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.