indicative of no liver injury (Antonelli-Ushirobira *et al*., 2010). Other liver enzyme activities too realized no significant decrease thereby suggesting that the acute administration of the test material did not alter the hepatocytes and consequently the metabolism of the rats.

Albumin is synthesized by the hepatocytes, and as such, it represents a major synthetic plasma protein, and its determination can act as a criterion for

assessing the synthetic capacity of the liver (Muhammed *et al*., 2011). Decrease in plasma proteins therefore tends to reflect chronic damage. The common pattern seen following significant hepatocellular damage is a reduction in albumin accompanied by a relative increase in globulins, which leads to A/G ratio reduction (Woodman, 1996). Serum urea and creatinine are known as the usual markers of renal function (Muhammed *et al*., 2011). Any rise in their levels is only observed if there is marked damage to functional nephrons (Lameire *et al*., 2005). The renal function tests such as blood urea and serum creatinine did not show any variation when compared to controls. There was also no change in serum electrolytes sodium, potassium, chloride and bicarbonate indicating that the green mussel formulation did not produce any change in renal function.

Acute toxicity study of the green mussel formulation did not showed any change in cholesterol, triglycerides, HDL, LDL, VLDL cholesterol levels. No significant changes were observed in cholesterol, LDL, and VLDL levels suggestive that the green mussel formulation had no effects on the lipid and carbohydrate metabolism of the rats. The liver is the site of cholesterol disposal, synthesis, glucose synthesis and generates free glucose into the blood from hepatic glycogen stores (Kaplan *et al*., 1995). In the present study, lowering of triglycerides was observed, which was not significant.

Histopathological analysis of the brain, spleen, kidney and liver did not show any pathological lesions in the organs of animals treated with the green mussel formulation suggesting that daily oral administration of the green mussel formulation for 90 days caused no detrimental changes or morphological disturbances.

The above observations concluded that the green mussel formulation did not produce any toxicity to wistar rats when administered for two weeks.

6D.2.3 Sub-chronic toxicity study of green mussel formulation

6D.2.3.1 General conditions and behavior

No treatment related signs of mortality were observed in the animals over the administration periods (maximum dose of 2000 mg/kg BW). The animal behavior, feed intake, and the normal body weight changes were not altered during the short-term and sub-chronic toxicity studies. In addition, the administration of the green mussel formulation at different doses did not produce any treatment-related changes in clinical signs such as mental state, external appearance and daily activities among the test groups when compared with the control. Any abnormal behavior or cases of diarrhea and soft feces were not observed during the period of study. No ophthalmological abnormalities were observed in any of the treatment groups prior to study initiation and near experimental completion. In general, the experimental animals from all treatment groups appeared healthy at the conclusion of the study period, and did not induce any clinical signs of toxicity in sub-chronic regimens.

6D.2.3.2 Body weight

The administration of the green mussel formulation during 90 days of long-term sub-chronic toxicity studies did not produce any abnormal change in the body weight of male and female rats when compared to the control (**Table 6D.1**). As expected, rats gained weight with time. In male rats, the gain in mean body weights for the treated groups at 0.5-2.0 g/kg BW was comparable with those in the control group throughout the study. Similarly, the mean body weights

of the treated female rats were comparable with those in the control group throughout the study. Overall, there were no changes in body weight or body weight gain in the animals attributable to the administration of the green mussel formulation than that of the control group. Any changes observed were sporadic and considered incidental, unassociated with test article administration.

Table 6D.1 Body weight, food and water consumption during sub-chronic (90 days) toxicity studies after the administration of green mussel formulation

	Male				Female			
Days	Control[a]	2.0 g/kg[b]	1.0 g/kg[b]	0.50 g/kg[b]	Control[a]	2.0 g/kg[b]	1.0 g/kg[b]	0.50 g/kg[b]
Body weight (g)								
0	230.8±1.4	254.9±2.5	263.6±14.2	284.4±0.5	185.8±1.5	218.5±15.6	195.8±1.4	190.9±5.2
14	239.6±4.5	261.8±1.5	270.4±2.2	290.1±1.4	192.4±2.4	224.4±12.5	202.1±2.6	196.8±6.2
42	252.4±6.6	273.9±6.5	281±18.6	300±1.5	204±2.6	232.6±11.6	212.1±3.2	206.9±2.6
70	263.8±8.5*	283.6±1.5*	290.2±19.5	308.4±1.6*	214.2±2.6*	240.9±11.4*	220.7±1.2*	216.2±2.4*
91	271.58±14.2*	290.8±1.6*	296.7±2.1*	314.5±1.2*	221.1±0.9*	246.6±0.5*	226.8±0.5*	222.5±2.9*
Food consumption (g)								
0	72.4±5.6	74.4±2.5	70.6±6.5	68.7±1.2	59.5±0.6	55.4±1.6	58±2.3	62.7±0.9
14	66.4±6.5	73.3±3.6	62.6±6.3	61.3±1.5	62.5±1.2	49.3±2.6	54.6±2.6	57.7±1.2
42	67.3±6.9	71.6±3.9	61.4±4.2	60.6±2.5	47.6±1.1	50.6±2.7	48.3±1.5	50±1.3
70	66.6±7.9	72.7±3.8	62.3±3.2	62.5±2.1	47.9±1.5	51.3±2.4	49±2.6	49.7±1.4
91	68.3±8.5	74.7±3.6	63.2±2.6	65.5±2	48.7±1.3	50.8±2.1	49.7±2.8	50±1.5
Water consumption (mL)								
0	100±4	90±2	100±2	110±3	100±2	80±2	110±4	90±2
14	90±3	70±5	110±3	100±1	80±4	80±1	70±5	90±2
42	90±2	110±3	90±1	100±2	100±3	80±4	90±1	90±3
70	110±3	110±1	100±5	100±3	100±2	100±1	90±2	110±3
91	100±4	100±2	110±1	100±4	100±1	110±2	100±2	100±3

*Data presented as mean± standard deviation (n = 5). Significantly different from control: $P < 0.05$.
[a]Control group received 1 mL distilled water.
[b]Sample group received three doses of green mussel formulation (2.0, 1.0 and 0.5 g/kg rat).

6D.2.3.3 Food and water consumption

The average food intake of male rats was nearly 66-80 g/day/cage and female was 51-62 g/day/cage. It was observed that average food intake of untreated control rats decreased from about 72 g to 68 g after 90 days study.

The same trend was observed for medium and low dose group (1.0 and 0.5 g/kg, respectively) of males and females. Administration of the green mussel formulation did not produce any significant difference in the food consumption of both genders of rats when compared to normal animals of the high dose group ($P > 0.05$) throughout the experimental period. The summarized food intake of the rats recorded after oral administration of the green mussel formulation to rats is shown in **Table 6D.1**. Similarly, the water consumption did not alter in male and female rats attributable to administration of the green mussel formulation when compared to normal animals during chronic and sub-chronic toxicity studies. Changes in water consumption during the treatment period are presented in **Table 6D.1**. Sporadic statistically significant changes in water consumption were considered spurious, unassociated with the test article administration.

6D.2.3.4 Relative organ weight

Fig.6D.1 presents the relative weights of the vital organs (in g) of rats (both genders). The weights of liver, kidney, spleen and brain recorded at the end of the sub-chronic study (day 91) did not show significant differences ($P > 0.05$) in any of the treatment groups compared with the control groups (**Fig.6D.1**). Furthermore, gross examination of the vital organs of all rats revealed no detectable abnormalities. The evaluation of the organs weights of treated rats group supported the safety of the green mussel formulation.

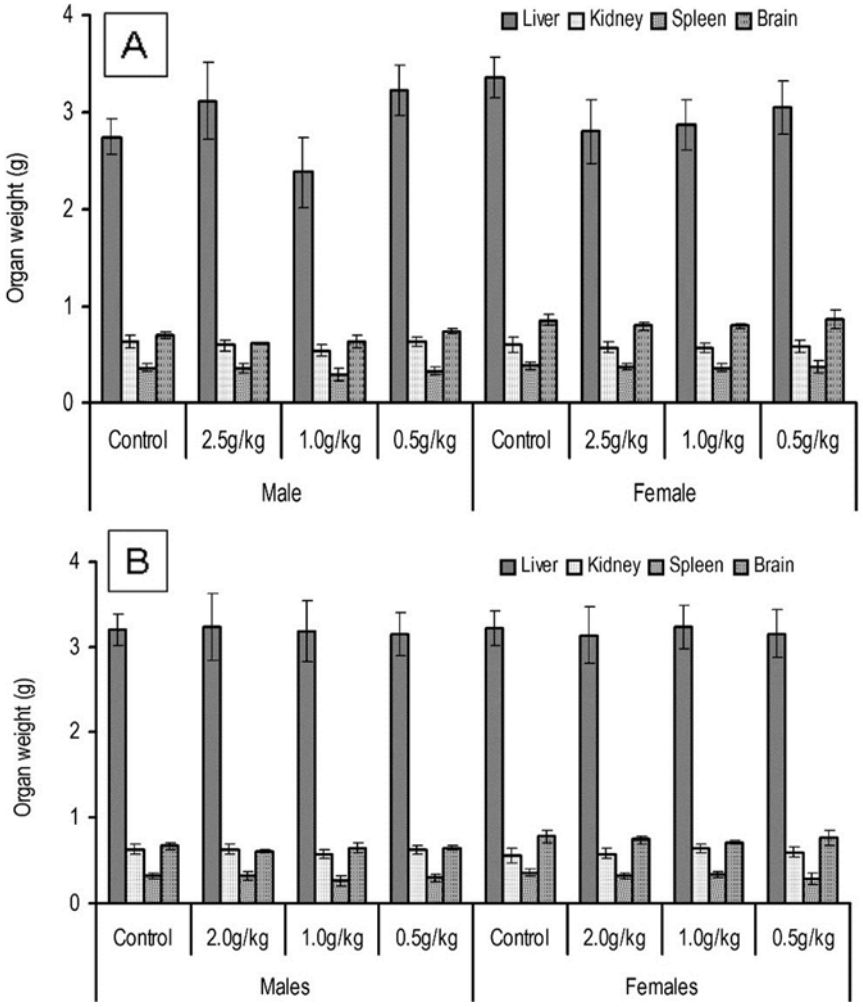


Fig.6D.1 Mean organ weights (in grams) of male and female rats administered green mussel formulation after (**A**) acute and (**B**) sub-chronic toxicity studies

6D.2.3.5 Hematological parameters

The effect of the green mussel formulation on haematological parameters such as haemoglobin, RBC and WBC count, platelet count and differential counts after sub-chronic toxicity (90 days) studies are presented in **Table 6D.2**. No treatment-related biologically significant effects of the green mussel formulation treatment at dose levels of 0.5-2.0g/kg in these haematology

parameters were apparent in both genders of rats when compared to untreated animals (**Table 6D.2**) ($P > 0.05$), and remained within physiological range throughout the treatment period (90 days). No test article-related changes in blood cell morphology were observed during the period of study.

Table 6D.2 Hematology analyses data of male and female rats administered with the green mussel formulation after sub-chronic (90 days) toxicity studies

Treatments		Lymphocytes (mm^3)	Eosinophills (mm^3)	Neutrophills (g/dL)	HGB (mm^3)	WBC (10^6/cmm)	RBC (10^5/cmm)
Male	Control[a]	5828.60± 159.14	998.20 ± 62.90	3213.20±17.13	15.44 ± 0.05	10040 ± 58.6	7.87 ± 0.07
	2.0g/kg[b]	4638.00±154.50	752.60±31.57	2329.40±83.04*	14.60 ± 0.64	7720 ±26.70	7.41 ± 0.29
	1.0g/kg[b]	3643.80±187.47*	618.60±33.24*	2017.60±88.02*	14.34 ± 0.73	6280 ±30.90	7.29 ± 0.58
	0.5g/kg[b]	4912.20±114.07	773.60±15.38	2754.20±78.98	14.64 ± 0.70	8440 ±174.70	7.27 ± 0.08
Female	Control[a]	5220.40± 231.0	888.80± 37.78	2730.80±80.31	13.38 ± 0.94	8840 ±148.9	6.37 ± 0.47
	2.0g/kg[b]	3856.40±167.93*	645.40±28.94	2298.20±114.25	14.12 ± 0.44	6800 ±97.7	6.97 ± 0.03
	1.0g/kg[b]	4975.20±269.33	770.00±31.75	1974.80± 83.87*	13.86 ± 0.83	7720±107.5	6.86 ± 0.21
	0.5g/kg[b]	2122.80±101.52*	321.20±14.67*	1096.00 ± 50.93*	13.54 ± 0.53	3540±101.7*	6.77 ± 0.07

*Data presented as mean± standard deviation (n = 5). Significantly different from control: $P < 0.05$.
[a]Control group received 1 mL distilled water.
[b]Sample group received three doses of green mussel formulation (2.0, 1.0 and 0.5 g/kg rat).
HGB: hemoglobin, WBC - total white blood cell count, RBC - red blood cell

6D.2.3.6 Serum biochemical parameters

Table 6D.3 summarizes the serum biochemical parameters used as the biomarkers of the liver and renal functioning, during the course of sub-chronic toxicity studies. The activity of the marker enzymes of the liver (SGOT, SGPT and ALP) were not significantly different ($P > 0.05$) in all dose groups of treated rats as compared to untreated control, and albumin/globulin (A/G) ratio was not altered in the treated animals of both genders (**Table 6D.3**). Sub-chronic oral administration of the green mussel formulation (for 90 days) did not cause any significant changes in hepatic function parameters such as total protein, albumin, total bilirubin and globulin in both sexes of rats during long term sub-chronic toxicity studies. However, no change in serum proteins and albumin were observed in the acute and sub-chronic studies, which show that the green mussel

formulation do not inhibit protein synthesis in the rats. This is supported by the microscopic examination showing the normal hepatocytes without any lesions in the liver. Bilirubin is a metabolic breakdown product of blood heme. Any course that might induce abnormally increased levels of bilirubin accumulating in human serum or plasma usually signify the presence of a variety of diseases with liver dysfunctions, ranging from jaundice to infectious hepatitis (Yao, 2004). Our results proved the report that long-term and high dose administration of the green mussel formulation did not significantly alter the bilirubin concentrations, which is an indication that it do not interfere with the metabolism of bilirubin in the liver. In liver, bilirubin also affects the protein synthesis. There was no obvious alteration of protein content in the liver at the experimental doses administered with the test material in comparison to the control group. It is of note that under normal circumstances, bilirubin-albumin conjugate protects the cells against the potential toxicity of bilirubin. Any imbalance in the formation of the conjugate resulting in the decreased protein content in the liver and increased total bilirubin in serum to have a detrimental effect leading to injury of the liver. Since, there was no adverse effect on plasma levels of total bilirubin, total protein and A/G ratio in the green mussel formulation treated animals when compared to control, it may be concluded that the test article did not alter the renal and hepatic function.

The renal function parameters such as serum creatinine and blood urea did not show any significant variation ($P > 0.05$) in treated animals compared to controls (**Table 6D.3**). There were no statistically significant differences in the levels of serum electrolytes such as chloride, potassium, sodium and bicarbonate after the treatment of green mussel formulation ($P > 0.05$), indicating no expressive changes in the general metabolism after consumption of the green mussel formulation by rats (**Table 6D.4**). The green mussel formulation did not produce any significant changes in the total cholesterol, HDL, LDL and VLDL, indicating no expressive changes in the general lipid metabolism after consumption of the test article by rats (**Table 6D.4**).

Table 6D.3: Serum biochemical analysis data of male and female rats administered with the green mussel formulation after sub-chronic (90 days) toxicity studies

		SGOT	SGPT	ALP	Bilirubin	Total protein	Albumin	Globulin	A/G ratio	Urea	Creatinine
		(U/L)	(U/L)	(U/L)	(mg/dL)	(g/dL)	(g/dL)	(g/dL)		(mg/dL)	(mg/dL)
Male	Control[a]	182.00±3.14	68.40±1.5	356.20±2.84	0.20±0.01	7.82 ±0.22	3.50±0.07	4.32 ±0.42	0.81	46.80 ± 0.96	0.68 ± 0.08
	2.0g/kg[b]	188.60±1.5	73.80±10.53	254.40± 3.18*	0.20 ±0.07	7.44±0.16	3.54 ±0.03	3.90 ±0.02	0.91	50.80 ± 0.15	0.62 ± 0.04
	1.0g/kg[b]	171.00±14.93	78.40±0.02	332.80±2.49	0.18 ±0.04	7.06±0.06	3.54 ±0.26	3.52 ±0.34	1.02	42.00 ± 0.54	0.62 ± 0.08
	0.50g/kg[b]	146.00±20.73*	68.80±0.42	254.40±2.44*	0.18 ±0.04	7.44±0.26	3.54 ±0.41	3.90 ±0.51	0.93	32.00 ± 0.87*	0.62 ± 0.13
Female	Control[a]	195.40±10.01	86.40±1.57	267.40±23	0.22 ±0.04	7.52±0.26	4.14 ±0.21	3.38 ±0.08	1.23	77.00 ± 5.94	0.78 ± 0.03
	2.0g/kg[b]	132.60±3.04*	63.40±1.99	340.20±4.94	0.16 ±0.05	7.22±0.21	3.94 ±0.27	3.28 ±0.3	1.20	49.00 ± 2.16*	0.56 ± 0.09
	1.0g/kg[b]	161.25±30.13	64.00 ±0.81	344.75±5.01	0.20 ±0.01	7.72±0.02	4.08 ±0.2	3.64 ±0.18	1.12	55.80 ±0.95	0.68 ± 0.04
	0.50g/kg[b]	145.20±10.96*	65.60±1.35	287.60±3.02	0.20±0.02	7.16±0.05	4.22 ±0.03	2.94±0.06	1.45	56.20 ± 0.03	0.68 ± 0.11

*Data presented as mean± standard deviation (n = 5). Significantly different from control: $P < 0.05$.
[a]Control group received 1 mL distilled water.
[b]Sample group received three doses of green mussel formulation (2.0, 1.0 and 0.5 g/kg rat).
SGOT - serum glutamic oxaloacetic transaminase, SGPT - serum glutamic pyruvic transaminase, ALP - alkaline phosphatase, A/G ratio – Albumin/ Globulin ratio

Table 6D.4: Serum biochemical analysis data of male and female rats administered with the green mussel formulation after sub-chronic (90 days) toxicity studies

		Na^+	K^+	Cl^+	HCO_3^+	Cholesterol	Triglycerides	HDL	LDL	VLDL
		(m.mol/L)	(m.mol/L)	(m.mol/L)	(m.mol/L)	(mg/dL)	(mg/dL)	(mg/dL)	(mg/dL)	(mg/dL)
Male	Control[a]	147.80 ± 0.66	5.50 ± 0.35	105.22 ± 0.47	25.80 ± 0.84	77.40 ± 0.19	137.50± 1.01	32.80 ± 0.42	18.60 ± 0.36	26.00 ± 0.89
	2.0g/kg[b]	145.72 ± 2.96	6.05 ± 0.96	104.88 ± 1.19	27.20 ± 1.09	74.20 ± 0.72	138.80± 1.8	34.60 ± 0.14	11.80 ± 0.21	27.80 ± 0.95
	1.0g/kg[b]	144.84 ± 0.34	6.24 ± 0.83	104.48 ± 0.80	26.40 ± 0.14	76.20 ± 0.73	152.80± 31.24	33.20 ± 0.30	13.75 ± 0.30	30.60 ± 0.06
	0.50g/kg[b]	146.06 ± 2.22	5.15 ± 0.22	104.48 ± 0.44	27.60 ± 0.89	75.20 ± 3.70	126.00± 0.22	34.80 ± 0.84	15.20 ± 2.68	25.20 ± 0.79
Female	Control[a]	139.98 ± 0.68	4.88 ± 0.10	101.32 ± 1.78	25.60 ± 1.14	75.60 ± 0.44	114.20± 01.94	32.20 ± 0.30	20.60 ± 6.87	22.80 ± 0.30
	2.0g/kg[b]	136.94 ± 0.73	5.03 ± 0.57	104.38 ± 0.79	27.40 ± 0.52	63.40 ± 3.36	123.40± 0.21	31.00 ± 0.71	7.80 ± 0.49	24.60 ± 0.89
	1.0g/kg[b]	138.18 ± 0.66	5.58 ± 0.61	103.54 ± 0.95	26.60 ± 0.89	75.20 ± 0.68	140.60± 0.35	32.80 ± 0.08	16.40 ± 0.28	26.00 ± 0.64
	0.50g/kg[b]	141.14 ± 0.59	5.85 ± 0.88	104.84 ± 0.83	27.20 ± 0.84	72.80 ± 0.03	145.60± 0.70	29.20 ± 1.64	14.40 ± 0.91	29.20 ± 0.15

Data presented as mean± standard deviation (n = 5).
[a]Control group received 1 mL distilled water.
[b]Sample group received three doses of green mussel formulation (2.0, 1.0 and 0.5 g/kg rat).
HDL - high-density lipoprotein, LDL - low-density lipoprotein, VLDL – very low-density lipoprotein

6D.2.3.7 Histopathological analysis

Necropsy of the treated animals after sacrifice did not show any morphological changes of the internal organs or any gross pathological abnormalities during sub-chronic toxicity studies. There were no macroscopic findings considered to be related to the treatment of the green mussel formulation (**Fig. 6D.2**).

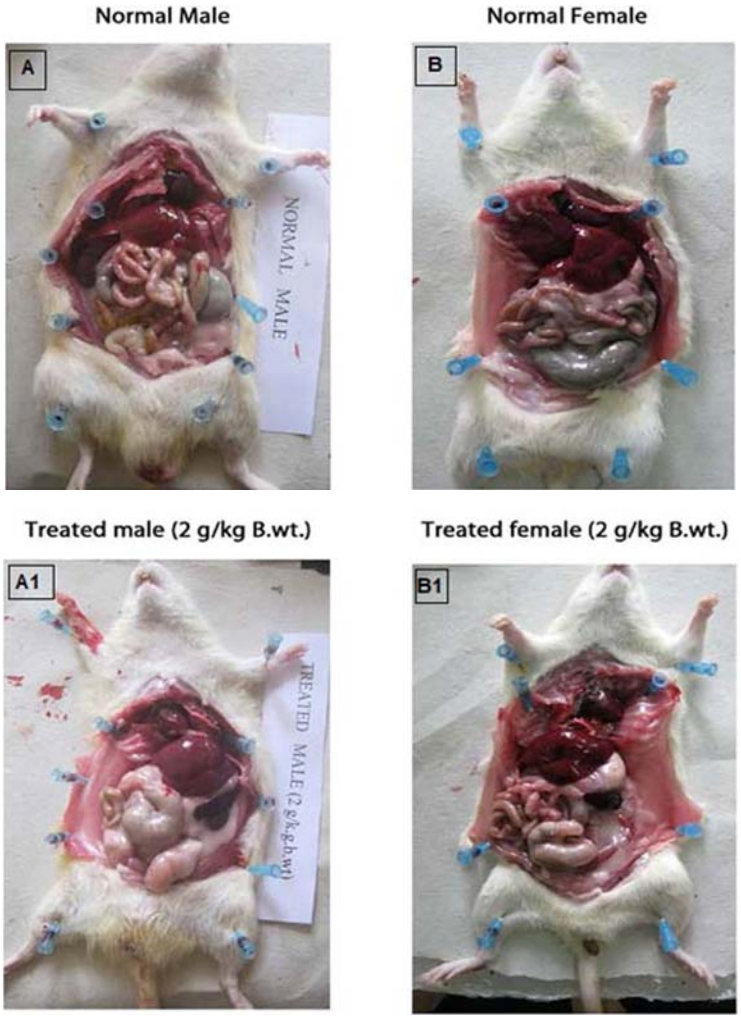


Fig. 6D.2 The cross section of the male and female rats after sub-chronic toxicity study of 90 days. (**A**) Normal male; (**A1**) Green mussel formulation (2.0g/kg) treated male; (**B**) Normal female (**B1**) Green mussel formulation (2.0 g/kg) treated female.

Gross examination of vital organs such as brain, kidney, spleen and liver of rats, and microscopic examination of tissue sections prepared from these organs did not observe any histopathological alteration in any treated rats during sub-chronic toxicity studies. The normal and treated sections of brain (**Fig. 6D.3**A & A1), kidney (**Fig. 6D.3**B & B1), liver (**Fig. 6D.4**A & A1), and spleen **(Fig. 6D.4**B & B1**)** showed normal appearance compared to control rats after sub-chronic toxicity study. The treated section of brain showed normal glial cells. Astrocytes, interstitial tissue of the brain and the portion of cerebellum also showed normal appearance compared to control rats (**Fig. 6D.3**A1). The treated section of the kidney showed normal glomeruli with normal Bowman's capsule. Glomeruli showed normal cellularity with renal tubules and interstitial tissue demonstrated the normal appearance (**Fig. 6D.3**B1). The section of liver tissue showed normal portal triads and biliary duct. A few lymphocytic collections were seen in the portal area, which was normal. Central venous systems also appeared normal. Hepatocytes showed normal morphology and they were arranged in cords. Sinusoidal space and Kupffer cells also appeared normal (**Fig. 6D.4**A1). The section of spleen showed normal lymphoid follicles with germinal centers. Sinusoidal spaces are dilated and they were lined by normal endothelial cells. Some areas showed hemorrhaged congestion with many siderophages (**Fig. 6D.4**B1). No other macroscopic or microscopic lesions in organs examined were observed.

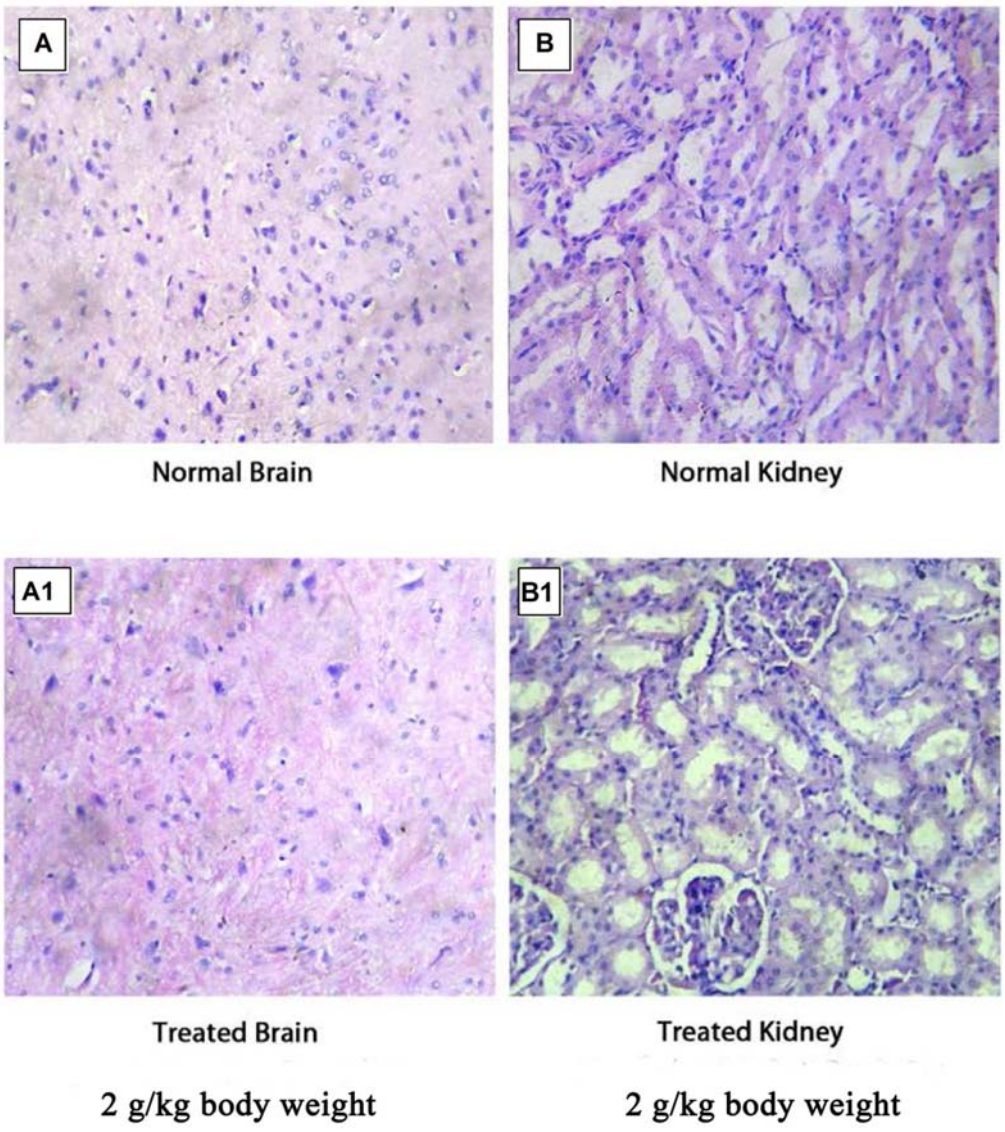


Fig. 6D.3 Photomicrograph of histopathological sections of the brain and kidney on day 90 of sub-chronic toxicity test. (**A**) Normal liver, (**A1**) brain sections from experimental rats after 90 days of treatment with 2.0g/kg of the green mussel formulation showing apparently normal glial cells, (**B**) photomicrograph of kidney section from experimental control rats, (**B1**) kidney sections from experimental rats after 90 days of treatment with 2.0g/kg of the green mussel formulation showing normal glomeruli.

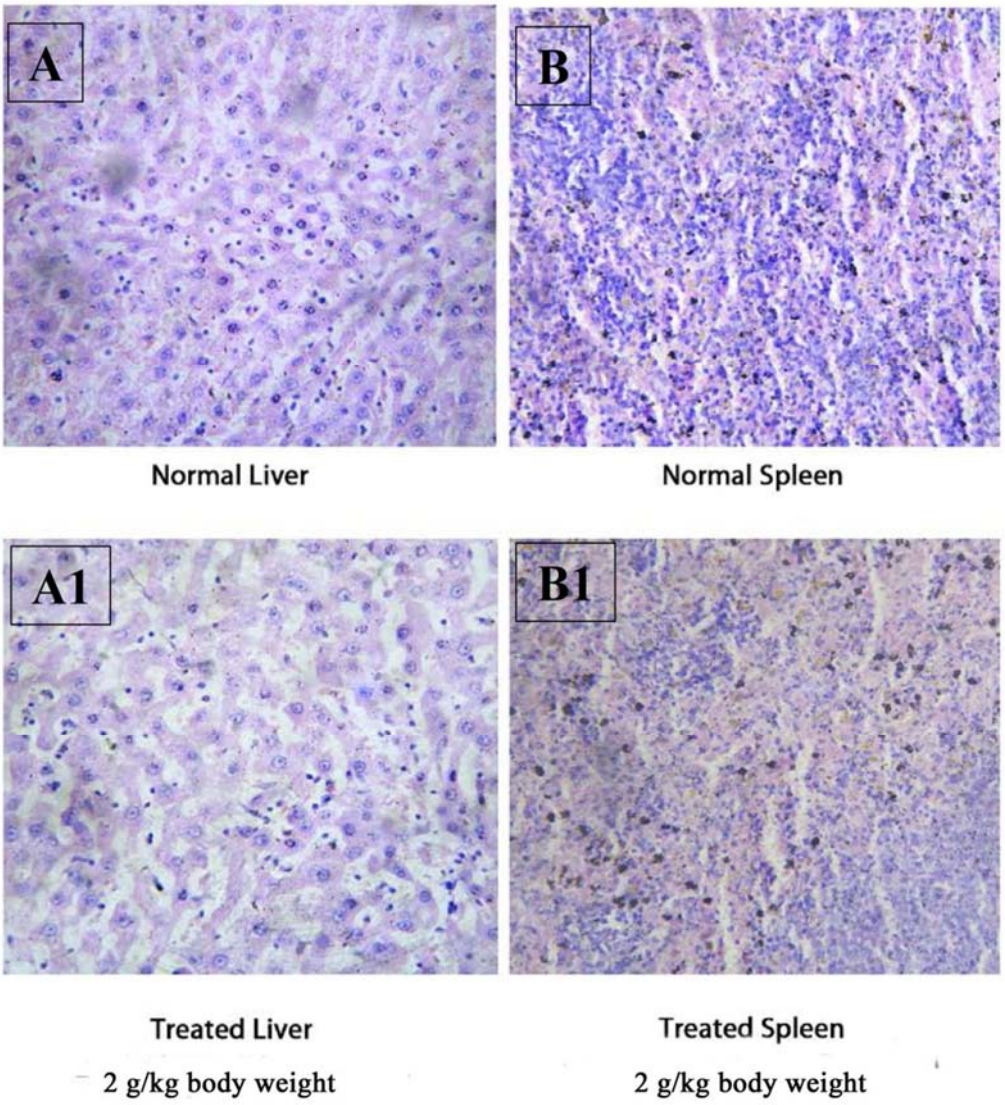


Fig. 6D.4 Photomicrograph of histopathological sections of the liver and spleen on day 90 of sub-chronic toxicity test. (**A**) Normal liver, (**A1**) photomicrograph of liver sections from experimental rats after 90 days of treatment with 2.0g/kg of the green mussel formulation showing apparently normal morphology of hepatocytes, (**B**) photomicrograph of spleen section from experimental control rats, (**B1**) spleen sections from experimental rats after 90 days of treatment with 2.0g/kg of the green mussel formulation showing normal lymphoid follicles with germinal centers.

6D.3 Conclusion

The acute and sub-chronic toxicity studies of the green mussel formulation using Wistar rats was carried out to understand its effect on various parameters such as mortality, weight change, food consumption, hematological, liver, and renal functions, serum electrolytes, and lipid profile. The results indicated that the green mussel formulation did not produce any change in food consumption, water consumption and body weights in rats, indicating that it has no toxicity to these animals. Also it did not produce any biochemical changes related to hepatic and renal function. This formulation did not produce any change in hematological and serum biochemical parameters. Necropsy of the treated animals showed normal appearance of various tissues. The no observable adverse effect level (NOAEL) was 2000 mg/kg BW. The toxicological studies demonstrated that the green mussel formulation is safe to consume without any adverse effects in the body

SUMMARY

Mollusks contribute significantly to the total marine fish catch of the world. Marine bivalves are abundant in coastal and estuarine waters of India. The bivalve fishery is constituted mainly by clams, mussels and oysters. Molluskan fishery is not well-organized along the Indian coast and they are exploited in large quantities by traditional methods and sold live in the market for human consumption. Mussels, oysters and octopus form good protein food with considerable quantities of poly unsaturated fatty acids. Cephalopods are important marine organisms as a food and are the store house of many biologically important metabolites. Diets that include seafood on a regular basis may represent some health benefits regarding nutritionally relevant compounds, such as, minerals, *n*-3 fatty acids and vitamin E. Marine organisms are considered as unique biostore of active compounds, having enormous therapeutic potential which led to the growing interest in investigation of natural lead molecules with potential antioxidant and anti-inflammatory characteristics.

In the present study we have considered *Perna viridis* and *Crassostrea madrasensis* among the bivalves and *Octopus dolffusi* as the candidate cephalopod. These species are abundantly available at the south west coast of India and are the preferred food items among the people living in the Malabar Coast. Based on this background, the present work is focused in the isolation of potential anti-inflammatory and antioxidant principles from these candidate bivalve mollusks and cephalopods, and their characterization by different spectroscopic techniques. The products containing active anti-inflammatory and antioxidant principles with suitable stabilizers are developed from the

candidate bivalve mollusks and cephalopods in an attempt to deliver potent medication and nutraceutical supplements.

The selected mollusks such as *P. viridis, C. madrasernsis* and *O.dolffusi* were evaluated for the comparative analysis of their spatial and seasonal disparities in the biochemical composition. The present study provides comprehensive knowledge of different biochemical and fatty acid variation of cultured and wild bivalves collected from two different locations of south west coast of India (Kochi and Kozhikode) during different seasons of the year. The higher PUFA content of the samples collected from this mussel harvesting area also contribute to their potential to exhibit anti-inflammatory activities. ***P. viridis,*** **by virtue of realizing a balanced** EAA/NEAA ratio with considerable amount of lysine and threonine, is a well-balanced and high-quality protein source, and a candidate species to supplement these amino acids of cereals. The optimum and balanced quantities of vitamins, mineral nutrients, and low cholesterol contents added to the good qualities of this particular species, and therefore, proved to be a desirable item in the human diet in the south-western region of India.

The Indian edible oyster *Crassostrea madrasensis* is one of the most important and promising economic oyster species being farmed for human consumption. Growth conditions play vital role in physiological mechanisms of oysters guiding fatty acid metabolism. High levels of PUFA (21.7-36.4%) including *n*-3 PUFAs (12.5-23.2 %), low levels of *n*-6 PUFA (maximum of 7% of total fatty acids) observed in both wild and cultured oysters. The ratios of essential/non-essential amino acids (EAA/NEAA) indicated that both oyster tissues are good sources of well-balanced proteins. Octopus was found to be the good protein source with low in fat content, and considered as the most interesting inhabitants of the seas. Greater levels of EPA and DHA in the

lipids implied the presence of antioxidant system in the edible muscle of *O. dolffusi*. Greater levels of PUFA including *n*-3 PUFAs, important in the human diet for their platelet anti-aggregating and blood pressure-reducing properties, lesser levels of *n*-6 PUFA, and relatively high *n*-3/*n*-6 PUFA ratio values characterized *P. viridis*. The higher PUFA content also contributed to their potential to exhibit anti-inflammatory activities, and therefore, proved to be a highly desirable item for use as food and nutrceutical supplement.

The present study demonstrated the importance of *P. viridis*, *C. madrasensis* and *O. dolffusi*, in terms of their potential radical scavenging and anti-inflammatory properties. Extracts both aqueous and solvent fractions of different polarities were generated from these species and screened for the promising antioxidant and anti-inflammatory activities. The present findings implied that the methanolic extracts derived from these candidate bivalve species could be used to isolate potentially valuable antioxidative and anti-inflammatory compounds. This forms a first comprehensive report on the antioxidative and anti-inflammatory properties of these seafood species as a novel source of antioxidants and anti-inflammatory leads.

The crude extracts were subjected to undergo repeated purifications with the aid of various chromatographic techniques and a total of ten compounds with potential antioxidant and anti-inflammatory activities were isolated from these mollusks. The chemical structures of the pure compounds, as well as their relative stereochemistries, were established by means of detailed spectroscopic experiments. Chromatographic separation of *P. viridis* extract led to the isolation of six new derivatives. The molecular structures of the purified compounds was proposed on the basis of comprehensive analysis of the ^{1}H NMR, ^{13}C NMR, including 2D-NMR experiments (^{1}H–^{1}H–COSY, HMQC, HMBC, and NOESY), and mass spectra. The chloroform partitioned methanolic

extract of *Perna viridis* was chromatographically fractionated over silica columns to yield 7-hydroxy-2-vinyl-1,2,3,4,4a,4b,5,6,7,8,10,10a-dodecahydro-4-phenanthrenone (**1**), 1,1,4b-trimethyl-7-vinyl-1,2,3,4b,5,6,7,8,8a,9,10,10a-dodecahydro-3-phenanthrenone (**2**), 10,14-dihydroxy-5,5-dimethyl-6,6a,6b,7,8,8a,9,10,11,12b-decahydro-5H-benzo[h]naphtho[1,2-c]chromene-8a-carbaldehyde (**3**), 8a-acetyl-10-hydroxy-5,5-dimethyl-6,6a,6b,7,8,8a,9,10,11, 12b,14,14a-dodecahydro-5H-benzo[h]naphtho[1,2-c]chromen-14-one (**4**), (1,5-dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl(3E,7E)-9-ethyl-5-hydroxy-3-methyl-3,7,9-decatrienoate (**5**) and (4E)-2,3,4,7,8,9,10,11,12,13,14,15,16, 17-tetradecahydro-10,13-dimethyl-17-(6-methylheptan-2-yl)-1H-cyclopenta[a] phenanthren-3-yl 6-oxooct-4-enoate (**6**). The scavenging effect of the purified compounds decreased with the order: **3**>**4**>**1**>**2**>**5**>**6**. These compounds could effectively inhibit COX-2 as well as LOX-5. This study established the potential of marine derived compounds as potential natural lead antioxidative and anti-inflammatory molecules for use in pharmaceutical and food industries. The knowledge on the structural features responsible for antioxidative and anti-inflammatory activities will guide us to synthesize the molecules in commercial scale for use as new generation antioxidant and anti-inflammatory leads.

The ethylacetate:methanol extract of *Crassostrea madrasensis* was fractionated chromatographically to yield methyl 12-hydroxy-2,9,9-trimethyl-10-[(2-phenylacetyl)oxy]-1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,12b,14a, 14b-icosahydro-2-picenecarboxylate (**7**) and methyl 1,12-dihydroxy-2,9,9-trimethyl-10-[(2-phenylacetyl)oxy]-1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a, 12b,14a14b-icosahydro-2-picenecarboxylate (**8**). The structures of the compounds, as well as their relative stereo chemistries, were established by means of spectral data analyses, including 2D-NMR experiments. Among the compounds isolated from

Crassostrea madrasensis, compound **7** showed maximum DPPH radical-scavenging ability (72.5 percent) than **8**. No significant differences in the $ABTS^{\cdot+}$ radical-scavenging activity, COX-2 and 5-LOX inhibition activities were observed for **7** and **8**.The effective antioxidant property of these compounds indicated that they have potential as natural antioxidant lead molecules in the food industry.

The ethylacetate:methanol extract of *Octopus dolffusi* yielded two pure compounds, namely, 1-[17-(1,5-dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10, 11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl] 5-methyl 3-[(E)-1-amino-3-ethyl-6-oxo-3-heptenyl]pentanedioate (**9**) and 4-ethyl-6-vinyl-2,3,6,7,7a,8,9,9a-octahydroazuleno[1,8-*bc*]pyran-2,9-dione (**10**). The structures of the compounds, as well as their relative stereo chemistries, were established by means of spectral data analyses, including 2D-NMR experiments. Both compounds (**9** and **10**) isolated from *Octopus dolffusi* showed similar effect in inhibiting the DPPH and $ABTS^{\cdot+}$ radicals. The *in vitro* anti-inflammatory properties with respect to COX-2 and 5-LOX inhibitory activities were also found to be similar for **9** and **10**.

Considering the importance and easy availability of *P. viridis*, a nutraceutical formulation has been formulated to inhibit 5-LOX and COX-2 with COX-1/COX-2 ratio below the safety threshold limit leading to selective activity against inflammatory mediators. However, it is to be noted that the active ingredients of *P. viridis* extract are lipidic in nature, and therefore, are susceptible to peroxidation (or oxidation) due to the presence of olefinic bonds in their structure. The present study explored the natural alternatives, such as, different compositions of oleoresins (*Rosmarinus officinalis* (ROO) and *Curcuma longa* (CLO) and other natural antioxidant additives in different proportions were blended with the *P. viridis* extract (FDPE) in different

combinations (GME_1, GME_2, GME_3 and GME_4). These experimental combinations were subjected to accelerated shelf life study for a period of 90 days (d_{90}) to find their individual and synergistic effects, which were able to reduce the free radicals causing oxidation reactions thereby deteriorating the nutritional compositions of the mussel extract. The dual inhibitory activity of Indian green mussel, *P. viridis* against pro-inflammatory enzymes cyclooxygenase (COX-2) and lipoxygenase (5-LOX) showed its better therapeutic profile and fewer side effects than synthetic NSAIDs. From the present study, it is revealed that different additives from natural origin like *Curcuma longa*, *Rosmarinus officinalis* etc., added in different combinations can retain the anti-inflammatory activity of *P. viridis* extract by stabilizing its active anti-inflammatory ingredients. The FT-IR spectra at various time intervals showed the possible degradation/retention of the anti-inflammatory principles of *P. viridis vis a vis* different combinations, in a time-dependent manner demonstrating that the combinations GME_2 and GME_4 have the potential to retain the active principles throughout the experimental period under shelf. The phenolic compounds with multiple centres of unsaturation in the antioxidant additives used to prepare the formulation intercepted the free radical chain reaction on unsaturated fatty acids during accelerated storage resulting in the storage stability of the long chain C20-22*n*-3 PUFAs. The study also highlights the utility of measuring pro-inflammatory prostaglandins (PGE_2 and $PGF_{2\alpha}$) as an index of antioxidant status and lipid peroxidation. The results obtained by HPLC were further validated using tandem LC/MS/MS and ^1H NMR analyses to understand and compare the signal pattern and intensity of the PG in GME_0 *vis-à-vis* the samples fortified with antioxidant additives after 90 days of accelerated shelf-life study.

The present work involved the extraction and isolation of different anti-inflammatory principles from *P. viridis* for the enrichment of the potentanti-inflammatory formulation GME_2. Specifically the anti-inflammatory lecithin and polysaccharides along with glycolipoprotein and *n*-3 polyunsaturated fatty acids were isolated from this bivalve mollusk in an attempt to enrich the anti-inflammatory activity of the formulations.

The oligosaccharide (compound **11)** was characterized as di-(N-acetyl-β-D-mannosamine) -(1→4)-2-N-acetyl-β-D-galactosamine-(1→) - (4-N-acetyl *p*-phenoxyl) motifs. IR spectrum of (→4)-2, 4 di-(N-acetyl-β-D-mannosamine) -(1→4)-2-N-acetyl- β -D-galactosamine-(1→) - (4-N-acetyl *p*-phenoxyl) unit revealed the broad and intense stretching at 3400 cm^{-1} is characteristic of hydroxyl groups and the weak stretching at 2930 cm^{-1} is attributed to the C–H bond. The fraction also exhibited an obvious characteristic absorption at 925 and 800 cm^{-1} corresponding to the existence of mannose. The characteristic absorptions at 850 cm^{-1} in the IR spectra indicated the presence of α-glycosidic linkages. The ^1H NMR spectrum (500 MHz) showed anomeric proton signals at δ 5.28-5.35 and δ 4.5 in a molar ratio of about 2:1, which were assigned to the two types of sugar units. The structure characteristics of oligosaccharide, especially the chain linkage and conformation of the sugar units, were completed and confirmed by 1D and 2D NMR. The *in vitro* anti-inflammatory activities were studied by using indomethacin and aspirin as positive controls and polysaccharide fraction registered significantly greater COX-2 inhibition (74.8%) than aspirin and indomethacin (58.6% and 59%, respectively, 5mg/mL).

The compound **12** contain C-28 carbon lysolecithin isolated from the green mussel, whereas the ultraviolet absorbance at λ_{max} (log ε) 244 (3.11) nm (in MeOH) has been assigned to the choline and glycerol systems. Its mass spectrum exhibited a molecular ion peak at m/z 550 ($C_{28}H_{57}NO_7P$). ^1H NMR

in conjugation with ^{13}C NMR recorded the signature peaks of choline, glycerol and fatty acid portion of the lysolecithin. ^{13}C NMR spectra displayed three close peaks at δ 54.31(C-1), δ 54.21(C-2) and δ 53.56(C-3) that were attached to the nitrogen represented singlet at δ 3.19 (s) with an integral value of 9. The lysolecithin fraction (68.9 %) exhibited strong, concentration dependent inhibition of COX-2, and was greater inhibition than the traditional NSAID COX inhibitor, indomethacin, at comparable concentrations.

The isolated naturally derived anti-inflammatory components, such as, lecithin and polysaccharides along with glycolipoprotein and *n*-3 polyunsaturated fatty acids were added in various compositions to the said anti-inflammatory combination GME_2 to prepare different experimental combinations (Q_1 to Q_2). These enriched combinations (Q_1 through Q_2) of the freeze dried *P. viridis* extract (FDPE) supplemented with the antioxidative oleoresins for stabilizing its active anti-inflammatory ingredients. Among the different treatments of enriched green mussel extracts Q_1 exhibited significant inhibition of pro-inflammatory COX-1, COX-2 and 5-LOX enzymes, indicating their potential for use as effective green alternatives to the synthetic drugs with multiple adverse effects on human health. Significant *in vivo* activities also indicated better therapeutic profile and fewer side effects of the anti-inflammatory formulation Q_1 as compared to the synthetic non-steroidal anti-inflammatory drugs. Therefore, Q_1 proved to be the effective new generation green alternatives to the synthetic non-steroidal anti-inflammatory drugs for use against a cascade of mammalian inflammatory diseases.

The acute and sub-chronic toxicity studies of the potential green mussel formulation using wistar rats was carried out to understand its effect on various parameters such as mortality, weight change, food consumption, haematological, liver, and renal functions, serum electrolytes, and lipid profile. The results indicated that the green mussel formulation did not produce any

change in food consumption, water consumption and body weights in rats, indicating that it has no toxicity to these animals. Also it did not produce any biochemical changes related to hepatic and renal function. This formulation did not produce any change in haematological and serum biochemical parameters. Necropsy of the treated animals showed normal appearance of various tissues. The no observable adverse effect level (NOAEL) was 2000 mg/kg BW. The toxicological studies demonstrated that the green mussel formulation is safe to consume without any adverse effects in the body.

In recent years, the use of antioxidants and anti-inflammatory agents of natural origin is considerably enhanced, by the concern about the adverse side effects of these popularly used synthetic antioxidants and NSAIDS. Presently the anti-inflammatory concentrates of natural origin, available in the market are mostly imported and highly priced. Therefore it is not cost-effective to use them as health food additives. The anti-inflammatory combination enriched product, might be a cheaper alternative to the imported supplements, and has been used as nutraceuticals. Hence, the enriched anti-inflammatory formulations are effective new generation green alternatives to the synthetic NSAIDs for use against a cascade of mammalian inflammatory diseases. The optimized procedure to prepare the stabilized anti-inflammatory combinations developed in the present study would contribute to the commercial application to produce the stabilized anti-inflammatory as nutraceuticals and dietary supplements.

REFERENCE

Abad, M., Ruiz, C., Martınez, D., Mosquera, G., Sanchez, J.L. (1995). Seasonal variations of lipids classes and fatty acids in flat oyster, *Ostreaedulis*, from San Cibran (Galicia, Spain). Comparitive Biochemistry Physiology, 110C, 109–118.

Ackman, R. G. (1995). Composition and nutritive value of fish and shellfish lipids. In A. Ruither (Ed.), Fish and fishery products (pp. 117–156). UK: CAB International.

Albentosa, M., Labarta, U.,Fernandez-Reiriz, M.J., Perez- Camacho, A. (1996). Fatty acid composition of *Ruditapesdecussates* spat fed on different microalgae diets. Comparitive Biochemistry Physiology, 13A, 113–119.

Alkanani, T., Parrish, C.C., Thompson, R.J., Mckenzie, C.H. (2007). Role of fatty acids in cultured mussels, *Mytilus edulis*, grown in Notre Dame Bay, Newfoundland. Journal of Experimental Marine Biology and Ecology, 348, 33–45.

Almansa, E., Domingues, P., Sykes, A., Tejera, N., Lorenzo, A., Andrade, J. P. (2006). The effects of feeding with shrimp or fish fry on growth and mantle lipid composition of juvenile and adult cuttlefish (Sepia officinalis). Aquaculture, 256, 403–413.

Ames, B. N., Shinenaga, M. K.,and Hagen, T. M (1993). Oxidants, antioxidants and the degenerative diseases of aging. Proceedings of the national Academy of Sciences, USA, 90, 7915-7922.

Anand, P.T., Chellaram, C., Kumaran, R., Shanthini, C. F. (2010). Biochemical composition and antioxidant activity of *Pleuroploca trapezium* meat. Journal of Chemical Pharmacological Research, 2, 526-535.

Anbuselvi, S., Chellaram, C., Jonesh, S., Jayanthi, A., Edward. J.K.P. (2009). Bioactive potential of coral associated gastropod, *Trochus tentorium* of Gulf of Mannar, southeastern India. Journal of Medicinal Science, 9, 240-244.

Andersen, R.J., Faukner, D.J., He, C., Van Duyne, G.D., Clardy, J. (1985). Metabolites of the marine prosobranch mollusk *Lamellaria* sp. Journal of American Chemical Society, 107, 5492–5495.

Anil, Chatterji., Zakir, A., Ansari, Baban., Ingole, S., Bichurina, M. A., Sovetova, Marina.,Yuri, A. Boikov. (2002). Indian marine bivalves: Potential source of antiviral drugs current science, 82, 10.

Ansell, A.D. (1974). Seasonal changes in biochemical composition of the bivalve, *Abra alba* from the clyde sea area. Marine. Biology, 25,13-20

AOAC International. (2005). Official Methods of Analysis.18th 642 edition. Association of Official 643 Analytical Chemists, Gaithersburg, MD.

AOAC. (1990) Official methods of analysis (15th ed.) Arlington VA *Association of Official Analytical Chemists*.

Appel, L.J., Sacks, F.M., Carey, V.J., Obarzanek, E., Swain, J.F., Miller III, E.R., Conlin, P.R., Erlinger, T.P., Rosner, B.A., Laranjo, N.M., Charleston, J., McCarron, P.,Bishop, L.M. (2005). Effects of protein, monounsaturated fat, and carbohydrate intake on blood pressure and serum lipids: results of the omniheart randomized trial. JAMA 294, 2455–2464.

Appeldoorn, R.S. (1983). Variation in growth rate of Mya arenaria and its relationship to the environment as ana1ysedT'through Principal Components Analysis and the parameter of the von Bertalanffy equation. Fisheries Bulletin. U.S, 81,75-84.

Appukuttan, K.K. (2007). The Molluscan fishery of India and its livelihood Enhancement potential, IFP Souvenir.

Appukuttan,K.K. (2008). Molluscan resources and management strategies**.** Proceedings of National Seminar Environment Management on livelihood, 6-12.

Arapov, J., Ezgeta–Balic, D., Peharda, M., Nincevi Gladan, Z. (2010). Bivalve feeding- how and what they eat? Ribarstvo, 68, 3, 105-116.

Arnao, M. B., Cano, A., Acosta, M. (2001). The hydrophilic and lipophilic contribution to total antioxidant activity. Food Chemistry, 73, 239–244.

Astorga Espana, M. S., RodriguezRodriguez, E. M., Romero, C. D. (2007). Comparison of mineral and trace element concentrations in two molluscs from the Strait of Magellan (Chile). Journal of Food Composition and Analysis, 20, 273–279.

Baccaglini, PI., Hogan, PG. (1983). Some rat sensory neurons in culture express characteristics of differentiated pain sensory cells. Proceedings of National Academic Science USA 80, 594–598.

Baker, J. T., Murphy, V. (1981). Handbook of Marine Science: Compounds from Marine Organisms. Vol. II, CRC Press, Boca Raton, Florida.

Balcazar, J.L., Blas, I.D., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., Muzquiz, J.L. (2006). The role of probiotics in aquaculture. Veterinary Microbiology, 114**,** 173-186.

Ballantyne, J. S., Hochachka, P. W., Mommsen, T. P. (1981). Studies on the metabolism of the migratory squid, *Loligo opalescens*: enzymes of tissues and heart mitochondria. Marine Biology Letters, 2, 75–85.

Ballot, C., Kluza, J., Martoriati, A. (2009).Essential role of mitochondria in apoptosis of cancer cells induced by the marine alkaloid Lamellarin D. Molecular Cancer Therapeutics, 8, 12, 3307–3317.

Barrento, S., Marques, A., Teixeira, B., Mendes, R., Bandarra, N., Vaz-Pires, P., Nunes, M.L. (2010). Chemical composition, cholesterol, fatty acid and amino acid in two populations of brown crab *Cancer pagurus*: Ecological and human health implications. Journal of Food Composition and Analysis, 23, 716–725.

Baruchel Wainberg. (1992). The role of oxidative stress in disease progression in individuals infected by the human immunodeficiency virus. Journal of Leukocyte Biology. 52, 111-114

Baylac, S., Racine, P. (2003). Inhibition of 5–lipoxygenase by essential oils and other natural fragrant extracts. International. Journal Aromatheraphy, 13, 138–142.

Benkendorff, K. (2010). Molluscan biological and chemical diversity: Secondary metabolites and medicinal resources produced by marine molluscs. Biological reviews of the Cambridge Philosophical Society, 85, 757–775.

Benkendorff, K., C.M. McIver and C.A. Abott, (2011). Bioactivity of the murex homeopathic remedy and of extracts from an Australian murcid mollusc against human cancer cells. Evidence-based Complementary and Alternative Medicine, 1:12.

Benkendorff, K., McIver, C. M., Abbott, C.A., (2009). Bioactivity of the *Murex* homeopathic remedy and of extracts from an Australian muricid mollusc against human cancer cells. Evidence-based Complementary and Alternative Medicine. doi:10.1093/ecam/nep042**.**

Benson, A. J., Marelli, D. C., Frischer M. E., Danforth J. M., Williams, J. D. (2001). Establishment of the green mussel, *Perna viridis* (Linnaeus 1758) (Mollusca: Mytilidae) on the West Coast of Florida, Journal of Shellfish Research, 20, 1, 21–29.

Bernay, B., Floch, M.B., Gagnon, J. Henry, J. (2006).Ovarian jelly-peptides (OPJs), a new family of regulatory peptides identified in the cephalopod *Sepia officinalis*. Peptides, 27, 1256–1268.

Bligh, E. G., Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology, 37, 900–917.

Blunt, J.W., Copp, B.R., Keyzers, R.A., Munro, M.H., Prinsep, M.R (2012). Marine natural products. Natural product report, 29, 2, 144.

Blunt, J.W., Copp, B.R., Keyzers, R.A., Munro, M.H., Prinsep, M.R (2013). Marine natural products. Natural product report, 30, 2, 237-323.

Blunt, J.W., Copp, B.R., Keyzers, R.A., Munro, M.H., Prinsep, M.R (2014). Marine natural products. Natural product report, 31, 2, 160-258.

Bohn, J. A., Be Miller, J. N. (1995). (1– ›3)-β-D-Glucan as biological response modifiers: A review of structure–functional activity relationships. Carbohydrate Polymers, 28, 3–14.

Booth, J.D. (1983) Studies on twelve common bivalve larvae and bivalve spawning seasons. New Zealand Journal of Marine Fresh- water Research, 17, 231-265.

Boucaud-Camou, E. (1990). La Seiche, un animal davenir. La Peche Maritime, 69, 1342, 321–329.

Boyle, P. R. (1990). Cephalopod biology in the fisheries context. Fisheries Research, 8, 303–321.

Brien, S., Lewith, G., Walker, A. F. (2006). Bromelain as an adjunctive treatment for moderate–to–severe osteoarthritis of the knee: a randomized placebo–controlled pilot study. Quarterly journal of medicine, 99, 12, 841–50

Brown, J.R., Hariwick, E.B. (1988a). Influences of temperature, salinity and available food upon suspended culture of the Pacific oyster, Crassostrea. Absolute and allometric growth. Aquaculture, Z9: 231-251.

Caggiula, A.W., Mustad, V.A. (1997). Effects of dietary fat and fatty acids on coronary artery disease risk and total and lipoprotein cholesterol concentrations:epidemiologic studies. American Journal of Clinical Nutrition, 65, 1597S–1610S.

Calder, P. C. (2006). Polyunsaturated fatty acids and inflammation. Prostaglandins leukotrines essential fattyacids, 75, 197– 202.

Calder, P.C. (2004). *n*-3 fatty acids, inflammation and immunity - relevance to postsurgical and critically ill patients. Lipids, 39, 1147–61.

Calder, P.C. (2009). Polyunsaturated fatty acids and inflammatory processes: New twists in an old tale. Biochimie, 91, 791–795.

Camus, Lionel., Gulliksen, Bjorn., Depledge, MH., Jones, MB. (2005). Polar bivalves are characterized by high antioxidant defences. Polar Research, 24, (1-2), 111 - 118.

Cancio, I., Ibabe, A., Cajaraville, M.P., (1999). Seasonal variation of peroxisomal enzyme activities and peroxisomal structure in mussels Mytilusgalloprovincialis and its relationship with the lipid content. Comparative Biochemistry and Physiology. Part C, 123, 2, 135–144.

Candela C.G., Lopez L., Kohen V. (2011). Importance of a balanced omega 6/omega 3 ratio for the maintenance of health: Nutritional recommendations. Nutricion Hospitalaria, 26, 323–329.

Capillas, C.R., Moral, A., Morales, J., Montero, P. (2002). Characterisation of non-protein nitrogen in the Cephalopods volador (*Illex coindetii*), pota (*Todaropsis eblanae*) and octopus (*Eledone cirrhosa*). Food Chemistry, 76, 2, 165-172.

Caponio, F., Summo, C., Pasqualone, A., Gomes, T. (2011). Fatty acid composition and degradation level of the oils used in canned fish as a function of the different types of fish. Journal of Food Composition and Analysis, 24, 1117–1122.

Carbone, M., Li, Y., Irace, C., Mollo, E., Castelluccio, F., Di Pascale, A., Cimino, G., Santamaria, R., Guo YW., Gavagnin, M. (2011). Structure and cytotoxicity of phidianidines A and B: first finding of 1,2,4-oxadiazole system in a marine natural product. Organic. Letters, 13, 2516–2519.

Chakraborty, K., Paulraj, R. (2008). Enrichment of eicosapentaenoic acid from sardine oil with Δ5-olefinic bond specific lipase from *Bacillus licheniformis* MTCC 6824. Journal of Agricultural Food Chemistry, 56, 1428-1433.

Chakraborty, K., Paulraj, R. (2009). Selective enrichment of n-3 polyunsaturated fatty acids with C18–C20 acyl chain length from sardine oil using Pseudomonas fluorescens MTCC 2421lipase. Food Chemistry, 114, 142–150.

Chandran, B., Rameshkumar, G., Ravichandran, S. (2009). Antimicrobial activity from the gill extraction of Pernaviridis (Linnaeus, 1758). Global journal of biotechnology & biochemistry, 4, 2, 88-92.

Chapmann, A.D. (2009). Numbers of Living Species in Australia Conservation of Island Biodiversity: Importance, Challenges and Opportunities. Centre for Applied Biodiversity Science. Conservation International, Arlington, 16.

Chatterji, Anil., Ansari, Zakir., Ingole, Baban., Bichurina, M. A., Marina Sovetova., Boikov Yuri. (2002). Indian marine bivalves: Potential source of antiviral drugs. Current science, 82, 10.

Chellaram, C, Prem Anand, T., Kuberan, G., Alex John, A, Priya, G., Arvind, Kumar. (2012). Anti -inflammatory and analgesic effects of coral reef associated gastropod, *Trochus tentorium* from Tuticorin coastal waters, southeastern India. African journal of biotechnology, 11, 80, 14621-14626.

Chellaram, C., Edward, J.K.P. (2009). Anti-inflammatory potential of coral reef associated gastropod, *Drupamargariticola*. Indian journal of science and technology, 2, 75-77.

Chellaram, C., Edward, J.K.P. (2009a). *In vivo* anti - inflammatory bustle of reef associated mollusc, *Trochus Tentorium*. Advanced biotechnology, 9, 32 – 34.

Chellaram, C., Edward, J.K.P. (2009b). Antinociceptive assets of coral associated gastropod, *Drupa margariticola* . International journal of pharmacology, 5, 236 – 239.

Chellaram, C., Gnanambal, K.M., Edward, J.K.P. (2004). Antibacterial activity of the winged oyster , *Pteria chinensis* (Pterioida: Pteridae). Indian journal of marine science, 33,369-372.

Cheung, S., Tai. J. (2007). Anti-proliferative and antioxidant properties of rosemary (*Rosmarinus officinalis*). Oncology reports, 17, 1525-1531.

Chow, C.K. (2008). Fatty Acids in Foods and their Health Implications, third ed. CRC Press.

Cimino G and M. Gavagnin, (2011). Tritoniopsins A–D, Cladiellane-Based Diterpenes from the South China Sea Nudibranch Tritoniopsis elegans and its Prey Cladiella krempf. Journal of natural products, 74, 1902–1907.

Cleland, J., Bernstein, S., Ezeh, A., Faundes, A., Glasier, A., Innis, J. (2006). Family planning: the unfinished agenda. The Lancet, 18,1810-27.

CMFRI (2003). Central Marine Fisheries Research Institute. Annual Report (2001-2002).CMFRI, Cochin

CMFRI (2009). Central Marine Fisheries Research Institute. Annual Report (2007-2008).CMFRI, Cochin.

CMFRI (2014). Central Marine Fisheries Research Institute. Annual Report (2012-2013).CMFRI, Cochin.

Cobb, C.S., Ernst, E. (2006). Systematic review of a marine nutriceutical supplement in clinical trials for arthritis: the effectiveness of the New Zealand green-lipped mussel *Perna canaliculus*. Clinical rheumatology, 25, 3, 275-84.

Conner, W. E. (2000). Importance of n_3 fatty acids in health and disease.The American Journal of Clinical Nutrition, 17,1, 171S–175S.

Cordain, L., Eaton, S.B., Sebastian, A., Mann, N., Lindeberg, S., Watkins, B.A., Okeefe, J.H., Brand-Miller, J. (2005). Origins and evolution of the Western diet: health implications for the 21st century. American Journal of Clinical Nutrition, 81,341-54.

Coudray, C., Feillet-Coudra, C., Rambeau, M., Tressol, J.C., Gueux, E., Mazur, A., Rayssiguier Y. (2006). The effect of aging on intestinal absorption and status of calcium, magnesium, zinc, and copper in rats: A stable isotope study. Journal of Trace Elements in Medicine and Biology, 20, 73–81.

Culkin, F., Morris, R.J. (1970). The fatty acids of some cephalopods. Deep-Sea Research, 17, 171–174.

Culkin, F., Morris, R.J., (1970). The fatty acids of some cephalopods. Deep-Sea research, 17, 171–174.

Cuzzocrea, S, Riley, D, Caputi, AP, Selvemini, D (2001). Antioxidant therapy: A new pharmacological approach in shock, inflammation, and ischemia/ reperfusion injury. Pharmacology, 53, 135-159.

Czarnecki R.,Grzybek J. (1995). Antiinflammatory and vasoprotective activities of polysaccharides iso- lated from fruit bodies of higher fungi P.1. Polysaccha-rides from *T rametes gibosa* (Pers.: Fr) Fr. (Polypora- ceae). Phytotherapy research, 9, 123-12

de Heer, M.I., Mulder, P., Korth, H.G., Ingold, K.U., Lusztyk, J. (2000). Hydrogen atom abstraction kinetics from intramolecularly hydrogen bonded ubiquinol-10 and other (poly) methoxy phenols. Journal American chemical society, 122, 2355-2360

De Moreno, J.E.A., Moreno, V.J., Brenner, R.R. (1976). Lipid metabolism of the yellow clam, *Mesodesma mactroides*: Composition of the lipids. Lipids, 11, 7,334-340.

De Moreno, J.E.A., Pollero, R.J.V., Moreno, J., Brenner, R.R. (1980). Lipids and fatty acids of the mussel (*Mytilus platensis d'Orbigny*) from South Atlantic waters. Journal of Experimental Marine Biology and Ecology, 48, 263–276.

Defer, D., Bourgnon, N., Fleury, Y. (2009). Screening for antibacterial and antiviral activities in three bivalve and two gastropod marine molluscs. Aquaculture, 293, 1-7.

Demple, B. (1999). Radical ideas: genetic responses to oxidative stress. Clinical and experimental pharmacology physiology, 26, 64– 68.

Den Brok, M.W., Nuijen, B., Garcia, J.L., Miranda, E., Calvo, P., Manada, C., Beijnen, J.H. (2006). Compatibility and stability of the novel anticancer agent ES-285 × HCl formulated with 2-hydroxypropyl-beta-cyclodextrin in infusion devices. Die Pharmazie, 61, 21–24.

Derby C.D., (2014). Cephalopod Ink: Production, Chemistry, Functions and Applications Mar. Drugs, 12, 2700-2730.

Derby, CD., Kicklighter,CE., Johnson, PM., Zhang, X. (2007). Chemical composition of inks of diverse marine molluscs suggests convergent chemical defenses. Journal of chemical ecology. 33, 1105–1113.

Donghong, Liu., Ningbo, Liao., Xingqian, Ye., Yaqin, Hu., Dan, Wu., Xin, Guo., Jianjun, Zhong., Jianyong, Wu., Shiguo, Chen., (2013). Isolation and Structural Characterization of a novel antioxidant mannoglucan from a marine bubble snail, *Bullacta exarata* (Philippi) Marine drugs, 11, 4464-4477.

Donia, M., Hamann, M.T. (2003). Marine natural products and their potential applications as anti-infective agents. Lancet Infectious Diseases,. 3, 6, 338–348.

Dridi, S., Salah Romdhane, M., Elcafsi, M.H. (2007). Seasonal variation in weight and biochemical composition of the Pacific oyster, *Crassostreagigas* in relation to the gametogenic cycle and environmental conditions of the Bizert lagoon, Tunisia. Aquaculture, 263, 238–248.

Duan, X.J., Zhang, W.W., Li, X.M., Wang, B.G. (2006). Evaluation of antioxidant property of extract and fractions obtained from a red alga. *Polysiphoniaurceolata.* Food Chemistry, 95, 37– 43.

Edwards, V., Benkendorff, K., Young, F. (2012). Marine compounds selectively induce apoptosis in female reproductive cancer cells but not in primary-derived human reproductive granulosa cells, Marine Drugs, 10, 1, 64–83.

Eklund M., Bauer, E., Wamatu, J., Mosenthin R. (2005). Potential nutritional and physiological functions of betaine in livestock. Nutrition Research Reviews, 18, 31–48.

Elliot, R.H., Strunin, L. (1993). Hepatotoxicity of volatile anaesthetics. British journal of Anaesthaesia, 70, 339-349

Emelyanov, A., Fedoseev, G., Krasnoschekova, O., Abulimity, A., Trendeleva, T., Barnes, P.J. (2002). Treatment of asthma with lipid extract of New Zealand green-lipped mussel: a randomised clinical trial. European Respiratory Journal, 20, 596-600

Engelhardt, G., Homma, D., Schlegel, K., Utzmann, R., Schnitzler, C. (1995). Anti–inflammatory, analgesic, antipyretic and related properties of meloxicam, a new non–steroidal anti–inflammatory agent with favourable gastrointestinal tolerance. Inflammation research, 44, 423–433.

Evans, P.H. (1993).Free radicals in brain metabolism and pathology.British Medical Bulletin, 49, 577-587

Fahmy, S.R., Soliman, A.M. (2013). In vitro antioxidant, analgesic and cytotoxic activities of Sepia officinalis ink and Coelaturaaegyptiaca extracts. African journal of pharmacy and pharmacology, 7, 22, 1512–1522.

Fang, Y.Z., Yang, S., Wu, G.Y. (2002). Free radicals, antioxidants, and nutrition. Nutrition, 18, 872-879.

FAO, 2012. Fish and fishery products: world apparent consumption statistics based on food balance sheets. FAO Yearbook, Fishery and Aquaculture Statistics. Food and Agriculture Organization of the United Nations, Rome.

FAO. (2004). The State of World Fisheries and Aquaculture. FAO Fisheries Department. Food and agriculture arganization of the United Nations. Rome, Italy, 153p.

Faulkner, D.J. (2003). Marine Natural Products. Natural Product Reports, 17, 7-55.

Fernandes, E., Costa, D., Toste, S.A., Lima, J.L.F.C., Reis S. (2004). *In vitro* scavenging activity for reactive oxygen and nitrogen species by nonsteroidal anti-inflammatory indole, pyrrole, and oxazole derivative drugs. Free Radical Biological Mededicine, 37, 1895–1905.

Fernández-Reiriz, M.J., Pérez-Camacho, A., Ferreiro, M.J., Blanco, J., Planas, M., Campos, M. J. (1989). Biomass production and variation in the biochemical profile (total protein, carbohydrates, RNA, lipids and fatty acids) of seven species of marine microalgae. Aquaculture,83, 17–37.

Folch, J., Lees, M., Stanley, G.H.S. (1957). A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry, 226, 497–509.

Fontana, A., d' Ippolito, G., D'Souza, L., Mollo, E., Parameswaran, P.S., Guido, Cimino. (2000). New acetogenin peroxides from the Indian sponge Acarnus bicladotylota. Journal of natural products, 63, 2435-2437.

Forsythe, J.W., Toll, R.B. (1991). Clarification of the Western Atlantic Ocean pygmy octopus complex: the identity and life history of *Octopus joubini* (Cephalopoda: Octopodinae).Bulletin Marine Science, 49, 88–97.

Freites, L., Labarta, U., Fernandez-Reiriz, M.J. (2002). Evolution of fatty acid profiles of subtidal and rocky shore mussel seed (*Mytilusbgalloprovincialis*, Link.). Influence of environmental parameters. Journal of Experimental Marine Biology and Ecology, 268, 185–204.

Fry, D.E, (1992). Multiple system organ failure. In:Fry (ed) Multiple system organ failure Mosby Year Book, St Louis, Missouri, 3-14.

Fu, X., Hong, E.P., Schmitz, F.J. (2000). New polypropionates pyrones from the Phillippine Sacoglossan *Placobranchus osellatus*. Tetrahedron, 56, 8989 -8993.

Fuentes, A., Fernández-Segovia, I., Escriche, I., Serra, J. A. (2009). Comparison of physico-chemical parameters and composition of mussels (*Mytilus galloprovincialis* Lmk.) from different Spanish origins. Food Chemistry, 112, 295–302.

Gamache, D.A., Povlishock, J., Ellis, E. F. (1986). Carrageenan induced brain inflammation. Journal of neurology, 65, 679- 685.

García García, J., Rodríguez, L.M., García García, B. (2004). Cost analysis of octopus ongrowing installation in Galicia. Spanish Journal of Agriculture and Research, 2, 531–537.

Gaspic, Z.K., Ujevic, I., Baric, A. (2002). The Mediterranean blue mussel as an environmental indicator of metal pollution in the coastal area of Eastern Adriatic. Fresenius Environmental Bulletin, 11, 620-625.

Gautam, R., Jachak, S.M. (2009). Recent developments in anti-inflammatory natural products. Medical reaearch review, 29, 5, 767-820.

Gavagnin, M., Spinella, A., Cimino, G., Marin A. (1994). Polypropionates from the Mediterranean mollusk *Elysia timida.* Journal of natural products, 57, 298-304.

Genoveffa Nuzzo, 1., Maria Letizia Ciavatta, 1., Robert, Kiss., Véronique, Mathieu., Helene, Leclercqz., Emiliano, Manzo 1., Guido, Villani., Ernesto, Mollo 1., Florence, Lefranc. (2012). Chemistry of the Nudibranch *Aldisa andersoni*: Structure and Biological Activity of Phorbazole Metabolites. Marine Drugs, 10, 1799-1811.

Geronikaki, A.A., Gavalas, A.M. (2006). Antioxidants and inflammatory disease: synthetic and natural antioxidants with anti-inflammatory activity. Combinatorial chemistry and high throughput screening, 9, 425-42.

Gestal, C., Costa, M., Figueras, A., Novoa, B. (2007). Analysis of differentially expressed genes in response to bacterial stimulation in hemocytes of the carpetshell clam Ruditapes decussatus: identification of new antimicrobial peptides. Gene, 406, 134- 143.

Gibson, R.A. (1983). Australian fish an excellent source of both arachidonic acid and *n*−3 polyunsaturated fatty acids. Lipids, 18, 743–752.

Gill, N., Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry, 48, 909-930.

Girones-Vilaplana, A., Valentao, P., Andrade, P.B., Ferreres, F., Moreno, D.A. (2012). Phytochemical profile of a blend of black chokeberry and lemon juice with cholinesterase inhibitory effect and antioxidant potential. Food Chemistry 134, 2090–2096.

Gockse, M.A., Tasbozan, O., Celik, M., Tabakoglu, S.S. (2004). Seasonal variation in proximate and fatty acid compositions of female common sole (*Solea solea*). Food Chemistry, 88,419–423.

Gorinstein, S., Martin-Belloso, O., Lojek, A., Ciz, M., Soliva-Fortuny, R., Park, Y.S., Caspi, A., Libman, I., Trakhtenberg, S., (2002). Comparative content of some phytochemicals in Spanish apples, peaches and pears. Journal of the Science of Food and Agriculture, 82, 1166-1170.

Grabley, S., Thiericke, R. (1999). Bioactive agents from natural sources: trends in discovery and application. Advances in Biochemical Engineering/Biotechnology, 64, 101-154.

Gulçin I., Buyukokuro glu, M.E., Oktay, M., Kufrevio glu, O. I. (2002a). On the in vitro antioxidant properties of melatonin. Journal of Pineal Research, 33, 167-171.

Haefner, B. (2003). Drugs from the deep: marine natural products as drug candidates. Drug Discovery Today. 8, 12, 536–544.

Halliwell, B. (1994). Free radicals and anti-oxidants:a personal view. Nutrition review, 52, 253-265.

Halliwell, B. (2006). Reactive Species and Antioxidants. Redox Biology Is a Fundamental Theme of Aerobic Life. Plant Physiology, 141, 2, 312–322.

Hamasaki, K., Takeuchi, T. (2001). Dietary value of Artemia enriched with ω-yeast or shark eggs as feed for planktonic larvae of *Octopus vulgaris*. Saibai-Giken, 28, 65–68.

Harikumar, G.,.Rajendran, G., (2007). An Over View of Kerala Fisheries - with Particular Emphasis on Aquaculture IFP Souvenir.

Hayashi, K., Yamamoto, S. (1987). Distribution of diacyl glyceryl ethers in the different tissues and stomach contents of gonatid squid Berryteuthis magister. Nippon Suissan Gakkaishi., 53, 1057-1063.

Heinrikson, L., and Meredith, S.C. 1984. Amino acid analysis by reverse-phase high performance liquid chromatography: precolumn derivatization with phenylisothiocyanate. Anal. Biochem. 136: 65-74

HMSO, UK. (1994). Nutritional aspects of cardiovascular disease report on health and social subjects London, 46.

HMSO. 2001. Report on Health and Social Subjects, Department of Health, Nutritional Aspects of Cardiovascular Disease, London, 46, 37-46.

Hochachka, P.W., Fields, H.A. (1983). Arginine,glutamate and proline as substrates for oxidation and for glycogenesis in cephalopod tissues. Pacific Science, 36, 2, 325-335.

Hochachka, P.W., Hartline P.H.,. Fields, J.H.A. (1977). Octopine as an end product of anaerobic glycolysis in the chambered nautilus. Science, 195, 72-74.

Hoffman, MA., Blessing, JA., Lentz, SS. (2003). A phase II trial of dolastatin- 10 in recurrent platinum-sensitive ovarian carcinoma: a Gynecologic Oncology Group study. Gynecol Oncol., 89(1), 95-8.

Hunt S. & Breuer S. W. (1971) Isolation of a new naturally occurring halogenated amino acid:

Imai, T.; Sakai, S. Study on the breeding of the Japanese oyster, *Crassostreagigas* Tohoku. Journal of Agricultural Research 1961, 12, 125-171.

Inzce, L.S., Lutz, R.A., Walting, L. (1980). Relationships between effects of environmental temperature and seston on growth and mortality of Mytilus edulis in a temperate nothern estuary. Marine Biology, J1, 147-156.

Ira, Bhatnagar., Se-Kwon, Kim. (2010). Marine Antitumor Drugs: Status, Shortfalls and Strategies. Mar. Drugs, 2010, *8*, 2702-2720.

Ireland C, Scheuer PJ. Science 1979, photosynthetic marine mollusk: *in vivo* C incorporation into metabolites of the sacoglossan *Placobranchus ocellatus*. Science205: 922-923.

Irina, Budge, S. M., Parrish, C.C. (1998). Lipid biogeochemistry of plankton, settling matter and sediments in Trinity Bay,Newfoundland. II. Fatty acids. Organic Geochemistry, 29, 1547–1559.

Iwasaki, M., Harada, R.(1985). Proximate and amino acid composition of the roe and muscle of selected marine species. Journal of food science, 50,1585-1587.

Jaggi, R., Wurgler, U., Grandjean, F., Weiser, M. (2004). Dual inhibition of 5-lipoxygenase/cyclooxygenase by a reconstituted homeopathic remedy; possible explanation for clinical efficacy and favourable gastrointestinal tolerability. Inflammation Research, 53, 150–157.

Jaisamut, S., Prabpai, S., Tancharoen, C., Yuenyongsawad S., Hannongbua, S., Kongsaeree P., Plubrukarn, A. (2013). Journal of Natural Products, 76, 2158–2161.

James, M.J., Gibson, R.A., and Cleland, L.G. (2000). Dietary polyunsaturated fatty acids and inflammatory mediator production. American Journal of Clinical Nutrition, 71, 343S-8S

Jangaard, P.M., Ackman, R.G. (1965). Lipids and component fatty acids of the Newfoundland squid, Illex illecobrosus (Le Sueur).Journal of Fisheries Research Board, 22, 131–137.

Jensen, A. (1978). Chlorophyll and carotenoids. In: Hellebust JA, Craigie JS (edn) Physiological and biochemical methods. Cambridge University Press London, 59-70.

Joseph, M. M., Joseph, S. (1983). Some aspects of experimental culture of the oyster Crassostrea madrasensis (Preston). Proceedings of Symposium Coastal Aquaculutre, 2,451-455.

Juana, Rovirosa., Aurelio, San-Martín., (2006). A novel metabolite from the Chilean mollusk *Siphonaria lesson* Quimica Nova, 29, 1.

Kalogeropoulus, N., Andrikopoulos, N. K., & Hassapidou, M. (2004).Dietary evaluation of Mediterranean fish and mollusks pan-fried in virgin olive oil. Journal of the Science of Food and Agriculture, 84, 1750–1758.

Kamboj, V.P. (1999). Bioactive Agent from the Ocean Biota. In: Ocean Science Trends Future Directions, Somayajulu, B.L.K. (Ed.). Indian National Science Academy, New Delhi, India,

Kanda, A., Minakata, H., (2006). Isolation and characterization of a novel small cardioactive peptide-related peptide from the brain of *Octopus vulgaris*. Peptides, 27, 1755–1761

Kao, Y.C**,** Lichtenberger, L.M**.** (1993). Effect of 16,16-dimethyl prostaglandin E_2 on lipidic organelles of rat gastric surface mucous cells. Gastroenterology, 104, 103–113,

Kao, Y.C., Lichtenberger, L.M. (1991). Phopholipid and neutral lipid containing organelles of rat gastroduodenal mucous cells. Posiible orgin of the hydrophobic mucosal linning. Gastroenterology, 101, 7–21.

Karakoltsidis, P.A., Zotos, A., Constantinides, S.M. (1995). Composition of the commercially important mediterranean finfish, crustaceans, and molluscs. Journal of Food Composition and Analysis, 8, 258–273.

Keys, A., Menotti, A., Karnonen, M. J., Aravanis, C., Blackburn, H., Buzina, R. (1986). The diet and 15-year death rate in the seven countries study. American Journal of Epidemiology, 124, 903–915.

Kharlamenko, V.I., Zhukova, N.V., Khotimchenko, S.V., Svetashev, V.I., Kamenev, G.M. (1995). Fatty acids as markers of food sources in a shallow-water hydrothermal ecosystem (Kraternaya Bight, Yankich Island, Kurile Islands). Marine Ecology-Progress Series, 120, 231-241.

Kim, I.G., Jung, I.L., Oh, T.J., Kim, K.C., Shim, H.W. (2002). Polysaccharide-enriched fraction isolated from *Duchesneachrysantha*protects against oxidative damage. Biotechnology Letters, 24, 1299–1305.

Kim, R. S., Labella, F. S. (1987). Comparison of analytical methods for monitoring autoxidation profiles of authentic lipids. Journal of Lipid Research, 28,1110-1117.

King, I., Childs, M.T., Dorsett, C., Ostrander, J.G., Monsen, E.R. (1990). Shellfish: proximate composition, minerals, fatty acids, and sterols. Journal of American Dietetic Association, 90, 677–685.

Kisters, K. (2004). Increased Calcium and decreased Magnesium concentrations and an increased calcium/magnesium ratio in spontaneously hypertensive rats versus Wistar-Kyoto rats: relations to arteriosclerosis.. American Journal of Hypertsion, 17,59-62.

Koduru, S., Jimoh, F.O., Grierson, D.S., Afolayan, A.J. (2007). Antioxidant activity of two steroid alkaloids extracted from *Solanum aculeastrum*. Journal of Pharmcology and Toxicology, 2, 160-167.

Kohl, A., Kerr.R. (2003). Pseudopterosin Biosynthesis: Aromatization of the Diterpene Cyclase Product, Elisabethatriene. Marine Drugs, 1, 54-65.

Kourounakis, A.P, Galanakis, D., Tsiakitzis, K. (1999). Synthesis and pharmacological evaluation of novel derivatives of anti-inflammatory drugs with increased antioxidant and anti-inflammatory activities. Drug Development Research, 47, 9-16.

Kreuzer, R. (1984). Cephalopods: handling, processing and products. FAO Fish Technical Paper, 254, 108.

Kripa, V., Mohamed, K.S. (2008). Green Mussel, Perna viridis, Farming in Kerala, India – Technology Diffusion Process and Socioeconomic Impacts. Journal of the World Aquaculture Society, 39, 5, 612-624.

Kris-Etherton, P. M., Harris, W. S., Appel, L. J. (2002). Fish consumption, fish oil, omega-3 fatty acids and cardiovascular disease. Circulation, 106, 2747–2757.

Kulkarni, S.K, Mehta, A.K, Kunchady, J. (1986). Anti-inflammatory actions of clonidine, guanfacine and B-HT 920 against various inflammagen -induced acute paw edemasin rats. International Journal of Pharmaceutical Archive, 279, 324-334

Kumar, S.S (2003). Studies on the cowries (Mollusca: Gastropoda: Cypraeidea) of Gulf of Mannar, Southeast coast of India, Ph.D Thesis submitted to Manonmaniam Sundaranar University, Tirunelveli.

Kuriakose, P.S., Appukuttan, K.K. (1980).Work details for rope culture of mussels,47-50.In K.N.Nayar.,S. Mahadevan., K,Alagarswami and P.T Meenakshisundaram(eds) Coastal aquaculture: mussel farming. Progress and Prospects. Central Marine Fisheries Research Institute Bulletin, 29.56

Kurihara, A., Okumura, S., Iwamoto, A., Takeuchi, T., (2006). Feeding pacific sandeel enhances DHA level in common octopus paralarvae. Aquaculture and Science. 54, 413–420.

Kurubail, R. Second International Workshop on COX–2. 1998, 28–31.

Lal, S. (1995). Macro and trace elements in fish and shellfish. In: Fish and fishery products: composition, nutritive properties and stability (edited by A. Ruiter). Wallingford: CAB International, 187-214.

Lall, S.P. 2002. The minerals. In: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition, 3rd ed. Academic Press, San Diego, CA, 259–308.

Larsen, L. N., Dahl, E., Bremer, J. (1996). Peroxidative oxidation of leuco-dichlorofluorescein by prostaglandin H synthase in prostaglandin biosynthesis from polyunsaturated fatty acids. Biochimica et Biophysica Acta, 1299, 47–53.

Laxmilatha, P., Rao, G. S., Patnaik, P., Rao, T. N., Rao, M. P., Dash, B. (2011). Potential for the hatchery production of spat of the green mussel *Perna viridis Linnaeu*s (1758). Aquaculture, 312, 1–4.

Leal, M.C., Puga, J., Serodio, J., Gomes, N.C., Calado, R. (2012). Trends in the discovery of new marine natural products from invertebrates over the last two decades-Where and what are we bioprospecting? Plos One, 7, 30580.

Learnal, Sudhakar., Prakash, Vincent. (2014). Purification and characterization of a novel C-Type hemolytic lectin for clot lysis from the fresh water clam *VilloritaCyprinoides*: A possible natural thrombolytic agent against myocardial infarction. Fish and shellfish immunology, 36, 2, 367–373.

Lee, P.G. (1994). Nutrition of cephalopods: fuelling the system. Marine and Freshwater. Behavioural Physiology, 25, 35–51.

Lemaire, P., Livingstone, D.R. (1993). Pro-oxidant/antioxidant processes and organic xenobiotic interactions in marine organisms, in particular the flounder *Platichthys flesus* and the mussel *Mytilus edulis*. Trends in Comparative Biochemistry **&** Physiology 1, 1119-l150.

Li, D., Zhang, Y., Sinclair, A.J. (2007). Seasonal variations of lipid content and composition in *Perna viridis*. Lipids, 42, 739–747.

Li, X.M., Li, X.L., Zhou, A.G., (2007). Evaluation of antioxidant activity of the polysaccharides extracted from Lyciumbarbarum fruits *in vitro*. European Polymer Journal, 43, 488–497.

Liu, R., Liu, Y., Zhou, Y.D., Nagle, D.G. (2007). Molecular-Targeted Antitumor Agents 15:Neolamellarins from the marine sponge *Dendrillanigra* Inhibit hypoxia-inducible Factor-1(HIF-1) activation and secreted vascular endothelial growth factor (VEGF) production in breast tumor cells. Journal of Natural Products, 70, 1741–1745.

Liu, Y., Liu, R., Mao, S.C., Morgan, J.B., Jekabsons, M.B., Zhou, Y.D., Nagle, D.G. (2008). Molecular-targeted antitumor agents. Furospongolide from a marine *Lendenfeldia*sp. sponge inhibits hypoxia-inducible factor-1. Activation in Breast Tumor Cells. Journal pf Natural Products, 71, 1854–1860.

Livingstone DR. (2001). Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Marine Pollution Bulletin, 42, 656–666.

Lowry, O. H., Roseborouch, N. I., Farrand, A. L., Randal, R. J. (1951). Protein measurement with the Folin-phenol reagent. J of Biological Chemistry, 263-275

Mallet, A.L., Haley, L.E. (1983). Growth rate and survivial in pure population matings and crosses of the oyster Crassostrea Virginica. Canadian Journal of Fisheries and Aquatic Science, 4Q, 948-954.

Manahan, D.T. (1990). Adaptions by invertebrate larvae for nutrient acquisition from seawater. Am. Zool. 30,147–160.

Manzo, E., Carbone, M., Mollo, E., Irace, C., Di Pascale, A., Li, Y. (2011). Structure and synthesis of a unique isonitrile lipid isolated from the marine mollusk *Actinocyclus papillatus*. Organic Letters, 2011,13,1897–9

Maoka, T. (2011). Carotenoids in marine animals. Marine. Drugs, 9 , 278 – 293.

Maria-Luisa., Lazo-de-la-Vega-Monroy., Cristina, Fernández-Mejía., (2013). Oxidative Stress in chronic degenerative diseases-A role of anti-oxidants book published under in Chapter 9 Oxidative stress in Diabetes Mellitus and the Role Of Vitamins with Antioxidant Actions.

Martins, J.G. (2009). EPA but not DHA appears to be responsible for the efficacy of omega-3 long chain polyunsaturated fatty acid supplementation in depression: Evidence from a meta-analysis of randomized controlled trials. Journal American College Nutrition, 28, 525–542.

McLean, C. H., Bulling, K. R. (2005). Differences in lipid profile of New Zealand marine species over four seasons. Journal of Food Lipids, 12, 313–326.

McPhee, S., Hodges, L. D., Wright, P. F., Wynne, P. M., Kalafatis, N., Harney, D. W., Macrides, T. A. (2007). Anti-cyclooxygenase effects of lipid extracts from the New Zealand greenlipped mussel, *Perna canaliculus*. Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology, 146, 3, 346-356

Menchaca, R., Martínez, V., Rodríguez, A., Rodríguez, N., Flores, M., Gallego, P., Manzanares, I., Cuevas. C. (2003). Journal of. Organic Chemistry. 68, 8859.

MihaelaIlie., DenisaMargina. (2012). Trends in the Evaluation of Lipid Peroxidation Processes, Lipid Peroxidation, Dr. Angel Catala (Ed.), ISBN: 978-953-51-0716-3, InTech, DOI: 10.5772/46075.

Mikheiskaya L., Molchanova V., Ovodova R., Santana V.F. (1988). Branched α-1,4-glucan from the gastropod mollusk *Strombus gigas*. Chemistry of Natural Compounds, 24, 29–32.

Miliou, H., Fintikaki, M., Tzitzinakis, M., Kountouris, T., Verriopoulos, G., (2006). Fatty acid composition of the common octopus, *Octopus vulgaris*, in relation to rearing temperature and body weight. Aquaculture, 256, 311–322.

Miller,T. E., Joanna ,Dodd., Ormrod, D. J., Geddes, R. (1993). Anti-inflammatory activity of glycogen extracted from Perna canaliculus (NZ green-lipped mussel) Agents and Action, 38, C139-C142.

Mimura, T., Maeda, K., Terada, T., Oda, Y., Morishita, K., Aonuma, S. (1985). Studies on biologicalactivities of melanin from marine animals. III. Inhibitory effect of SM II (low molecular weightmelanoprotein from squid) on phenylbutazone-

induced ulceration in gastric mucosa in rats, andits mechanism of action. Chemical and Pharmaceutical Bulletin, 33, 2052–2060.

Min-Hsiung, Pan., Yu-Ting, Huang., Chi-I, Chang., Chi-Tang, Ho., Bonnie Sun, Pan. (2007). Apoptotic inducing Epidioxysterols (EDS) identified in black clam (*Meretrixlusoria*). Food Chemistry, 102, 788–795.

Mitra, D., and Chatterji, A. (2002). A Indian green mussel (*Perna viridis*) as a source of anti-HIV activity; U S Patent US /0168416 A1.

Mitra, D., Chatterji, A. (2004). Indian green mussel (*Perna viridis*) as source of anti -HIV activity.; US Patent #6770302 3

Miyamoto, T., Sakamoto, K., Arao, K., Komori, T., Higuchi, R., Sasaki. T. (1996). Dorisenones, cytotoxic spongian diterpenoids, from the nudibranch *Chromodoris obsoleta*. Tetrahedron, 52, 8187

Montgomery, M.K., McFall-Ngai, M.J. (1992). The muscle derived lens of a squid bioluminescent organ is biochemically convergent with the ocular lens. The Journal of Biological Chemistry, 267, 20999-21003.

Moreira, M. E. C., Pereira, R. G. F. A., Dias, D. F., Gontijo, V. S., Vilela, F. C., de Moraes, G. O. I., Giusti-Paiva, A., Santos, M. H. (2013). Anti-inflammatory effect of aqueous extracts of roasted and green *Coffea arabica* L. Journal of Functional Foods, 5, 1, 466-474.

Morris, S.A., De Silva, E.D., Anderson, R.J. (1990). Chromodoranediterpenes from the tropical doridnudibranch, Chromodoriscavae. Canadian Journal of Chemistry, 69, 768-771.

Motoe, K., Ooizumi, T., Hayashi, S., Kawasaki, K. (1997). Seasonal changes in the contents of proximate composition, minerals and vitamins of firefly squid. Journal of Japanese Food Science and Technology, 44, 133–139.

Moxica, C., Linares, F., Otero, J.J., Iglesias, J., Sánchez, F.J. (2002). Cultivo intensivo deparalarvas de pulpo, *Octopus vulgaris* Cuvier, Bol. Inst. Esp. Oceanography, 18, 31–36.

Murphy, J.K., Moone, D.B., Mann, J.N., Nichols, D.P., Sinclair, J.A. (2002). Lipid, fatty acid, and sterol composition of New Zealand green lipped mussel (*Pernacanaliculus*) and Tasmanian blue mussel (*Mytilusedulis*). Lipids, 37, 6, 587–595.

Murphy, J.K., Moone, D.B., Mann, J.N., Nichols, D.P., Sinclair, J.A. (2002). Lipid, FA, and Sterol Composition of New Zealand Green Lipped Mussel (*Pernacanaliculus*) and Tasmanian Blue Mussel (*Mytilusedulis*). Lipids, 37, 6, 587–595.

Murugan, P., Leelavinothan, P., Chippada A. R. (2008). Effect of tetrahydrocurcumin on insulin receptor status in type 2 diabetic rats: studies on insulin binding to erythrocytes. Journal of Biosciences, 33, 1, 63–72.

Nabhitabhata, J., Nilaphat, P., Promboon, P., Jaroongpattananon, C., Nilaphat, G., Reunreng, A. (2005). Performance of simple largescale cephalopod culture system in Thailand. Phuket. Marine Biology Centre Research Bulletin, 6, 337–350.

Nagabhushanam, R., Bidarkar, D.S. (1978). Studies on seasonal changes in the biochemical constituents of the oyster *Crassostreacucullata*. Indian Journal Fisheries, 25,156–164.

Nagash, Y.S., Nazeer, R.A., Sampath Kumar, N.S. (2010). *In vitro* antioxidant activity of solvent extracts of mollusks (*Loligoduvauceli*and *Donaxstrateus*) from India. World Journal of Fish and Marine Sciences, 2, 240-245.

Narasimham, K.A.(1980)..Fishery and biology of the green mussel, *Perna viridis* (Linnaeus), 10-16. In K.N.Nayar.,S. Mahadevan., K,Alagarswami and P.T Meenakshisundaram(eds) Coastal aquaculture: mussel farming. Progress and Prospects. Central Marine Fisheries Research Institute Bulletin, 29.56

Nash,D.M., Eaton, C.A., Crewe, N.F. (1978). Lipid classes and fatty acid composition of squid. Technical Reports of the Fisheries and Marine Service of Canada, 833, 8.

Navarro, J C., Villanueva, R. (2000). Lipid and fatty acid composition of early stages of cephalopods: an approach to their lipid requirements. Aquaculture, 183, 161-177.

Navarro, J.C., Villanueva, R. (2003). The fatty acid composition of Octopus vulgaris paralarvae reared with live and inert food: deviation from their natural fatty acid profile. Aquaculture, 219, 613–631.

Nestel, P.J. (2000). Fish Oil and Cardiovascular Disease: Lipids and Arterial Function, Americal Journal of Clinical Nutrition, 71, 228S-231S

Ngoile, M.A.K, (1987). Fishery biology of the squid *Loligoforbesisteenstrup* (Cephalopoda: *Loliginidae*) in Scottish waters. Ph.D thesis, University of Aberdeen, 218

Nurjanah, Agoes., Mardiono, Jacoeb., Roni, Nugraha., Suhana, Sulastri., Nurzakiah.,Siti, Karmila. (2012). Proximate, Nutrient and Mineral Composition of Cuttlefish (Sepia recurvirostra) Advance Journal of Food Science and Technology. 4, 4, 220-224.

O'Dor, R. K., Mangold, K., Boucher-Rodoni, R.,Wells, M. J., Wells, J. (1984). Nutrient absorption, storage and remobilization in Octopus vulgaris. Marine Behavioral Physiology, 11, 239–258.

Okumura, S., Kurihara, A., Iwamoto, A., Takeuchi, T., (2005). Improved survival and growth in Octopus vulgaris paralarvae by feeding large type Artemia and Pacific sandeel, Ammodytes personatus. Aquaculture, 244, 147–157.

Okumus, I., Stirling, H.P. (1998). Seasonal variations in the meat weight, condition index and biochemical composition of mussels (*Mytilus edulis* L.) in suspended culture in two Scottish sea lochs. Aquaculture, 159, 249–261.

Okuzumi, M., Fujii, T. (2000). Nutritional and functional properties of squid and cuttlefish. Tokyo: National Cooperative Association of Squid Processors, 223.

Orban, E., Di Lena, G., Masci, M., Nevigato, T., Casini, I., Caproni, R. (2004). Growth, nutritional quality and safety of oysters (*Crassostrea gigas*) cultured in the lagoon of Venice (Italy). Journal of the Science of Food and Agriculture, 84,1929–1938.

Orban, E., Di Lena, G., Nevigato, T., Casini, I., Marzetti, A., Caproni, R. (2002). Seasonal changes in meat content, condition index and chemical composition of mussels (*Mytilus galloprovincialis*) cultured in two different Italian sites. Food Chemistry, 77,57–65.

Osman, H., Suriah, A.R., Law, E.C., 2001. Fatty acid composition and cholesterol content of selected marine fish in Malaysian waters. Food Chemistry 73, 55–60

Ozogul, Y., Duysak, O., Ozogul, F., Özkütük, A.S., Türeli, C., (2008). Seasonal effects in the nutritional quality of the body structural tissue of cephalopods. Food Chemistry, 108, 847–852.

Ozogul, Y., Ozogul, F. (2005). Fatty acid profiles of commercially important fish species from the mediterranean. Food Chemistry, 100(4), 1634-1638.

Ozyurt, G., Duysak, O., Akama, E., Tureli, C. (2006). Seasonal change of fatty acids of cuttlefish *Sepia officinalis* L. (molluska: cephalopoda) in the north eastern Mediterranean Sea. Food Chem., 95, 3, 382-385.

Paez-Osuna, F., Frias-Espericueta, M.G., Osuna-Lopez, J.I., Trace metal and concentrations in relation to season and gonadal maturation in the oyster *Crassostreairidiscens*. Marine Environmental Research, 1995, 40, 19–31.

Pan, Min-Hsiung., Huang, Yu-Ting., Chang, chi-I., Ho, Chi-tang, Pan., Bonnie, Sun. (2007). Apoptotic-inducing epidioxysterols identified in hard clam (*Meretrix lusoria*). Food Chemistry, 102, 3, 788-795.

Pardo, B., Paz-Ares, L., Tabernero, J., Ciruelos, E., Garcia, M., Salazar, R., Lopez, A., Blanco, M., Nieto, A., Jimeno, J., Izquierdo, M.A. Trigo, J.M. (2008). Phase I clinical and pharmacokinetic study of kahalalide F administered weekly as a 1-hour infu-sion to patients with advanced solid tumors. Clinical Cancer Research, 14, 1116–1123.

Parke, A. L., Ioannides, C., Lewis, D.F.V., Parke, D. V. (1991). Molecular pathology of drugs-disease interaction in chronic autoimmune inflammatory diseases. Inflammopharmacology, 1, 3-36

Parke, A., Parke, D.V (1995).The pathogenesis of inflammatory disease: surgical shock and multiple system organ failure. Inflammopharmacology, 3, 149-168.

Passi, S., Cataudella, S., Di Marco, P., De Simone, F., & Rastrelli, L. (2002). Fatty acid composition and antioxidant levels in muscle tissue of different Mediterranean marine species of fish and shellfish. Journal of Agricultural and Food Chemistry, 50, 7314–7322.

Pazos, A.J., Ruiz, C., Garcia-Martin, O., Abad, M., Sanchez, J.L. (1996). Seasonal variations of the lipid content and fatty acid composition of *Crassostrea gigas* cultured in El Grove, Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 114, 171–179.

Pazos, M., Sánchez, L., Medina, I., (2005). α-Tocopherol oxidation in fish muscle during chilling and frozen storage. Journal of Agricultural Food Chemistry, 53, 4000–4005.

Pereira, L. A., Bentley, K., Peeters, A., Churchill, M. J., Deacon. N. J. (2000). A compilation of cellular transcription factor interactions with the HIV-1 LTR promoter. Nucleic Acids Research, 28, 663-668.

Perez, E.A., Hillman, D.W., Fishkin, P.A., Krook, J.E., Tan, W.W., Kuriakose, P.A. (2005). Phase II trial of dolastatin-10 in patients with advanced breast cancer. Invest New Drugs, 23, 3, 257–61.

Periyasamy, N., (2012). Biochemical, antimicrobial and anticoagulant potential of *Acanthochitonamahensis* from Tranquebar, South east Coast of India. M phil thesis, Annamalai University, India.

Pettit, G.R., Kamano, Y., Herald, C.L., Tuinman A.A., Boettner F.E. (1987). The isolation and structure of a remarkable marine animal antineoplastic constituent: Dolastatin 10. Journal of American Chemical Society, 109, 6883-6885.

Phadke, J.D, Anderson, L.A (1988). Ethnopharmacology and Western Medicine. Journal of Ethnopharmacology, 25, 61.

Phillips, K.L., Nichols, P.D., Jackson, G.D. (2002). Lipid and fatty acid composition of the mantle and digestive gland of four Southern Ocean squid species: Implications for food-web studies. Antarctic Science, 14, 3, 212-220.

Plaza, M., Cifuentes, A., Ibañez., E. (2008). In the search of new functional food ingredients from algae. Trends in Food Science & Technology. 19, 31–39.

Power, A., Sheehan, D. (1996). Seasonal variation in the antioxidant defence system of gill and digestive gland of the Blue Mussel, Mytilusedulis. Comparative. Biochemistry and . Physiology C, 114, 99–103.

Provencio, M., Sánchez, A., Gasent, J., Gómez, P., Rosell, R. (2009). Cancer treatments: can we find treasures at the bottom of the sea*?* Clinical Lung Cancer*,* 10, 4, 295-300.

Purushan, K.S., Gopalan U.K., Rao T.S.S. (1983). On setting spat and growth of edible oyster *Crassostreamadrasensls* (Perston) in Cochin backwater. Proceedings of the symposium on coastal aquaculture, 2,444-450

Qasim, S.Z., Parulekar, A.H., Harkantra, S.N., Ansari, Z.A., Nair, A.(1977). Aquaculture of green mussel, *Mytilus viridis L*. Cultivation on ropes from floating rafts. Journal of Marine **Science**, 6,15-21

Qin Y (1993). The chelating properties of chitosan fiber. Journal of Applied Polymer Science, 49, 727 - 731.

Quan, L.D., Thiele, G., Tian, M J., Wang, D. (2008). The development of novel therapies for rheumatoid arthritis. Expert Opinion on Therapeutic Patents, 18, 7, 723–738.

Rajagopal, S., van der Velde, G.,Jenner, H.A. (2002). Effects of low level chlorination on zebra mussel, *Dreissenapolymorpha*. Water Research, 36, 3029–3034.

Ramasamy, P., Vino, AB., Saravanan, R., Subhapradha, N., Shanmugam, V., Shanmugam, A. (2011). Screening of antimicrobial potential of polysaccharide from cuttlebone and methanolic extract from body tissue of *Sepia prashadi* Wink worth, 1936. Asian Pacific Journal of Tropical Biomedicine, 244-248.

Ramesha, C.S., Pickett, W.C., (1986). Platelet-activating factor and leukotriene biosynthesis is inhibited in polymorphonuclear leukocytes depleted of arachidonic acid. Journal of Biological. Chemistry, 261, 7592–7595.

Rang, H.P., Dale, M.M. (2012). Rang and Dale's pharmacology:Edinburgh : Elsevier Churchill Livingstone

Rao, K.V.. Nayar. K.N. (1956). Rate of growth in spat and yearlings of the Indian backwater oyster *Ostrea madrasensis* Preston. Indian Journal of Fisheries 3, 2, 231-260.

Rasmussen, RS., Morrissey, MT. (2007). Marine biotechnology for production of food ingredients. Advances in Food and Nutrition Research. 52, 237–292.

Rasoarahona, J. R. E., Barnathan, G., Bianchini, J. P., Gaydon, E. M. (2005). Influence of season on the lipid content and fatty acid profiles of three tilapia species (Oreochromsis niloticus, O. macrochir and Tilapia rendalli) from Madagascar. Food Chemistry, 91(4), 683–694.

Reale, A., Ziino, M., Ottolenghi, F., Pelusi, P., Romeo, V., Condurso, C. (2006). Chemical composition and nutritional value of somemarine species from the Egadi Islands. Chemistry and Ecology, 22(1), S173–S179.

Reddy, M.V.R., Rao, M.R., Rhodes, D. (1999). Lamellarin α 20-sulfate, an inhibitor of HIV-1 integrase active against HIV-1 virus in cell culture. Journal of Medicinal Chemistry, 42, 11, 1901–1907

Regoli, F., Gorbi, S., Frenzilli, G., Nigro, M., Corsi, I., Focardi, S., Winston, G.W. (2002). Oxidative stress in ecotoxicology: from the analysis of individual antioxidants to a more integrated approach. Marine Environmental Research 54, 419-423

Reyes, F. New Bioactive Metabolites from Marine Invertebrates, 6th European Conference on Marine Natural Products, 19–23 July 2009, Porto, Portugal, Book of abstracts, p. 35.

Robards, D. M., Anthony, J. A., Rose, G. A., Piatt, J. F. (1999). Changes in proximate composition and somatic energy content forPacific sand lance (Ammodytes hexapterus) from Kachemak. Journal of Experimental Marine Biology and Ecology, 242, 245–258.

Rodhouse, P.G., Nigmatullin, C.M. 1996. Role as consumers. Philos. Trans. R. Soc. Lond. B 351, 1003–1022.

Rosa, R., Nunes, M. L., Sousa Reis, C. (2002). Seasonal changes in the biochemical composition of Octopus vulgaris, Cuvier, 1797, from three areas of the Portuguese coast. Bulletin of Marine Science, 71, 739–751.

Rosa, R., Pereira, J., Nunes, M. L. (2005). Biochemical composition of cephalopods with different life strategies, with special reference to a giant squid, Architeuthis sp. Marine Biology, 146, 739–751.

Rosas, C., Cuzon, G., Pascual, C., Gaxiola, G., Chay, D., Lopez, N., Maldonado, T., Domingues, P.M., (2007). Energy balance of Octopus maya fed crab or an artificial diet. Marine Biology. 152, 371–381.

Rosas, C., Tut, J., Baeza, J., Sanchez, A., Sosa, V., Pascual, C., Arena, L., Domingues, P.,Cuzon, G., (2008). Effect of type of binder on growth, digestibility, and energetic balance of Octopus maya. Aquaculture 275, 291–297.

Rovirosa, Juana., San-Martín, Aurelio. (2006). A novel metabolite from the Chilean mollusc *Siphonaria lesson*. Quimica Nova, 29.

Ruch, R.J., Cheng, S.J., and Klaunig, J.E. (1989). Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogen 10: 1003-1008.

Rudd. D., Benkendorff, K. (2014). Supercritical CO2 extraction of bioactive Tyrian purple precursors from the hypobranchial gland of a marine gastropod. Journal of suoercritical fluids, 94, 1-7.

Russo, G., Tringali. G. (1983). Hemagglutinating and antibacterial activity in hemolymph of Octopus vulgaris. Review of International Oceanographic Medicine. 70/71, 49-54.

Safer, A. M., Al-Nughamish, A. J. (1999). Hepatotoxicity induced by the antioxidant food additive, butylatedhydroxytoluene (BHT), in rats: an electron microscopical study. Histology and Histopathology, 14, 391– 406.

Salo-Vaananen, P., Ollilainen, V., Mattila, P., Lehikoinen, K., Salmela-Molsa, E., Piironen, V. (2000). Simultaneous HPLC analysis of fat-soluble vitamins in selected animal products after small-scale extraction**.** Journal of Agricultural Food Chemistry, 71, 4, 535-543.

Santos, L., Goncalves, M.V., Vassallo, D.V, Oliveira, E.M., Rossoni, L.V.(2006). Effects of high sodium intake diet on the vascular reactivity to phenylephrine on rat isolated caudal and renal vascular beds: Endothelial modulation. Life Sciences, 78, 2272–2279

Santos-Silva, J., Bessa, R.J.B., Santos-Silva, F. (2002). Effect of genotype, feeding system and slaughter weight on the quality of light lambs. II. Fatty acid composition of meat. Livestock Production Science, 77, 187–194

Santovito G, Piccinni E, Cassini A, Irato P and Albergoni V. (2005). Antioxidant responses of the Mediterranean mussel, *Mytilus galloprovincialis*, to environmental variability of dissolved oxygen. Comparative Biochemistry and Physiology, Part C Toxicology & Pharmacology, 140, 321–329.

Santovito, G., Piccinni, E., Cassini, A., Irato, P., Albergoni, V. (2005). Antioxidant responses of the Mediterranean mussel, Mytilusgalloprovincialis, to environmental variability of dissolved oxygen. Compartive Biochemistry Physiology C, 140,321-329.

Sasikumar, G., Krishnakumar, P.K., Thomas, S., Sampathkumar, G., Nagaraja, D., Bhat, G.S. (2007). Influence of environmental factors on growth rate of *Crassostrea madrasensis* (Preston) in suspended culture. Asian Fisheries Science, 20, 241-255.

Scartezzini, P., Antognoni, F., Raggi, M.A., Poli, F., and Sabbioni, C. (2006). Vitamin C content and antioxidant activity of the fruit and of the ayurvedic preparation of *Emblica officinalis* Gaertn. J. Ethnopharmcol. 104, 113–118.

Schroeder, C.I., Smythe, M.L., Lewis, R.J. (2004). Development of small molecules that mimic the binding of ω-conotoxins at the N-type voltage-gated calcium channel. Molecular Diversity, 8, 127–134.

Seeram, N.P., and Nair, M.G. (2002). Inhibition of lipid peroxidation and structure−activity-related studies of the dietary constituents anthocyanins, anthocyanidins, and catechins. Journal of Agricultural Food Chemistry, 50, 5308−5312.

Seixas, P., Rey-Méndez, M., Valente, L.M.P., Otero, A., (2008). Producing juvenile Artemia as prey for Octopus vulgaris paralarvae with different microalgal species of controlled biochemical composition. Aquaculture, 283, 83–91.

Shankaracharya, N. B. Tamarind – chemistry, technology and uses – a critical appraisal. Journal of . Food Technology, 1998, 35, 3, 193–208.

Shanmugam, A, Mahalakshmi, T.S, Barwin Vino, A. (2008). Antimicrobial activity of polysaccharide isolated from the cuttlebone of *Sepia aculeata* (orbingy, 1848) and *Sepia brevimana* (Steenstrup, 1875): an approach to selected antimicrobial activity for human pathogenic microorganisms. Journal of Fish Aquatic Science, 3, 5, 268-274

Sheehan, D., Power, A. (1999). Effects of seasonality on xenobiotic and antioxidant defence mechanisms of bivalve molluscs. Comparative Biochemistry and Physiology. Part C, 123, 193–199.

Shenoy, A.S. (1988). Octopus a delicacy in Japan. Journal of Sea Food Export, 20, 21-25.

Shuhan, Lei., Xiumei, Zhang., Peidong, Zhang., Yuzuru, Ikeda. (2013). Biochemical composition of cuttlefish *Sepia esculenta* eggs during embryonic development. Molluscan research 32, 1-2.

Simmons, T.L, Andrianasolo, E., McPhai, K., Flat, P., Gerwick, W.H., (2005). Marine natural products as anticancer drugs. Molecular Cancer Therapeutics, 4, 333-342.

Simopoulos, A.P. (2008). The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Experimental Biology and. Medicine, 233,674–688.

Sinanoglou, V. J., Miniadis-Meimaroglou, S. (1998). Fatty acid of neutral and polar lipids of (edible) Mediterranean cephalopods. Food Research International, 31, 467–473

Sinanoglou, V. J., Miniadis-Meimaroglou, S. (1998). Fatty acid of neutral and polar lipids of (edible) Mediterranean cephalopods. Food Research International, 31, 467–473.

Sinanoglou, V.J., Miniadis-Meimaroglou, S., (2000). Phospholipidsin Mediterranean cephalopods. Journal of Biosciences,55, 245-255.

Sinanoglou, V.J., Miniadis-Meimaroglou, S., (2000). Phospholipids in Mediterranean cephalopods. Journal of Bioscience,.55, 245-255.

Spencer, B. E. (2002). Molluskan shellfish farming 1st edn Blackwell Science, Malden, MA, USA.

Sreejamole, K L, Radhakrishnan C.K (2010) Preliminary qualitative chemical evaluation of the extracts from mussel *Perna virdis*, International Journal of Pharmacological Science Review and Research, 5, 2, 38-42.

Sreejamole, K.L., Radhakrishnan, C.K. (2013). Antioxidant and cytotoxic activities of ethyl acetate extract of the Indian green mussel *Perna viridis.* Asian Journal of Pharmceutical and Clinical Research, 6, 3, 197-201.

Storey, K.B., Storey, J.M., (1978). Energy metabolism in the mantle muscle of the squid, Loligo pealeii. Journal of Comparitive Physiology, 123, 169– 175.

Storey, K.B., Storey, J.M., (1983). Carbohydrate metabolism in cephalopod molluscs. In: Hochachka, P.W.(Ed.), The Mollusca. Metabolic Biochemistry and Molecular Biomechanics, vol. 1. Academic Press, New York, 91–136.

Sukhotin, A.A, Abele, D., Portner, H.O. (2002). Growth, metabolism and lipid peroxidation in *Mytilus edulis*: age and size effects. Marine Ecology: Progress Series, 226, 223–234

Sweeney, M.J., Roper, C.F.E. (1998). Classification, type localities, and type repositories of recent Cephalopoda. Smithson Control Zoology, 586, 561-599.

Sykes, A.V., Domingues, P.M., Correia, M., Andrade, J.P. (2006). Cuttlefish culture-state of the art and future trends. Vie Milieu, 56, 129–137.

Tai, Z., Cai, L., Dai, L., Dong, L., Wang, M., Yang, Y., Cao, Q., Ding, Z. (2011). Antioxidant activity and chemical constituents of edible flower of *Sophora viciifolia.* Food Chemistry, 126, 1648–1654.

Takashi, Maoka., Tetsuji, Etoh., Alexandra, V., Borodina., Alexande,r A. (2011). Soldatov A Series of 190-Hexanoyloxyfucoxanthin Derivatives from the Sea Mussel, *Mytilus galloprovincialis*, Grown in the Black Sea, Ukraine. Journal of Agricultural Food Chemistry, 59, 13059–13064.

Takaya, Y., Uchisawa, H., Matsue, H., Okuzaki, B.I.,Narumi, F., Sasaki, J.I., Ishida, K. (1994). An investigation of the antitumor peptidoglycan fraction from squid ink. Biolgical. Pharmcology Bulletin, 17, 846–849.

Takemoto, T., Daigo, K., Kondo, Y., Kondo. K. (1966). Studies on the constituents of *Chondriaarmata*. VIII. on the structure of domoic acid. Yakugaku Zasshi, 86, 874-877.

Tardy, C., Facompré, M., Laine, W. (2004). Topoisomerase I-mediated DNA cleavage as a guide to the development of antitumor agents derived from the marine alkaloid lamellarin D: triester derivatives incorporating amino acid residues. Bioorganic and Medicinal Chemistry, 12, 7, 1697–1712.

Tarley, C.R.T., Visentainer, J.V., Matsushita, M., de Souza, N.E. (2004). Proximate composition, cholesterol and fatty acids profile of canned sardines (*Sardinella brasiliensis*) in soybean oil and tomato juice. Food Chemistry, 88, 1–6.

Taussig, S.J., Batkin, S. (1998). Bromelain the enzyme complex of pineapple (*Ananas comosus*) and its clinical application. An update. Journal of Ethnopharmacology, 22,191-203.

Taylor, A.G., Savage, C. (2006). Fatty acid composition of New Zealand greenlipped mussels, Perna canaliculus: implications for harvesting for n-3 extracts. Aquaculture, 261, 430–439.

Teichert, R.W., Olivera, B.M. (2010). Natural products and ion channel pharmacology. Future Medicinal Chemistry, 2, 731–744.

Thanonkaew, A., Benjakul S., Visessanguan, W. (2006). Chemical composition and thermal property of cuttlefish (Sepia pharaonis) muscle. Journal of Food Composition Analysis, 19, 2-3, 127-133.

Tiffany, L.B., Bui, L.M. (2002). Improvement of Arthritic Signs in Dogs Fed Green-Lipped Mussel (*Pernacanaliculus*). Journal of Nutrition, 132, 634S-1636S.

Tocher, D.R. (1995). Glycerophospholipid metabolism. In: Hochachka, P.W., Mommsen, T.P. (Eds.), Biochemistry and Molecular Biology of Fishes, Metabolic Biochemistry, Amsterdam, 119–157.

Tran, H. T., Newman, R. A., Beck, D. E., Huie, R., Abbruzzese, J. L., Madden, T. A. (1997). Phase I, pharmacokinetic/pharmaco-dynamic study of dolastatin-10 in adult patients with advanced solid tumors. Proceedings of American Association of Cancer Research, 38, 306.

Tunstall-Pedoe, H. (2006). Preventing chronic diseases: a vital investment: WHO global report. Geneva: World Health Organization, International Journal of Epidemiology, 35, 4, 1107.

Uemura, D., Chou, T., Haino, T., Nagatsu, A., Fukuzawa, S., Zheng, SZ., Chen, H. (1995) Pinnatoxin A: a toxic amphoteric macrocycle from the Okinawan bivalve *Pinna muricata*. Journal of American Chemical Society, 117,1155.

Ulbricht, T.L.V., Southgate, D.A.T. (1991). Coronary heart disease: seven dietary factors. The Lancet, 338, 985–992.

Umehara, Masahiro., Negishi, Takayuki., Tashiro, Tomoko. (2012). Structure-related cytotoxic activity of derivatives from kulokekahilide-2, a cyclodepsipeptide in Hawaiian marine mollusk. Bioorganic and medicinal chemistry letters, 22, 24.

Utting, S.D. (1988). The growth and survival of hatchery reared Ostrea edulis L. spat in relation to envirmomental conditions at the on growing site. Aquaculture, 9,27-38.

Vaya, J., and Michael, A. 2001. Nutritional Antioxidants Mechanisms of Action, Analyses of Activities and Medical Applications. Current Medicinal Chemistry, Immunology Endocrine and Metabolic Agents.

Velayudhan, T.S., Kripa, V.,. Appukuttan. K.K., 1998. Production and economics of edible oyster cultured in an estuarine system of Kerala. Marine Fisheries Information Service. T & E Series, 154, 1-6

Venkatraman C., Venkataraman K. (2012). Diversity of Molluscan Fauna long the Chennai Coast International day for biological diversity.

Vetvicka, V., Yvin, J.C. (2004). Effects of marine β-1,3-glucan on immune reactions. Internation Immunopharmacololog, 4, 721–730.

Villanueva, R., Bustamante, P. (2006). Composition in essential and non-essential elements of early stages of cephalopods and dietary effects on the elemental profiles of I paralarvae. Aquaculture, 261, 225–240.

Villanueva, R., Riba, J., Ruiz-capillas, C., Gonzalez, A.V., Beata,M. (2004). Amino acid composition of earlystages of cephalopods and! effect of amino acid dietary treatments on Octopus vulgaris paralarvae. Aquaculture, 242, 455-478.

Villanueva, R., Riba, J., Ruiz-capillas, C., Gonzalez, A.V., Beata, M. (2004). Amino acid composition of earlystages of cephalopods and!effect of amino acid dietary treatments on Octopus vulgaris paralarvae. Aquaculture, 242: 455-478

Vine, K.L., Locke, J.M., Ranson, M., Pyne, S.G., Bremner, J.B. (2007). An investigation into the cytotoxicity and mode of action of some novel *N*-Alkyl-substituted isatins. Journal of Medicinal Chemistry, 50, 5109–5117.

Walne, P. R. (1976). Experiments on the culture in the sea of the Buttersh *Venerupisdecussata* L. Aquaculture, 8, 371-381.

Walsh, L.S., Turk, P.E., Forsythe, J.W., Lee, P.G., (2002). Mariculture of the loliginid squid Sepioteuthis lessoniana through seven successive generations. Aquaculture 212, 245–262.

Wanasundara, U.N., Shahidi, F. (1999). Concentration of omega-3 polyunsaturated fatty acids of seal blubber oil by urea complexation: optimization of reaction conditions. Food Chemistry, 65, 41-49.

Wang, J., Carbone, M., Gavagnin, M., Mandi, A., Antus, S., Yao, L.G. (2012). Assignment of absolute configuration of bis-γ-pyrone polypropionates from marine pulmonate molluscs. European Journal of Organic Chemistry, 1107–11.

Watanabe, M., Fuda, H., Jin, S., Sakurai, T., Ohkawa, F., Hui, S.P., Takeda, S., Watanabe, T., Koike, T., Chiba, H., Journal of Agricultural Food Chemistry, 2012, 60, 830–835.

Wei, G., and Ho, C. (2006). A stable quinone identified in the reaction of carnosol, a major antioxidant in rosemary, with 2, 2-diphenyl-1-picrylhydrazyl radical. Food Chemistry, 96, 471–476.

Wells, M.J., Wells, J. (1989). Water uptake in a cephalopod and the function of the so-called "pancreas". Journal of Experimental Biology, 145, 215–226.

Wendel, M., Heller, A.R. (2009). Anticancer actions of omega-3 fatty acids—Current state and future perspectives. Anticancer Agents Medicinal Chemistry, 9, 457–470.

Wesselinova, D. (2000). Amino acid composition of fish meat after different frozen storage period. Journal of Aquatic Food Product Technology, 9(4), 41–48

Westley, C.B., McIver, C.M., Abbott, C.A., le Leu, R.K., Benkendorff, K. (2010). Enhanced acute apoptotic response to azoxymethane-induced DNA damage in the rodent colonic epithelium by Tyrian purple purcursors: A potential colorectal cancer chemopreventative. Cancer Biological Therapeutics, 9, 34–42.

White, A.R., Kaye, C., Poupard, J., Pypstra, R., Woodnutt, G., Wynne, B. (2004)Augmentin amoxicillin/clavulanate in the treatment of community-acquired respiratory tract infection: a review of the continuing development of an innovative antimicrobial agent. Journal of Antimicrobial Chemotherapy, 53, 3–20.

Whitehouse, M.W., Macrides, T.A., Kalafitis, N., Betts, W.H., Haynes, D.R., Broadbent, J. (1997). Anti-inflammatory Activity of a Lipid Fraction (lyprinol) from the NZ Green- Lipped Mussel, Inflammopharmacology, 5, 237–246.

Winter, C. A., Risley, E. A., Nuss, G.W. (1962). Carageenan induced oedema in hind paw of the rats as an assay for anti-inflammatory drug. Proceedings of the Society for Experimental Biology and Medicine, 111, 544-547.

Witz, G. (1991). Active oxygen species as factors in multistage carcinogenesis. Proceedings of the society of Experimental Biology and Medicine, 198, 675-682.

Yang, J. H., Du, Y. M., Huang, R. H., Sun, L. P., Liu, H., Gao, X. H. (2005). Chemical modification and antitumour activity of Chinese lacquer polysaccharide from lac tree Rhus vemicifera. Carbohydrate polymers, 59, 101–107.

Yesim, Ozogul., Onder, Duysak., Fatih, Ozogul., Ali Serhat, Ozkutuk., Canan, Tureli., (2008). Seasonal effects in the nutritional quality of the body structural tissue of cephalopods. Food Chemistry, 108 847–852.

Yoon, S. J., Yu, M. A., Pyun, Y. R., Hwang, J. K., Chu, D. C., Juneja, L. R. (2003). The nontoxic mushroom Auricularia auricular contains a polysaccharides with anticoagulant activity mediated by antithrombin. Thrombosis Research, 112, 151–158

Youdim, K.A., Joseph, J.A. (2001). A possible emerging role of phytochemicals in improving age-related neurological dysfunctions: a multiplicity of effects. Free Radical Biological Medicine, 30, 583–94.

Zatylny, C., Gagnon, J., Boucard-Camou, E., Henry, J. (2002). ILME: a water-bourne pheromonal peptide released by the eggs of *Sepia officinalis*. Biochemistry & Biophysics Research Communications, 275, 217–222.

Zhang, H., Ye, L., Wang, K. (2010). Structural characterization and anti-inflammatory activity of two water-soluble polysaccharides from *Bellamya purificata*. Carbohydrate Polymers, 81, 953–960.

Zhen-fang, Zhou., Yue-wei, Guo. (2012). Bioactive natural products from Chinese marine flora and fauna Acta Pharmacologica Sinica, 33,1159–1169.

Zheng, W., Wang, S.Y. (2001). Antioxidant activity and phenolic compounds in selected herbs. Journal Agriculture and Food Chemistry, 49,11, 5165-5170.

Zhong, J.P., Wang, G., Shangm, J.H., Pan, J.Q., Li, K, Huang, Y., Liu H.Z. (2009). Protective effects of squid ink extract towards hemopoietic injuries induced by cyclophosphamine. Marine Drugs, 7, 9-18.

Zlatanos, S., Laskaridis, K., Feist, C., & Sagredos, A. (2006). Proximatecomposition, fatty acid analysis and protein digestibility-correctedscore of three Mediterranean cephalopods. Molecular Nutrition and Food Research, 50, 967–970

PUBLICATIONS

Si. No.	Title	Status
1.	Kajal Chakraborty, Selsa J Chakkalakal, Deepu Joseph Response of pro-inflammatory prostaglandin contents in anti-inflammatory supplements from green mussel *Perna viridis* L. in a time dependent accelerated shelf-life study. Journal of functional foods, 7, 527-540	Published
2	Kajal Chakraborty, Selsa J Chakkalakal, Deepu Joseph Effect of natural additives on the fatty acid signatures of green mussel *Perna viridis* L in a time dependent accelerated shelf life study. Journal of Food Quality, 37, 415–428.	Published
3	Kajal Chakraborty, Deepu Joseph, Selsa J Chakkalakal, Toxicity profile of a nutraceutical formulation derived from green mussel *Perna viridis*. BioMed Research International, 06/2014:471565	Published
4	Kajal Chakraborty, Selsa J Chakkalakal, Deepu Joseph. Nutritional and antioxidative attributes of green mussel (*Perna viridis* L.) from the southwestern Coast of India. Journal of Aquatic Food Product Technology.	*Accepted*
5	Kajal Chakraborty, Selsa J Chakkalakal, Deepu Joseph. D.Nutritional composition of edible oysters (*Crassostrea madrasensis* L.) from the south west coast of India. Journal of Aquatic Food Product Technology.	*Accepted*
6	Kajal Chakraborty, Selsa J Chakkalakal, Deepu Joseph. Antioxidative status of a nutrient enriched formulation of *Perna viridis* in a time series shelf life study. Journal of Aquatic Food Product Technology.	*Accepted*

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