Characterization and quantification of biochemical components of selected Molluscs species from the Kerala coast

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by

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Ph. D. Thesis under the Faculty of Marine Sciences

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DEDICATION

To my Parents, teachers, husband, friends and beloved son for always supporting, helping and standing by me.

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Certificate

This is to certify that the thesis entitled **"Characterization and quantification of biochemical components of selected Molluscs species from the Kerala coast"** is an authentic record of the research work carried out by Mrs. Ragi A. S. under my supervision and guidance at the Department of Chemical Oceanography, School of Marine Sciences, Cochin University of Science and Technology, Kochi-16, in partial fulfilment of the requirements for Ph. D. degree of Cochin University of Science and Technology and no part of this has been presented before for any degree in any university. I further certify that all the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the Doctoral Committee of the candidate have been incorporated in the thesis.

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Declaration

I hereby declare that the thesis entitled **"Characterization and quantification of biochemical components of selected Molluscs species from the Kerala coast"** is an authentic record of the research work carried out by me under the guidance and supervision of Dr. S. Muraleedharan Nair, Professor, Department of Chemical Oceanography, School of Marine Sciences, Cochin University of Science and Technology, and no part of this has previously formed the basis of the award of any degree, diploma, associateship, fellowship or any other similar title or recognition in any University/Institution.

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Preface

Molluscs are one of the largest invertebrate groups, which were included in the earliest listed groups of living organisms. Based on the species number, molluscs are the second largest phylum in the marine environment. They are divided into seven classes, of which the main four classes includes bivalvia, polyplacophora, cephalopoda and gastropoda, all of which include snails, clams, scallops, oysters, cuttlefish, and octopus. Gastropods are the most numerous molluscs in terms of classified species and account for 80% of the total mollusc species. This class has an extraordinary diversification of habitats which were live in gardens, woodland, deserts, and on mountains; in small ditches, great rivers and lakes; in estuaries, mudflats, the rocky intertidal, the sandy sub tidal, in the abyssal depths of the oceans including the hydrothermal vents, and numerous other ecological niches, including parasitic ones. Gastropods typically have a well-defined head with two or four sensory tentacles with eyes and a ventral foot. They include snails and slugs. Bivalves are another class of molluscs, have no head and they lack some usual molluscan organs like radula and odontophore. They include clams, oysters, cockles, mussels, scallops. Majority of gastropods and bivalves are filter feeders

Sea is a huge source of both biologically active and nutritious compounds. India has a long coastline of 7517 km with rich marine fishery resources consisting of especially of fishes, crustaceans, and molluscs. Molluscs are not a popular food in India due to the lack of awareness combined with the conventional food habit of the people. In our country, people from coastal region use oysters, clams, mussels and a few gastropods as nutritious food. There is comparatively less demand for edible gastropods in India because most of the people do not know the nutritive as well as medicinal value of molluscan shellfish. But in other countries, especially South Malaya, Madagasker, Corea, Africa etc., gastropod meat is largely used as delicious food. This gastropods and

bivalves are rich sources of amino acids, fatty acids, vitamins and other biologically active compounds. In this context the present study entitled: **"Characterization and quantification of biochemical components of selected Molluscs species from the Kerala coast"** has provide a basic knowledge on the medicinal use and nutritional importance of some gastropods and bivalves.

Two types of molluscs such as gastropods and bivalves were collected for the analysis. Three gastropods such as *Bursa spinosa*, *Murex trapa* and *Tibia curta* and two bivalves such as *Villoritta cyprinoids* and *Perna viridis* were collected from Ashtamudi estuary, major fishing zone of south India. Ashtamudi is the second largest estuarine system in Kerala, South India included in the list of wetlands of international importance, as defined by the Ramsar Convention (2002) for the conservation and sustainable utilization of wetlands. It is a palm-shaped extensive water body with a water spread area of about 32 km^2. The marine gastropod*, Telescopium telescopium*, a large marine gastropod mollusc in the family Potamididae belonging to the genus, *Telescopium*. *T. telescopium* was collected from Pappinissery mangrove ecosystem, situated in the Kannur district, Kerala, India, that was covering a distance of 7−8 km from the coastline.In this station an extensive mangrove area of about 20 ha was found where species of *Avicennia, Rhizophora, Kandelia* and *Acanthus* are common. The main objectives of the thesis are:

1) To estimate the proximate and mineral composition of the soft tissue of gastropods and bivalves.
2) Characterizations of amino acids and alkanes from the soft tissue.
3) To determine the percentage composition of steroids and fatty acids in the species.
4) Bio-activity screening of fatty acids and steroids.

The thesis is divided into eight chapters,

Chapter 1, the introduction of the thesis, describes the various application of natural products derived from terrestrial as well as marine origin. The pharmaceutical applications of marine natural products, as well as the compounds which are in the current clinical trials, are also discussed. Economic importance of gastropods and bivalves were discussed in this chapter. Furthermore, the reviews of the literature on natural products were discussed in this chapter. A general introduction to gastropods and bivalves and its uses along with the aim, scope of this study are detailed.

Chapter 2, the materials and methods, deals with the species collected and analytical methodology adopted for the analysis. This chapter also discuss the sampling sites as well as its geographical importance.

Chapter 3, describes general biochemical characterizations (total protein, carbohydrate, lipid, ash and moisture contents) of the gastropods and bivalves. Calorific value and mineral composition of the species were discussed in this chapter. Calorific value gives an idea about the edible nature of the species. Moreover, permissible limits of all minerals in mollusc species were incorporated in this chapter.

Chapter 4, deals with the Characterization of amino acids from the soft tissue of gastropods and bivalves. Total 15 amino acids were identified from these species, of which eight are essential and seven are non-essential. Also described about the taste activated value of each amino acids identified from species.

Chapter 5, describes the Characterization of n-alkanes from the soft tissue of gastropods and bivalves. Alkanes were ranging from C_{11} to C_{27} in molluscs except *T. telescopium* and *P. viridis*. In

T. telescopium and *P. viridis,* alkanes ranging from C_{11} to C_{28} were observed. Phytoplankton and algae are the food material of these gastropods and bivalves. Characterization of n- alkanes gives information about the food habits of these organisms.

Chapter 6, illustrates the Characterization of steroids from the soft tissue of gastropods and bivalves. Seven sterols and one sterone were identified in the present study. Screening of biological activity of identified steroids was checked using online software, PASS (Prediction of Activity Spectra of Substances).

Chapter 7, describes the Characterization of fatty acids from the soft tissue of gastropods and bivalves. Saturated fatty acids, branched chain fatty acids, mono unsaturated fatty acids, polyunsaturated fatty acids and non-methylene interrupted fatty acids were identified in the present study. Anti-microbial as well as the anti-cancerous screening of polyunsaturated fatty acids separated from the crude fatty acid mixture of *M. trapa* and *B. spinosa* were discussed in this chapter. This chapter gives a baseline awareness of pharmacological importance of the fatty acids identified from the species.

Chapter 8, provides a brief summary and conclusion on the achievements of the study. This chapter summarises the nutritional importance of the gastropods and bivalves, which highlight the popularity of molluscs as a conventional food source. This chapter also indicates the scope of future work.

All the chapters end with the respective references. List of publications were illustrated in the Appendix

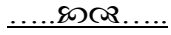
Contents

List of Abbreviations

AA	Amino acids
AAlk	Algal alkane
ACL	Average chain length
Ala	Alanine
ALA	Alpha-linolenic
Arg	Arginine
BCFAs	Branched-chain fatty acids
BS	*B. spinosa*
BSTFA	N, O-bis-(tri methyl silyl) tri fluoro acetamide
CH_3OH	Methanol
$CHCl_3$	Chloroform
CLC-Pred	Cell Line Cytotoxicity Predictor
CPI	Carbon preference index
Cys	Cysteine
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
DMEM	Dulbecco's modified eagles media
DMSO	Dimethyl sulfoxide
DPA	Docosapentaenoic acid
EAA	Essential amino acids
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
FA	Factor analysis
FAME	Fatty acid methyl esters
FAME	Fatty acid methyl ester
FBS	Fetal bovine serum
FDA	Food and drug administration
GC-FID	Gas chromatography - flame ionisation detector
GC-MS	Gas chromatography - mass spectrometry
GC-MS	Gas chromatography- mass spectrometry
GLA	Gamma-Linolenic acid

Glu	Glutamic acid
Gly	Glycine
H_2SO_4	Sulphuric acid
HCA	Hierarchical cluster analysis
HCC	Hepatocellular carcinoma
HCl	Hydrochloric acid
$HClO_4$	Perchloric acid
HEp-2	Human laryngeal carcinoma cell line
His	Histidine
HMW	High molecular weight n-alkanes
HPLC	High performance liquid chromatography
Ile	Isoleucine
IMP	Inosine monophosphate
KOH	Potassium hydroxide
LA	Linoleic acid
Leu	Leucine
LMW	Low molecular weight n-alkanes
Lys	Lysine
MeOH	Methanol
Met	Methionine
MRSA	Multidrug resistant *S. aureus*
MT	*M. trapa*
MTT	3-(4, 5 dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
MUFAs	Monounsaturated fatty acids
N_2	Nitrogen
NaOH	Sodium hydroxide
NCCS	National centre for cell science
NCI	National Cancer Institute
NEAA	Non-essential amino acids
NIST	National institute of standard technology
NMIs	Non-methylene interrupted fatty acids
PA	Palmitoleic acid
Pa	Probability to be active

PASS	Prediction Activity Spectra of Substances
PCA	Principal component analysis
Phen	Phenylalanine
PITC	Phenyl-iso-thiocyanate
Pro	Proline
PUFAs	Polyunsaturated fatty acids
PV	*P. viridis*
SCC	Squamous cell carcinoma
Ser	Serine
SFAs	Saturated fatty acids
Talk	Terrestrial alkane
TAR	Terrigenous to aquatic ratio
TAV	Taste active value
TC	*T. curta*
TEA	Triethylamine
TLC	Thin layer chromatography
TMTD	4,8,12- tri methyl tridecanoic acid
TT	*T. telescopium*
Tyr	Tyrosine
UV-Visible	Ultraviolet- visible
Val	Valine
VC	*V. cyprinoids*



Chapter 1

INTRODUCTION

Contents

Life on earth originated in the ocean, and it still continues in one or other form. So ocean is acknowledged as the mother of all living organisms. Oceans offer a huge biodiversity of flora and fauna (estimated to be over 5,00,000 species), which are more than twice of the land species (FAO, 2016). Quest for nutritive food sources is still in its higher research (Haldar et al., 2014; Alho & Reis, 2017). The source in search is mainly for highly nutritive and cheap cost food materials which support the commercial sector as a major stream of raw material (Solanki et al., 2017). Sea is the dependable resource for meeting these needs. Presently around 40% of the world's population lives within 100 km of the coast and it will increase to 70% in the next 50-100 years (FAO, 2016; UNEP, 2016). Thus, coastal ocean plays a vital role in human life and world economic system (Barbier et al., 2011; Murray et al., 2011; Yin et al., 2017). Coastal waters are most dynamic, nutrient rich and productive.

1.1 Coastal ecosystem

Coastal ecosystems are the place at which water and land joint together to create an environment with a distinct structure, diversity, and flow of energy. They cover approximately 8% of the total earth and are shelter for many different types of plants and animals (Victor & Lazarus, 2000; Frohlich-Nowoisky et al., 2016). Coastal ecosystem includes salt marshes, mangroves, wetlands, estuaries, coral reefs, and bays (Victor & Lazarus, 2000; Woodroffe, 2002; Din et al., 2017; Luna et al., 2017). Wetlands play a significant role in maintaining ecosystem functions globally. It is known as the most valuable natural ecosystem, because it significantly supports wildlife habitat. Wetlands provide water, food and shelter for all organisms, besides they have additional role in water quality improvement process, flood abatement process and carbon sequestration (Tiner, 2003; Costanza et al., 2006; Voyer et al., 2017). Wetlands produce fuel wood for cooking, fibres for textiles and paper making, thatch for roofing, and timber for building (Tiner, 2003; Smardon, 2014). The water found in wetlands is either freshwater or saltwater (Smith et al., 2007; Smardon, 2014). Swamps, marshes, bogs, and fens are the main and mangrove, carr, pocosin, and varzea are the subtype wetlands (Smardon, 2014; Din et al., 2017).

Estuarine systems are partially enclosed coastal body of brackish water with one or more rivers or streams flowing into it, and with a free connection to the open sea (Pritchard, 1967; Attrill & Rundle, 2002; Gireeshkumar, 2013). They are highly productive ecosystems, performing significant role in various ecological and biological functions (Dolbeth

et al., 2007; Treloar et al., 2017). Every estuary is unique in terms of their morphological features, climatic settings, tidal incursion and chemical processes, however all estuaries support diverse flora and fauna (Flemer & Champ, 2006; Garcia-Oliva et al., 2017; Pichler et al., 2017). Habitats of estuaries include freshwater and saltwater marshes, shallow open waters, swamps, mud and sand flats, sandy beaches, rocky shores, mangrove forests, river deltas, oyster reefs, tidal pools and sea grass beds (Flemer & Champ, 2006; Pichler et al., 2017). Estuaries are otherwise known as nurseries of the sea, because so many marine animals depend on them as a food source (Din et al., 2017; Garcia-Oliva et al., 2017). Many plants and animals are adapted to live in these unique environments. Estuaries are also a major layover point for migratory birds and animals (Boominathan et al., 2008; Garcia-Oliva et al., 2017). Moreover, coastal communities depend estuaries for tourism, shipping and transportation, and fishing (Treloar et al., 2017; Voyer et al., 2017).

Mangroves refer to any dozens of species of trees or large shrubs which grow within the intertidal zone in tropical and subtropical regions and have special adaptations to survive in this environment (Luna et al., 2017). Due to the halophytic nature, they provide better shelter for both marine and terrestrial fauna (Srikanth et al., 2016; Perri et al., 2017). Mangrove species have a number of mechanisms to remove or exclude salt from their tissues, and certain species have the ability to secrete salt from their leaves (Mendez-Alonzo et al., 2016; Srikanth et al., 2016). Furthermore, aerial roots of mangroves transport oxygen to underground roots located in the waterlogged anaerobic soil (Barbier, 2016; Méndez-Alonzo et al., 2016; Srikanth et al., 2016). Mangrove ecosystem contains

variety of migratory bird species and terrestrial animals (Gopal & Chauhan, 2006; Kirby et al., 2008; Roshnath & Sinu, 2017). Moreover underwater roots of mangroves support species such as algae, oysters and sponges which grow on the root surfaces and further increasing the available habitat niches (Méndez-Alonzo et al., 2016; Din et al., 2017). Mangrove forests play an important role in coastal people's trade and consumption, by providing fish, molluscs and crustaceans, also they are good source for fuel, timber, honey, medicines and fodder (Barbier et al., 2011; Barbier, 2016; Barbier, 2017). It has high economic values in different types of ecosystem services such as forestry, fisheries and tourism, also it protect from storm (Salem & Mercer, 2012; Barbier, 2017).

Salt marshes serve as the transition from the sea to the land, where fresh and salt water mixing occurs (Adam, 2002; Silliman, 2014). They play a vital role in the aquatic food web and the transport of nutrients to coastal waters. Also, they provide coastal protection for terrestrial animals (Woodroffe, 2002; Silliman et al., 2005). Salt marsh ecology composed of a variety of plants such as sedges, rushes and grasses which involves complex food webs comprising primary producers, primary consumers and secondary consumers (Vernberg, 1993; Dittami et al., 2017; Whitfield., 2017). Primary producers include vascular plants, diatoms, macro algae, phytoplankton and epiphytes. Primary consumers comprise zooplankton, molluscs, macrozoa and insects. Plants and animals living in this area have its own biochemical adaptations against physical stressors such as high salinity, intense temperature, and low oxygen in waterlogged soils (Silliman & Bertness, 2002; Silliman et al., 2005;

Rog & Cook, 2017). Salt marshes are one of the most strong and resistant ecological communities because, they protect other shoreline ecosystems from human activity (Silliman et al., 2005; Silliman, 2014; Roberts et al., 2017).

Coral reefs have an important role in world economic system (Costanza et al., 1997; Bellwood et al., 2004). They contribute fisheries in three ways; fishing in shallow coastal waters, fishing directly on the reef and fishing in offshore. These three types of fisheries support food webs, life cycles and productivity. Approximately one third of the world's fish species are observed in coral reefs (Jones et al., 1999; Jones et al., 2005; Birkeland, 2017). Moreover, coral reefs support booming tourist industries in many countries (Perri et al., 2017). Pennekamp state coral reef park from Florida, USA, 45 protected coral reef areas (including national parks) in Caribbean countries, coral reef from Oman and Cayman Islands are the major coral reef based tourism places in the world (Burt et al., 2016). Lakshadweep archipelago a Union Territory of India, is a major coral reef based tourism place in India (Nair et al., 2017). Furthermore, coral reefs are natural protective barriers, retarding storm waves, preventing beach erosion, allowing place for flourishing mangroves, and providing safe landing sites for boats (Adger et al., 2005; Pierre et al., 2017).

1.2 Resources from coastal ecosystem

Coastal resources comprise a wide variety of plants and animals such as fish, crustaceans, molluscs, seaweeds and other aquatic plants. There are approximately 29,000 species of fishes, 80,000-100,000 species of molluscs and 200,000 species of arthropods have been

reported to occur in sea (Hanly et al., 2017; Padghane et al., 2017). According to FAO (2016), there are approximately 49,861,891 tons of finfish, 16,113,194 tons of molluscs, 6,915,073 tons of crustaceans and 893,568 tons of other marine organisms were produced all over the world.

Phytoplanktons are the primary producers in the marine world and they play a vital role in food web (Iglesias-Rodriguez et al., 2008). Marine algae and seaweeds are the replenishable sources gifted to mankind for existence on this world (Ghosh et al., 2012). They flourish on water body, rocks, corals or any natural or manmade substrata, which have been widely used as a food as well as remarked resource of bioactive compounds (Manivannan et al., 2009; Cho et al., 2011). Coastal environment contains 6000 species of red seaweeds, 2000 species of brown seaweeds and 1200 species of green seaweeds (Bajpai, 2017). Large plants, sea graces and mangroves have an important role in the protection of land from natural calamities (Murray et al., 2011). Sea weeds such as *Gracilaria, Laminaria, Sargassum*, sea cucumber, sea urchin are widely used as aquaculture feeds (Qi et al., 2010). Algae, including both macroalgae (seaweeds) and microalgae (phytoplankton) are used alternatives to fishmeal in fish feeds (Ghosh et al., 2012; Tacon & Metian, 2015)

Fishery is the major marine resource, as it provides food security, job opportunities, income and livelihoods as well as traditional cultural identity (Hanly et al., 2017; Whitfield, 2017). They constitute almost half of the total number of vertebrates in the world. Coastal ecosystems, such as estuaries, marshes, shallow bays, wetlands, mangroves, coral reefs and seagrass beds, play a major role in the life cycles of many

economically important fish species by providing breeding, nursery and feeding grounds (Nagelkerken et al., 2002; Whitfield, 2017). Fishery resources display massive diversity of size, shape and biology and they live in almost all conceivable aquatic habitats (Tacon, 1995; Tacon & Metian, 2015; Hanly et al., 2017). Tunas and tuna-like species are collectively the most valuable fishery resources exploited in the high seas (FAO, 2011). Most wetland functions increase with the size of the area, as in the case of fish production (Alho & Reis, 2017). Crayfish, sardines, tuna, salmon and catfish are the major nutritional fish variety. The global total capture fishery in 2014 amounted to 93.4 million tonnes, whereby 81.5 million tonnes and 11.9 million tonnes came from marine and inland waters respectively (FAO, 2016). Thus, the net-export value of fisheries in developing countries is higher than the total of rice, coffee, sugar and tea together (Sahle et al., 2017). Fishery provides nutritional as well as pharmacologically important foods (Allison et al., 2009).

Approximately, 40% of fish are used for other purposes such as fishmeal to feed fish grown in captivity (FAO, 2016). In such cases, molluscs and crustaceans are widely used as an alternative source of nutritional food. Crab variety of crustaceans group includes king, blue and snow crab. It is also an excellent source of many nutrients including protein, vitamin B12, and vitamin C (Correia-da-Silva et al., 2017). Sea cucumber is a marine benthic organism which can be found in various ecosystems such as sea grass, coral reef, mangrove and mud flat. They have high value of nutrition and are used for traditional food, medicine and functional food, but recently it is listed as marine endangered species due to the over usage of this species (Zaenuri et al., 2016).

Like fish, molluscs are also delicious and protein rich food among the sea foods (Jagadis, 2005). In the geological time scale, molluscs evolved about 600 million years ago during the Cambrian period (Chen et al., 2004). The name 'Mollusca" was first used by Linnaeus in the year 1757 (Dodge, 1959; Kabat, 1990). Molluscs are soft-bodied animals and the word "mollusc" is derived from the Latin word "mollis" means "soft". Molluscs are the second largest phylum, having the architectural skill for developing their own shelter (Zhang & Zhang, 2006). The shelter or shell is made up of calcium carbonate and it provides protection for them (Weiss et al., 2002). Molluscs are the first living creatures to have hard shells and are widely distributed throughout the marine and estuarine ecosystems (Ruppert & Barnes, 1994). Majority of them are aquatic and a few are adapted to terrestrial environment. Structurally, molluscs are a heterogeneous group of animals with different structural form and they represents large variety of organisms such as snails, slugs, whelks, octopuses, squid, clams, scallops, oysters and chitons (Kabat, 1990). There are approximately 6000 species of gastropods and 15,000 species of bivalves have been reported to occur in sea (Prasanna et al., 2017). Gastropods and bivalves constitute approximately 98% of the total mollusc community (Jeyasanta & Patterson, 2017). Gastropods are the largest group of the molluscs, includes snails, conchs, abalones, whelks, sea slugs and garden slugs. Bivalves have a hinged, two-part shell joined by strong muscles and it includes clams, oysters and scallops (Ponder & Lindberg, 1997; Kocot et al., 2011). Both gastropods and bivalves are filter feeders and sedentary in nature (Turon et al., 1997). Gastropods, bivalves and oysters are a rich source of protein and omega-3 fats and

shrimps are a low-fat, low-calorie and high iodine content shellfish (Shanmugam & Sambasivam, 2007; Periyasamy et al., 2014; Husain et al., 2017).

Molluscs have a tremendous role in Indian tradition and economic system (Thomas, 2015a). They are used as medicines, currency, ornaments, and as mascots to wars (Mayer, 2015). In India, several industrial cottages export mollusc's shells to foreign countries in the form of polished shells, utilitarian objects and hand-crafted shells. Moreover in Indian coast, they are used as a symbol of social status and great pride (Boominathan et al., 2008). Peoples in coastal area make necklaces, rings, ear rings, studs, table lambs, bathi stands, eve chains, bangles, ash trays, key chain pendants and curtains using this molluscs shell (Gutierrez-Zugasti & Cuenca-Solana, 2015). In Southeast Asia, the shell-craft industries still use thousands of tons of shells annually for making ornaments (Boominathan et al., 2008).

1.3 Biodiversity of molluscs

Molluscs have the ability for colonizing all possible habitats, which extended from deep sea to high mountains. The annual production of marine mollusc in 2012 is 15.2 million metric tonnes and it accounted for about 22.8% of the total (inland and marine) aquaculture production (FAO, 2014). World's over all annual mollusc production is varying from 0.97-2.7% (FAO, 2016). Douglass (2017), studied the distribution and abundance of *Fasciolaria trapezium* along the Southwest Madagascar. Latama & Nessa (1994), detected the gastropods abundance around Kodingareng Keke Island. Padula et al. (2017) studied the species

abundance of molluscs in south western Atlantic region. Molluscs such as *Amathina tricarinata, Petricola hemprichi* and *Cardites akabana* widely distributed in Iskenderun Bay, SE Turkey (Çeviker & Albayrak, 2006). Fernandes & Pimenta (2017) recorded 243 species of gastropods from the Eastern Brazillian shelf zone. Saeedi et al. (2017) reported 379 bivalve species and 895 shelled gastropods from Antarctica and Sub-Antarctic Region. Jeyabaskaran et al. (1996) studied the distribution of molluscan cryptofauna of Karaichalli Island. Ng et al. (2017) observed 130 muricid species in the South China Sea. A survey in the year 2009 identified 24 bivalves and 29 gastropod species from the Gulf of Tehuantepec (Rios-Jara et al., 2010) and 15 species of bivalves and 13 species of gastropods at different depths of Palk Strait, south east coast of India (Karthikeyan et al., 2009).

Bivalve and gastropod resources of 12 major centres are Karwar, Mangalore, Calicut, Kochi, Kollam, Kanyakumari, Goa, Tuticorin, Rameswaram, Mandapam, Chennai and Visakhapatnam (Shoji et al., 2005; Abdellaoui et al., 2017). Large quantities of two varieties of gastropod species such as *Turbo* and *Trochus* were observed in Andaman Islands (Mohamed & Venkatesan, 2017). Shyam et al. (2017), studied the distribution of *Architectonica laevigata, Chicoreus virgeneus, C. ramosus and Murex tribulus* in Indian waters. Appukuttan & Babu Phillip in the year 1994 described the gastropods in Neendakara and Sakthikulangara of Quilon area and its present status is established by Mohamed & Venkatesan (2017). 103 mollusc species were observed in Tuticorin, south east coast of India (Jeyasanta & Patterson, 2017). Sonak (2017), reported 13

species of bivalves and only one species of gastropod in the estuarine system of Goa, west coast of India.

1.4 Major biochemical constituents of molluscs

Nutritional value of an organism is reflected in the biochemical composition (Renitta, 2005; Margret, 2015). Biochemical analyses play a vital role on physical growth and development, physical activity, maintenance of normal body function and health (Haldar et al., 2014; Olenchock et al., 2017; Shabana et al., 2017). Like all organisms, carbohydrate, proteins and lipids are the major biochemical constituents in molluscs. Biochemical composition of molluscs is affected by water, temperature, nutrient availability and reproductive cycle (Renitta, 2005; Margret, 2015; Olenchock et al., 2017; Shabana et al., 2017). Carbohydrates are the major sources of energy in all human diets, consisting of carbon, hydrogen and oxygen units combined in varying configurations. Carbohydrate is essential for all organisms, but its ratio is less when compared to proteins and lipids in all animals, especially in aquatic animals (Periyasami et al., 2014; Margret, 2015; Olenchock et al., 2017).

Proteins are the fundamental biomolecules of cell in all the living organisms and its quality is usually assessed by using amino acid composition. It is a good source of energy for the development of muscles and other tissues in the body (Fagbuaro et al., 2006). Analysing the composition of amino acids helps to assess the nutritive value of an organism. Nitrogen requirement of an organism is also supported by the proteins. Also, they serve a major role in the production of hormones,

enzymes and haemoglobin. Amino acids are the building blocks of proteins which are essentially organic compounds consisting of amino as well as acidic groups. They contribute a considerable role in the health of the human nervous system, hormone production, cellular structure and muscular structure (Furst & Stehle, 2004). There are 20 naturally occurring amino acids and they comprises valine, phenylalanine, threonine, leucine, tryptophan, methionine, isoleucine, lysine, histidine, arginine, cysteine, glycine, glutamine, proline, tyrosine, alanine, asparagine, glutamic acid, aspartic acid and serine (Furst & Stehle, 2004). The absence of any of these amino acids will affect the capability of tissue to grow, be repaired or be maintained (Reeds, 2000). Molluscs have the ability to synthesize one or more of the essential amino acids (Peters et al., 1997; Monroig et al., 2016).

Lipids are the substances which dissolve in alcohol but not in water. They include alkane, alkene, steroids, fatty acids, etc. (Chakravarty et al., 2015; Giftson & Patterson, 2016). Alkanes and alkenes are the least polar lipid component, composed only of carbon and hydrogen. They are mainly present in petroleum compounds. Moreover, certain eukaryotes and prokaryotes derived hydrocarbons from fatty acids. They are usually straight chain molecules, but some methyl-branched alkanes found in insect species (Fujibayashi et al., 2016; Wang et al., 2016). Odd chain alkanes from C_{15} to C_{33} are the principal lipid fraction of plants (Desurmont et al., 2016; Machado et al., 2017). Most of the species in the marine world contain alkanes ranging from C_{15} to C_{20}, of which C_{17} predominant (Ahmed et al., 2014; Li et al., 2017; Machado et al., 2017). Most of the alkanes present in the molluscs are mainly derived from its-

own environment, since they are filter feeders (Gomiero et al., 2015; Nesvacil et al., 2016). Also, during peroxidation processes minute amounts of alkanes are formed from fatty acids (Foo et al., 2017).

Steroids are predominant lipid components with saturated tetracyclic hydrocarbon group. They are highly diverse group of metabolically active compounds with a fused cyclopentanophenanthrene ring with a 3-hydroxyl moiety. The "usual" sterols observed in marine organisms have a 3β-hydroxy-Δ5- (or Δ0-) cholestane nucleus and a C8-C10 side chain (Ekins et al., 2007; Dagorn et al., 2014; Ozogul et al., 2015). There are over 200 such sterols, occurring in marine organisms as complex inseparable mixtures, of which cholesterol is usually dominated (Blunt et al., 2014; Joy et al., 2017). The quality and quantity of steroid composition may vary between mollusc species. Bivalves contains large variety of sterols like brassicasterol, crinosterol, 24-methylenecholesterol, sitosterol, stigmasterol, etc. (Pereira et al., 2013; Sun et al., 2014), but cholesterol is the only sterol occurring in cephalopoda and pelecypoda. Numerous other sterols were detected in the gastropod, but in such small quantities.

Principal constituents of lipids are fatty acids. Fatty acids are an organic compound having a hydrocarbon chain with a terminal carboxyl group. They are classified on the basis of chain length, degree of unsaturation, geometry and position of the double bonds. They are broadly classified into saturated fatty acids (SFAs), unsaturated fatty acids, branched chain fatty acids (BCFAs), monounsaturated (MUFAs) and polyunsaturated fatty acids (PUFAs) based on their structure. Each

variety of fatty acids shows its own characteristic property. Most of the sea foods, especially mollucsc are rich source of n3 fatty acids (Gomez-Estaca et al., 2017). Fatty acids are essential for life, because they play an important role in hormone production and transportation of fat soluble vitamins such as vitamin A, D, E and K (Ziboh, et al., 2002; Zhukova, 2014; Monroig et al., 2016). Also they are good source of energy, membrane constituents, as well as metabolic and signalling mediators (Colombo et al., 2016; Munekata et al., 2017).

Like the biochemical constituents, minerals are also essential component in living organisms for their biological metabolism and growth (Yusoff & Long, 2011; Hossen et al., 2015). Ca, Mg, Fe, Mn, Cu, Ni, Zn, etc. are essential for numerous biological functions such as fluid regulation, muscle movement, nerve functioning and bone structure (Margret et al., 2013). The knowledge of the biochemical and mineral composition of any newer species will be more significant, because it reflects the edible nature of those organisms.

1.5 Importance of molluscs

1.5.1 In food industry

Many marine molluscs are used as nutritive food source all over the world. Bivalves, cephalopods and gastropods contribute the majority of the molluscan fishery (FAO, 2015; FAO, 2016). Marine gastropods such as *Cookia sulcata, Chicoreus ramosus, Rapona venosa, Babylonia spirata* and bivalves such as *Crassostrea rhizophorae, P. viridis, Cerastoderma edule, Ostra edulis, Meretrix casta, Protothaca theca,* and *Mytilus galloprovincialis* are the major worldwide nutritionally

important molluscs (Periyasamy et al., 2011; Olmedo et al., 2013; Smoothey, 2013; Celik et al., 2014). Pictures of common mollusc used in food industry were depicted in Figure 1.1. Isotope studies of human bones from Atlantic coast of Europe revealed that marine molluscs were the major food resources in Mesolithic period (Dupont et al., 2009). Also, they were the major diet resource of peoples from Caribbean, San Francisco and Mediterranean region in Neolithic period (Colonese et al., 2011; Choy et al., 2012; Mannino et al., 2012).

Turban snails such as *Turbo militaris, Lunella torquata*, and *L. undulate* were used as a valued food in the coastal areas of Japan, Australia, Korea, and China (Smoothey, 2013; Mason et al., 2014; Saito & Aono, 2014). Peoples from Japan and Korea obtained approximately 50% of their total sea foods from molluscs. Moreover, marine molluscs played a minor role in Holocene sites in Korea (Krigbaum et al., 2013; Thomas, 2015b). Biochemical composition studies on marine gastropods such as *B. spirata, B. spinosa* and *Mytilus galloprovincialis* indicate that they are highly nutritional (Shanmugam et al., 2006; Babu et al., 2010). Marine shrimps were used as nutritive food in India. Also, they are used for making fish meal, because they have high energy value (Appukuttan & Babuphilip, 1994). Fresh water bivalve mollusc, *Unio elongatulus* are widely used as delicious food in Turkey (Ekin et al., 2011). Marine bivalves such as *Tegillarca granosa, C. ramosus* and *Xancus pyrum* were used for making sausage and pickle in India (Patterson et al., 1994). *Peronia verruculata* is a marine gastropod widely used as a nutritive food in the coastal areas of India (Solanki et al., 2017).


B. spirata


C. ramosus


C. sulcata


M. galloprovincialis


O. edulis


P. viridis


R. venosa


C. edule

Figure 1.1: Some nutritionally important molluscs

1.5.2 As traditional medicine

A large number of mollusc species have been widely used all over the world, as a source of traditional medicines for the treatment of inflammatory diseases (Alessandri et al., 2013). Also, they were extensively used as indigenous medicines for the treatment of obesity, multiple sclerosis, chronic obstructive pulmonary disease, asthma, rheumatoid arthritis, neurodegenerative disease, atherosclerosis and inflammatory bowel disease (Nathan & Ding, 2010; Benkendorff et al., 2015). In Latin American countries, molluscs were widely used as ethnomedicine for the

treatment of asthma, stomach pain, osteoporosis, skin ulcers, influenza, pain relief tuberculosis and pneumonia (Alves & Alves, 2011). The operculum of gastropod species such as *Chicoreus ramous*, *Lentigo lentiginous* and *Chicoreus virgineus* was traditionally used by Meddle East countries for the treatment of skin disease, wounds in stomach, arthritis, and eye and ear diseases (Lev, 2007). Marine garsropods such as *Hexaplex trunculus, Bolinus brandaris, Charonia tritonis* and *Stramonita haemastoma* were used by Roman as anti-inflammatory agents (Voultsiadou, 2010). Marine molluscs were used as a traditional medicine for chest infection, knee pain and treatment for tropical ulcers in North east Brazil, Korea and Europe (Voultsiadou, 2010; Kim & Song, 2013). Bivalves such as *Crassostrea rhizophorae, Neoteredo reynei, Lyrodus pedicellatus, Anomalocardia flexuosa, Megalobulims oblongus* and *Cassis tuberosa* were commonly used in Latin America for the treatment of osteoporosis, flu, asthma and tuberculosis (Alves & Alves, 2011). Both the shell and flesh of two bivalves such as *Pecten* spp. and *Mytilus galloprovincialis* were used as traditional medicines in Ancient Greece for the treatment of cystitis, sores and wounds (Voultsiadou, 2010). Shell and flesh of two bivalves viz., *Anodontities trapesialis* and *Mytilus unguiculatus* were used in Argentina and Korea as a ethnomedicines for the treatment of fever, skin wounds and injuries (Kim & Song, 2013).

In India, molluscs have been used in the preparation of Ayurveda medicines since long time. Sankhabhasma is an Ayurveda medicine prepared from molluscs and used against rickets and asthma (Babu et al., 2010). In India, *Monetaria moneta* (gastropod) has been used for the treatment for asthma and *Zimbabwe* snail shells were used to treat topical

ulcers (Krishna & Singh, 2012). Also, *Bellamya* sp. (gastropod) was used for treating asthma, joint pain arthritis and rheumatism (Prabhakar & Roy, 2009). Moreover, extracts prepared from powdered oyster shell as well as cowry shell are used in homeopathic medicines (Babu et al., 2012). *Filopaludina* sp. was commonly used in all over the India, for the treatment of conjunctivitis and asthma (Prabhakar & Roy, 2009; Krishna & Singh, 2012). Pictures of common mollusc used as traditional medicines, were shown in Figure 1.2. Pearl oyster (*Pinctada margaritifera*) was used all over the India, for the treatment of asthma and tuberculosis (Gopal et al., 2008). Operculum of *P. cochllidium* is used in unani drugs (Babu et al., 2010).

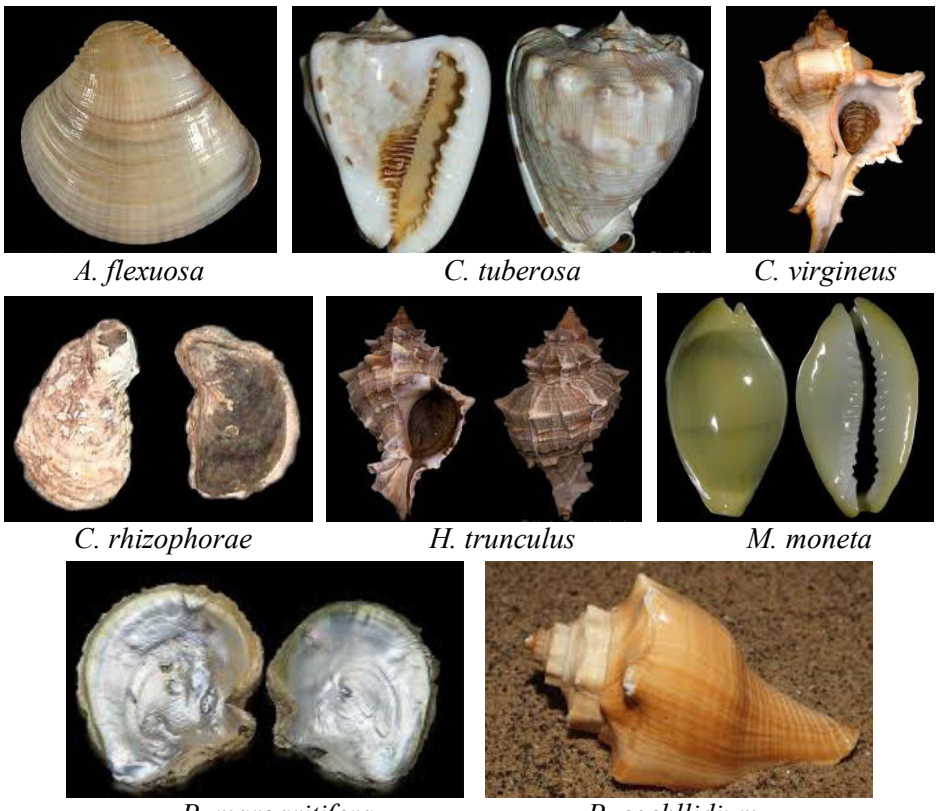


Figure 1.2: Molluscs commonly used as traditional medicines

1.5.3 In pharmaceutical industry

Marine invertebrates such as echinodermata, porifera, mollusca, cnidarian and arthropoda have the ability to produce secondary metabolites, including terpenes, amino acids, aliphatic hydrocarbons, steroids, alkaloids, carbohydrates, fatty acids and peptides (Senthilkumar & Kim, 2013). Molluscs use these secondary metabolites for both communication and protection from predators (Benkendorff, et al., 2015). Also, they use these bioactive compounds to protect them against microbial pathogens. Moreover, molluscs acquired secondary metabolites for healing wounds in the microbial-rich marine environment (Dang et al., 2015). These marine natural products or secondary metabolites have wide range of therapeutic properties such as antihypertensive, anti-inflammatory, anticoagulant, antioxidant, antimicrobial, wound healing and immune modulating, anticancer and other medicinal properties (Perdicalis et al., 2013; Senthilkumar & Kim, 2013). Thus, a number of marine derived natural products are now used in the preparation of novel drugs (Ahmad et al., 2018). Pictures of common mollusc used in pharmaceutical industry were illustrated in Figure 1.3. In the last three decades, researchers have keen interest on the secondary metabolite isolated from molluscs and a total of approximately 1,145 natural products were isolated from mollusc species (Benkendorff, 2010; Benkendorff, et al., 2015; Mayer et al., 2017). There are at least 18 other compounds originally found in molluscs and associated cyanobacteria that are currently in clinical trials (Mayer, 2017; Ahmad et al., 2018).

Ziconotide (an effective drug for the treatment of chronic pain) (Mayer et al., 2010; Patel et al., 2017) and Brentuximab vedotin (effective drug for the treatment of lymphoma and Hodgkin's disease) (Brown et al., 2017) were the two drugs isolated from molluscs, also they were tested and approved by Food and Drug Administration (FDA) (Mayer et al., 2017). Ziconotide is first isolated from the cone snail *Conus magus* (Schroeder et al., 2004) and has been commercialised under the name of Prialt (Atanassoff et al., 2000; Svenson, 2013; Ahmad et al., 2014). Brentuximab vedotin was first isolated from marine gastropod, *D. auricularia* (Senter & Sievers, 2012). Tetrodotoxin, a highly potent neurotoxin has been specifically reported from marine gastropod (*Charonia lampas*) from Portugal (Rodriguez et al., 2008). Moreover, it was present in mussels (*Mytilus edulis*) and oysters from England, Greece and Netherlands (Turner et al., 2015; Vlamis et al., 2015; Turner et al., 2017). Zalypsis is an alkaloid first isolated from the skin and mucus of the Pacific nudibranch *Jorunna funebris* and has potent ant myeloma activity (Malve, 2016). Elisidepsin and glembatumumab vedotin were the peptides isolated from mollusc (*E. rufescens*) and they were widely used for the treatment of breast cancer and melanoma (Hamann et al., 1996; Ott et al., 2016). Pinatuzumab vedotin and Tisotumab vedotin were also isolated from marine molluscs and were used as drug for chronic lymphocytic leukaemia (Advani et al., 2016; Ruiz-Torres et al., 2017). Lyprinol and Seatone were isolated from green lipped mussels and they were used as promising anti-inflammatory agents (Ahmad et al., 2018). Biolane is a drug isolated from marine mollusc and widely used for healing the injury of soft tissues in healthy

people (Chen et al., 2017). PCSO-524 is a drug isolated from the lipid extract of *P. canaliculus* (Ahmad et al., 2018). Kahalalide F, a good anti-cancer agent was extracted from marine mollusc *Elysia rufescens* (Hamann et al., 1996; Jo et al., 2017). Keenamide A (a cytotoxic drug) was isolated from the marine mollusc *Pleurobranchus forskali* (Wesson & Hamann, 1996; Ruiz-Torres et al., 2017). Spisulosine ES-285 (a cytotoxic drug) derived from marine mollusc *Spisula polynyma* (Jo et al., 2017). Dolastatin 10 and Dolastatin 15 (linear peptides having anti-cancer properties) were isolated from *Dollabella auricularia* of Indian Ocean (Mayer & Gustafson, 2003; Yamada et al., 2010).

C. magus *S. polynyma* *E. rufescens*

Figure 1.3: Molluscs commonly used in pharmaceutical industry

1.5.4 Other industrial uses

Molluscs shells have 33 to 40% calcium, of which 90 to 98% are in the form of calcium carbonate. So they are widely used as the major source of raw material for the lime industries. Shell grit forms an important ingredient in the preparation of dental cream, talcum powder and in carbide industry (Boominathan et al., 2008). Shells of the *Placuna Bruguiere, Spirula Lam* and of cockles (*Chiefly Cardiidae*), were used in the manufacture of tooth pastes (Boominathan et al., 2008). Elicina is a

cosmetic skin repair cream which is isolated from mucus secretions of brown snail *Helix aspersum* (Ahmad et al., 2018). Pictures of common mollusc used for industrial purpose were shown in Figure 1.4. Dye produced from the sea snail (murex) was used in textile industry (Sukenik et al., 2017).

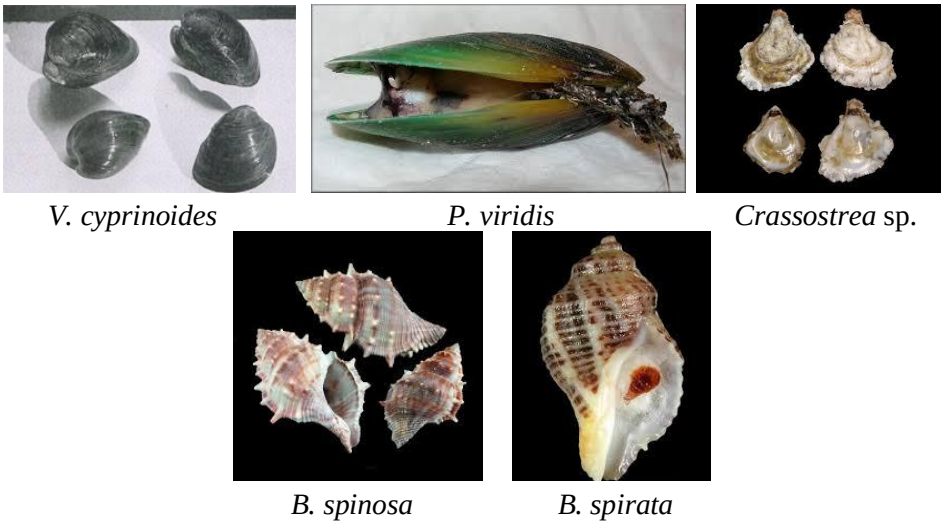
P. Bruguiere *C. Cardiidae* *H. aspersum*

Figure 1.4: Molluscs commonly used in industrial purpose

1.5.5 As ornaments

Molluscs possess a valuable role in world economy, as they are used widely in shell craft industry for making ornaments especially beads and pendants (Vanhaeren et al., 2006; Mayer, 2015; Gutierrez-Zugasti & Cuenca-Solana, 2015). Marine gastropods are widely used as ornaments in Singapore (Ng et al., 2014). Also molluscs have a marvellous impact on Indian tradition and economy, since they are used in making ornaments and currency (Thomas, 2015a). More over pearl oysters are major economically important species in Lakshadweep, Gulf of Mannar and Andaman (Venkatraman et al., 2004; Boominathan et al., 2008). Marine molluscs were widely used in the ornamental industry in Kanyakumari, Rameshwaram, Mahabalipuram, Fortcochi, Kollam, etc. (Appukuttan, 2008). Furthermore, ornaments made by molluscan shells are

becoming highly priced objects in Indian and foreign markets (Thomas, 2015a). Pictures of common mollusc used for making ornaments were shown in Figure 1.5. Marine molluscs such as *C. discoidea, C. cucullata, P. malabarica, K. opima, Meretrix* sp., *V. cyprinoides, P. viridis, Crassostrea* sp., *B. spinosa* and *B. spirata* were commonly used as ornaments in coastal areas of Kutch, Dwarka, Bombay, Ratnagiri, Jaytapur, Kerala, Tamilnadu, Karwar, etc. (Thomas, 2015b).



V. cyprinoides *P. viridis* *Crassostrea* sp.

B. spinosa *B. spirata*

Figure 1.5: Molluscs commonly used as ornaments

1.5.6 As sentinel organism

Many species of molluscs are used for the mussel watch program because they fulfil the qualities of an ideal sentinel organism (Sures, 2004): they have a wide geographical distribution, are sensitive to many contaminants but tolerant to a large range of abiotic factors, can be maintained and experimented on the laboratory, and have a sedentary nature

that helps in definitely representing the local pollution (Turon et al., 2014). Levels of all the elements in these species have great relevance because they can help to monitor the variation in the concentration and accumulation of all elements in the biota. In recent years, investigators have focused their attention on placing other possible organisms such as gastropods and bivalves for assessing trace metal pollution (Jakimska et al., 2011; Bille et al., 2015). In the past two decades, researchers have been using different gastropod molluscs, namely *B. nanum, D. trunculus,* and *C. gallina* (Usero et al., 2004), and bivalves, namely *P. grandis, C. angulata, S. plana, P. longirostris, U. tangeri, M. kerathurus, C. virginica, R. ovate, R. venosa*, and *N. didyma*, as sentinel organisms (Apeti et al., 2005; Bonneris et al., 2005; Gupta & Singh, 2011). Pacefic oyster, *Crassostrea gigas* was used for monitoring environmental conditions of the Bizert lagoon (Dridi et al., 2007). Pictures of common mollusc used for monitoring purpose were shown in Figure 1.6. Marine mussel, *M. galloprovincialis* was widely used in Ghazaouet harbour as sentinel organism (Touahri et al., 2016). *M. galloprovincialis, M. edulis* and *M. trossulus* were used for monitoring Cu in Southern Europe Baltic and North Atlantic coast of America (Brooks et al., 2015). *Mytilus galloprovincialis* were used for the assessment of pollution level in Gulf of Trieste and Bay of Brest (Lopez-Galindo et al., 2014; Rocha et al., 2015; Lacroix et al., 2017). Mussels (*Mytilus* spp.) have been widely used for monitoring purpose mainly due to their wide distribution, and also because they tend to accumulate the pollutants contained in the water column, reaching very high concentrations (Kimbrough et al., 2008; Lacroix et al., 2015; González-Fernandez et al., 2016).

M. galloprovincialis *M. edulis* *M. trossulus*

Figure 1.6: Molluscs commonly used as sentinel organism

1.6 Aim, scope and objective of the study

Malnutrition is a serious problem across the world. According to FAO, there were 795 and 194.6 million undernourished people in the world and India respectively (FAO, 2016). Fishery and aquaculture resources would provide alternative source of food. Dietary resources from many marine organisms (both flora and fauna) are currently in high demand, because they are considered healthy, nutritional and possess pharmacological values. Coastal zone is an essential part of marine life, because approximately 95% of the world's marine production originates from coastal ecosystems (Din et al., 2017). Moreover, 40% of the world's population lives in coastal area. Coastal and marine environments can begin up to 100 km inland, extend to the continental shelf, and include ocean systems with waters up to 50 meters in depth (Barbier, 2017). India is blessed with a coast line of 7,516.6 km and has rich marine fishery resources consisting chiefly of fishes, crustaceans and molluscs (Basha & Rao, 2017). Basic resources comprise fuel, water and fertile land, which are essential requirements for housing, energy and food security. Seafood prominently includes fish and shellfish. Shellfish

includes various species of molluscs, crustaceans, and echinoderms. According to FAO (2014, 2016), the apparent fish consumption (per capita) has increased from of 9.9 Kg to 20 Kg. Also, fish and fish products are now transferred to different places, and hence its quality is becoming much more important, which reflects on its price. So various another sources such as crustaceans and molluscs were used for balancing the scarcity of fishery products. In this context, low fat-high protein marine molluscs were investigated to fulfill these demands.

The present study aims for the characterization and quantification of major biochemical components of some molluscs (specifically gastropods and bivalves) collected from certain areas of the Kerala coast. Furthermore, this study targets initial screening of bioactive compounds isolated from the same species. Detailed literature collections on gastropods and bivalves encouraged this effort and are observed to be novel. From this angle, detailed biochemical compositions are required to assess the nutritional quality of both gastropods and bivalves. General biochemical composition or proximate analysis includes the quantification of total carbohydrates, proteins, lipids, ash content and calorific value. From these data, the extent of nutritional index of molluscs can be evaluated. Major secondary metabolites such as amino acids, fatty acids and steroids were determined and these conveyed the nutraceutical and therapeutical qualities of both gastropods and bivalves. The therapeutical qualities were supported with their antimicrobial and cytotoxic activities too. Six species of molluscs were collected from various locations in Kerala coast. It includes four gastropods viz., *Telescopium telescopium, Murex trapa, Bursa spinosa, Tibia curta* and two bivalves viz., *Perna*

viridis, *Villoritta cyprinoids* The major objectives of the present study can be summarized as follows:

- Evaluation of the general proximate compositions such as total proteins, lipids, carbohydrates, ash, moisture and calorific values of four gastropods and two bivalves
- Investigation of the mineral compositions of gastropods and bivalves
- Characterization and quantifications of amino acids and alkanes
- *In silico* biological activity studies of steroids isolated from molluscs
- Extraction and characterization of fatty acids in both gastropods and bivalves
- Antimicrobial and cytotoxicity screening of polyunsaturated fatty acids separated from the crude extract of *M. trapa* and *B. spinosa*.

In short, the thesis highlights the scope of gastropods and bivalves as potential sources of proteins, carbohydrates, lipids, amino acids, fatty acids and steroids which are needed for human and animal nutrition. In the present scenario of over exploitation of fishery products, these species can be recommended as low fat, high protein dietary resource.

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Chapter 2

MATERIALS AND METHODS

Contents

2.1 Sampling location

Marine molluscs were collected from six locations in Kerala coast, according to the availability of the species. The sampling locations are shown in Figure 2.1. Gastropods *viz., Tibia curta, Murex trapa,* and *Bursa spinosa* and bivalves viz., *Perna viridis* and *Villorita cyprinoids* were collected from five different stations (S1, S2, S3, S4 and S5 respectively) of Ashtamudi estuary situated in Kollam district of Kerala state, India. S1 lies in the barmouth region of Neendakara (Latitude: 8° 56' 40"N. Longitude: 76° 32' 25"E) and S2 is the old Neendakara fishing harbour (Latitude: 8° 56' 13.1"N. Longitude: 76° 32' 30.9"E). S3 is located in Sakthikulangara barmouth (Latitude: 8° 55′ 30″N. Longitude: 76° 33′ 22"E). S4 and S5 are located in Dalavapuram (Latitude: 8° 94′ 87"N. Longitude: 76° 55′ 03"E) and Chavara Thekumbhagom (Latitude: 8° 96′ 67"N. Longitude: 76° 56′ 33"E) respectively (Figure 2.1). Ashtamudi estuary is the second largest estuarine system in Kerala included in the list of wetlands of

international importance, as defined by the Ramsar Convention (2002) for the conservation and sustainable utilization of wetlands. It is a palm-shaped extensive water body with a water spread area of about 32 km^2 and eight prominent arms, adjoining the Kollam town. Neendakara is a famous fishing centre in South India and fishery is a major source of income for the fishermen community. Three sites viz., S1, S2 and S3 are from located near the Neendakara fishing harbour; hence most of the mechanised fishing trawlers operated in the Neendakara zone is on-board in this site. Oil spillage from mechanised boat is a major source of pollution on this site. S4 and S5 have located away from barmouth region; hence pollution is less compared to the other sites. Bivalves were collected from the fresh water region of Ashtamudi. A little variety of mangroves was found near Dalavapuram Bridge.

The gastropod *Telescopium telescopium* was collected from Pappinissery mangrove ecosystem (S6) (Latitude: 11° 56' 8" and Longitude: 75° 21' 13") (Figure 2.1), situated in Kannur district of Kerala state (India), that was covering a distance of 7 − 8 km from the coastline. On the eastern side of the Valapattanam highway bridge, an extensive mangrove area of about 20 ha was found where species of *Avicennia, Rhizophora, Kandelia* and *Acanthus* are common. The creeper *Derris trifoliata* was seen along with the isolated growths of *Aegiceros corniculatum*, while on the northern side isolated patches of *Kandelia candel* were also found. Land hermit crabs belong to the family coenobitidae were richly present in this area. These crabs were tucking inside the body of *T. telescopium* for protection and carry it with them wherever they go.

2.2. Molluscs species

A total of six species were collected, of which four species belonging to the gastropod variety and two belonging to the bivalve variety. Taxonomy of these species is given in Table 2.1. All the species (Figure 2.2) were identified by experts and were kept in the deep freezer.

Table 2.1: Hierarchical classification of molluscs selected for the present study

Species	Genus	Family	Class	Phylum	Kingdom
Murex trapa	*Murex*	*Muricidae*	Gastropoda	Mollusca	Animalia
Tibia curta	*Tibia*	*Strombidae*	Gastropoda	Mollusca	Animalia
Bursa spinosa	*Bursa*	*Bursidea*	Gastropoda	Mollusca	Animalia
Telescopium telescopium	*Telescopium*	*Potamididae*	Gastropoda	Mollusca	Animalia
Perna viridis	*Perna*	*Mytilidae*	Bivalvia	Mollusca	Animalia
Villoritta cyprinoids	*Villorita*	*Corbiculidae*	Bivalvia	Mollusca	Animalia

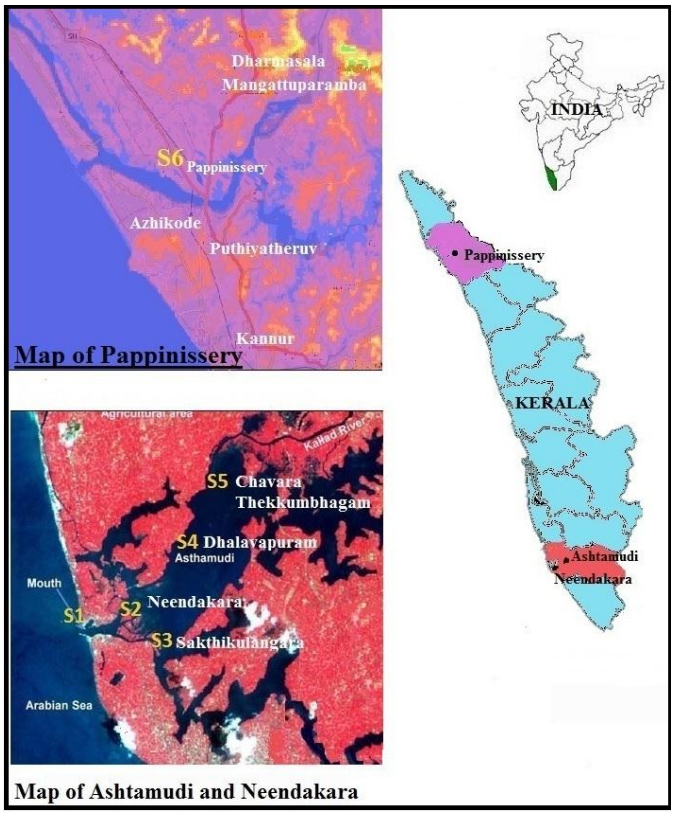

Figure 2.1: Map of Ashtamudi and Pappinissery estuary showing sampling locations

2.3 Sampling technique

The mangrove snails, *T. telescopium* were collected by hand picking, and the remaining gastropods (*B. spinosa, M. trapa, T. curta*) and bivalves (*P. viridis, V. cyprinoids*) were collected with the help of fishing net. All the samples were washed in tap water, and the soft bodies were carefully removed from the shell and stored in a deep freezer.

Figure 2.2: Species collected for the present analysis

2.4 Analytical methodology

The freeze dried tissue samples were ground well to fine powder under cold condition. The fine powdered tissue samples were kept in glass bottles and stored in the deep freezer until analysis. The dried samples were either used directly or extracted using an appropriate solvent for the determination of various parameters.

2.4.1 Biochemical composition

2.4.1.1 Protein

Total protein contents of marine molluscs were determined using a spectrophotometer (Lowry et al., 1951; Sadasivam & Manickam, 1996) after extracting the proteins with dilute alkali. 10 mg of dried, homogenised tissue sample was taken in a boiling tube; 15 mL of 1N NaOH was added and heated for 1 h. After cooling to room temperature, the volume was made up to 15 mL with 1N NaOH. 1 mL of the extract was pipetted into a separate stoppered test tube and 5 mL of freshly prepared alkaline copper tartrate reagent was added followed by 1 mL of 1:1 Folin–Ciocalteu reagent. It is mixed thoroughly and allowed to stand for 15 minutes for colour development. The absorbance was then read at 750 nm against a reagent blank using UV-Visible spectrophotometer (Analytik Jena Specord 200 plus). Bovine serum albumin was used as standard with multi point calibration yielding the correlation factor r^2 as 0.999. Results were expressed as percentage of dry weight of tissue.

2.4.1.2 Carbohydrate

Total carbohydrate contents of marine molluscs were determined using a spectrophotometer following the phenol-H_2SO_4 method (Sadasivam & Manikam, 1996) and the carbohydrate from tissue matrix was extracted using H_2SO_4. 10 mg of dried soft tissue were taken in a boiling tube, 15 mL 1N H_2SO_4 was added and heated in a boiling water bath for 3 h. After cooling to room temperature, the volume was made up to 15 mL with 1N H_2SO_4. 1 mL of the extract was pipetted into a separate stoppered test tube and 1 mL of 5% phenol was added followed by rapid addition of 5 mL of conc. H_2SO_4. After cooling the mixture in

the test tube, absorbance was measured at 490 nm against a reagent blank using UV-Visible spectrophotometer (Analytik Jena Specord 200 plus). Glucose was used as standard with multi point calibration yielding the correlation factor r^2 as 0.999. Results were expressed as percentage of dry weight of tissue.

2.4.1.3 Lipid

Total lipid contents of marine molluscs were determined using a spectrophotometer by sulfo-phospho-vanillin assay after its extraction using $CHCl_3$: CH_3OH (2:1) solvent mixture (Cheng et al., 2011). To 20 mg of dried soft tissue taken in a screw capped boiling tube, 15 mL of 2:1 $CHCl_3$: CH_3OH solvent mixture was added. The tube was loosely capped and heated in a water bath for 30 min at 60 °C. After cooling the solution, the volume was made up to 15 mL with the solvent mixture. 1 mL of the extract was pipetted in a separate stoppered test tube, allowed to dry completely in a desiccator overnight. The dried content was digested with 1 mL of conc. H_2SO_4 by boiling in a water bath for 10 min. After cooling the tube, 5 mL of phospho-vanillin reagent was added and allowed to stand for 15 min for colour development. The absorbance was measured at 520 nm against a reagent blank using UV-Visible spectrophotometer (Analytik Jena Specord 200 plus). Cholesterol was used as standard with multi point calibration yielding the correlation factor r^2 as 0.999. Results were expressed as percentage of dry weight of tissue.

2.4.2 Calorific value (Caloric content)

The caloric content of soft tissues were determined by converting the amounts of major biochemical contents into calorific values using

standard calorific equivalents, *viz*., 5.65 for proteins, 9.45 for lipids and 4.20 for carbohydrates (Dare & Edwards, 1975). The calorific values were expressed as Kcal g^{-1} on dry weight basis.

$$\textbf{Calorific value (KCal/g)} = \frac{(\mathbf{5.65 * P}) + (\mathbf{9.45 * L}) + (\mathbf{4.20 * C})}{\mathbf{100}}$$

Where P = Total protein content (%)

L = Total lipid content (%)

C = Total carbohydrate content (%)

2.4.3 Ash

The ash content of soft tissues from various molluscs represents the total inorganic materials present, was determined by ashing 1 g of the dried tissue powder taken in a porcelain crucible at 500 °C for 8 h in a muffle furnace (ASTA, 1999). The weight of the residue represented the ash content. The results were expressed as a percentage of dry weight of tissue.

2.4.4 Moisture

The moisture content of soft tissues was estimated by following the method of AOAC (1994). 10 g of tissue was well grounded using motor and pestle. It was spread uniformly in a petri dish and heated in a hot air oven at 100 ± 2 °C for about 16 h. Then, it was cooled and weighed. Repeated heating, cooling, and weighing were done until the difference in weight between 2 successive readings showed less than 1 mg. Then, the moisture content in percentage was calculated using the following formula.

$$\textbf{Moisture content} = \frac{\textbf{Amount of water in the body tissue}}{\textbf{Wet weight of the body tissue}} * \mathbf{100}$$

2.4.5 Minerals

Metals (micro and macro) such as calcium, magnesium, iron, copper, zinc, manganese, cobalt, chromium, lead, cadmium and nickel present in the soft tissue of molluscs were estimated using atomic absorption spectroscopy (Perkin Elmer-3110) after digestion using di-acid mixture ($HClO_4$: HNO_3) (Grasshoff, 1999).

About 0.5 g dried tissue sample was digested with a mixture of concentrated HNO_3 (AnalaR grade; BDH 69%) and $HClO_4$ (AnalaR grade; BDH 60%) in the ratio of 5:1. The samples were heated in a hot-block digester (Anton Paar Multiwave 3000) first at low temperature (40 °C) for 1 h and fully digested at 140 °C for at least 3 h. After digestion, the samples were evaporated and washed the residue with milli Q water and filtered through whatman 40 filter paper (Yap et al., 2002; Yap et al., 2009). The samples were made up to 25 mL and then analysed for metals using atomic absorption spectroscopy (Perkin Elmer-3110).

Metal concentrations were presented as mg kg^{-1}. All analyses were carried out in triplicate and the mean ± standard deviations were reported. The accuracy and precision of analyses were checked with TORT-2, LobsteZ Hepatopancreas, National Research Council, Canada and the triplicate measurements showed recovery between 92.75 − 99.79% for all the metals.

Table 2.2 Analysis of Standard reference material for heavy metals (TORT-2)

Metal (mg kg^{-1})	Certified Value	Obtained concentration (n=3)	% of recovery
Cd	42.3 ± 1.80	41.9 ± 1.45	99.05
Co	1.06 ± 2.10	1.01 ± 2.68	95.26
Cr	1.95 ± 0.24	1.92 ± 0.65	98.46
Cu	497 ± 22.0	496 ± 6.25	99.79
Fe	179 ± 8.0	177 ± 4.41	98.88
Mn	15.6 ± 1.0	14.47 ± 1.75	92.75
Ni	5.30 ± 0.24	5.16 ± 0.61	97.35
Pb	0.24 ± 0.018	0.23 ± 0.08	95.83
Zn	136 ± 6.0	133.64 ± 7.51	98.26

2.4.6 Extraction and fractionation of lipid compounds (fatty acids, alkanes, and steroids)

The method described by Folch et al. (1957) and Barnes and Blackstock (1973) were used for the extraction and fractionation of lipid compound classes (Figure 2.3). For the analysis of lipids, 30 g of finely powdered tissue samples was taken in a stoppered conical flask and add 70 mL of cold $CHCl_3$: CH_3OH (2:1) and shake overnight under cold condition. The extract was collected in a round bottom flask. The procedure was repeated for 3 to 4 times for the complete extraction. The extracts were combined and evaporated to dryness using a rotary evaporator (Heidolph, Germany). The extracted residue was subjected to mild alkaline hydrolysis using 0.5 M KOH/MeOH and heating gently (70 °C for 6 h). After the sample has been cooled, the neutral lipids were partitioned from the alkaline solution into 10 mL of hexane. The hexane layer was evaporated to dryness, and the extract was then re-dissolved into 1 mL n-hexane for further analysis. This was used for the analysis of neutral compounds.

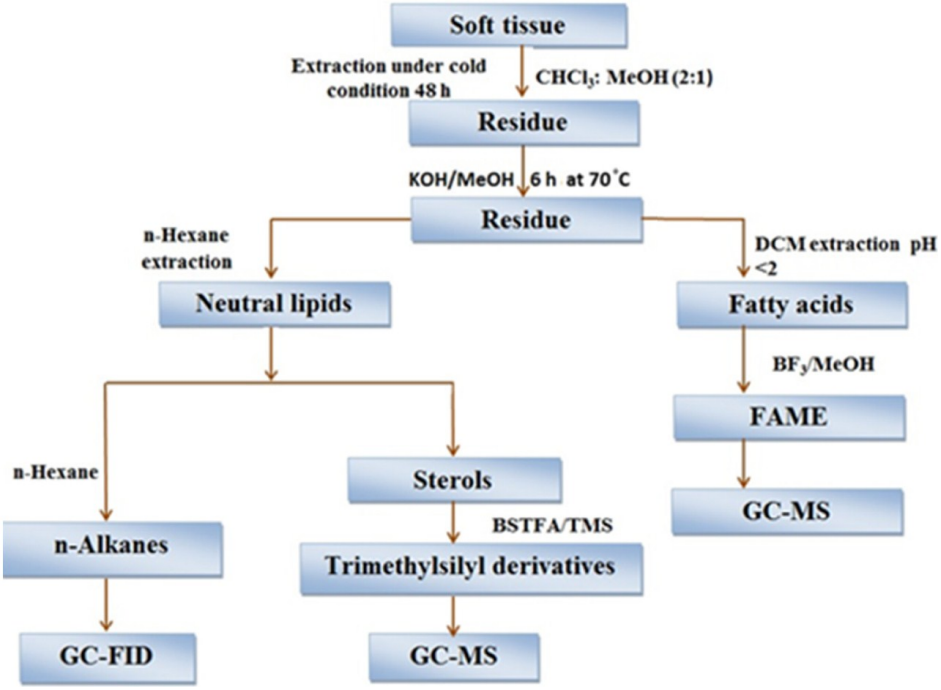

Figure 2.3: Pictorial representation of extraction and fractionation of lipids

The remaining aqueous layer containing the fatty acid salts was acidified to pH = 2, where the fatty acids in this polar-lipid fraction were partitioned separately into an additional 2 mL of hexane. The polar lipid fraction containing the fatty acids was evaporated to dryness using rotary evaporation and treated with 10 mL of 12% BF_3/MeOH (Sigma Aldrich) while heating at 70 °C for 30 minutes to form the fatty acid methyl esters (FAMEs). The FAMEs were subsequently partitioned from the reaction solution into 10 mL of hexane. The hexane layer was evaporated to dryness, and the extract was then re-dissolved into 1 mL n-hexane for chromatographic analysis.

The neutral fraction obtained by mild alkaline hydrolysis of the total lipid extract was separated into individual compound classes on

silica gel viz., saturated hydrocarbons, aromatic hydrocarbons, and polar compounds. The fractions were eluted with n-hexane, ethyl acetate: n-hexane (15%) respectively, and dried under nitrogen. Hexane fractions were dried and re-dissolved in 1 mL HPLC grade n-hexane for chromatographic analysis. Ethyl acetate fraction was converted to trimethylsilyl derivatives by reaction with N, O-bis-(tri methyl silyl) tri fluoro acetamide (BSTFA) and pyridine for 3 h at 70 °C.

2.4.7 Gas chromatographic analysis of fatty acids, alkanes and steroids

2.4.7.1 Fatty acids

Fatty acid compounds were separated from the lipid fraction using dichloromethane. Extracted fatty acids were analysed as fatty acid methyl esters (FAME) in gas chromatography - mass spectrometry (GC-MS) (Perkin Elmer Clarus 620 GC), with MS detector equipped with a non-polar HP ultra-double–fused silica capillary column (30 m length, 0.32 µm internal diameter, 0.25 µm film thickness). Operating conditions were as follows: ion source of electron voltage 70 eV kept at 200 °C. Spectra were scanned from 50 to 600 m/z with scan time of 1.50 s. Initially, the temperature was increased from 50 °C to 200 °C at a rate of 2 °C per min and held at 200 °C for 5 min. Then, the temperature was again increased from 200 °C to 280 °C at a rate of 10 °C per min and held at 280 °C for 10 min. Full data acquisition was obtained with the use of MS turbomass version 5.4.2. Quantification was achieved by calibration of FAMEs standards supplied by Sigma Aldrich (Supelco 37 Component FAME Mix, 18919-1AMP). Sample FAMEs were also injected under the above mentioned conditions, and their concentrations were determined by

comparing calibration plot of authentic standards (FAME 37 mix, Sigma Aldrich, USA).

2.4.7.2 Alkanes

n-Hexane fraction separated from neutral fraction was used for alkane analysis. The hydrocarbon concentration was determined by gas chromatograph (Perkin Elmer Clarus GC 620) equipped with flame ionisation detector (GC-FID) with a 30 m×0.25 mm i.d. DB-5 column, 0.25 μm film thickness. Oven temperature was held at 50 °C for 5 min and then increased to 300 °C at a rate of 3 °C per min and held for 5 min. The injector temperature was kept at 260 °C and the detector temperature was maintained at 325 °C. Nitrogen was used as carrier gas with a flow rate of 2 mL per min. Identification of individual compounds was achieved by comparison of GC retention times with those of standard compounds. Quantification was made based on the calibration with authentic standards (C7 - C40, Sigma Aldrich).

2.4.7.3 Steroids

Ethyl acetate fraction separated from neutral fraction was used for sterol analysis. Ethyl acetate fraction was converted to trimethylsilyl derivatives and analysed using a Perkin Elmer Clarus GC 620 GC, equipped with MS detector and a non-polar HP ultra-double-fused silica capillary column (30 m, 0.32 mm internal diameter, 0.25 μm film thickness). Operating conditions were as follows: ion source of electron voltage 70 eV kept at 200 °C. Spectra were scanned from 50 to 600 m/z with a scan time of 1.50 s. Initially, the temperature was increased from 50 °C to 220 °C at a rate of 10 °C per min and held at 220 °C for 5 min.

Then, the temperature was again increased from 220 °C to 290 °C at a rate of 1 °C per min and held at 290 °C for 10 min. The detector was held at 290 °C and helium was used as carrier gas. Full data acquisition was obtained with the use of MS Turbo Mass version 5.3.2. Individual compounds were identified by comparison of mass spectra with literature and library data, retention time of authentic standards and interpretation of mass spectrometric fragmentation patterns.

2.4.8 Characterization of amino acids

2.4.8.1 Extraction and derivatisation of amino acids

Total amino acids were extracted according to the standard protocol (Bidlingmeyer et al., 1984). Amino acids were extracted by adding 10 mL 6 M HCl to freeze dried homogenized tissue (50 mg) in a pre-cleaned and muffled (450 °C for 3 h) glass vials, and purging the headspace with N_2. The vials were kept in an oven at 110 °C for 24 h. The extracts were then centrifuged at a speed of 5000 rpm for 10 min (KUBOTA 6500, Japan) and for neutralisation, it was washed with distilled water, HCl content in extract was removed (Stevenson & Cheng, 1970; Cheng, 1975) using rotary evaporator (Heidolph, Germany). For the detection and quantification of the extracted amino acids, pre-column derivatisation with phenyl-iso-thiocyanate (PITC) (Bidlingmeyer et al., 1984) was used. In this technique, dried samples (HCl removed) were dissolved in 20 µmol L^{-1} of ethanol : water : triethylamine (TEA) (2:2:1) and dried again under vacuum. Then to the dried sample, 20 µl freshly prepared reagent consisted of ethanol : water: TEA: PITC (7:1:1:1) were added under nitrogen atmosphere and sealing them for 30 min at room

temperature. The reagents were then removed under vacuum at 45 °C to reduce the evaporation time without any significant sample difference in comparison to drying at lower temperatures (the dried derivatives could be kept dried and frozen for several weeks without significant degradation).

The individual amino acids (AAs) - aspartic acid (Asp), glutamic acid (Glu), serine (Ser), glycine (Gly), histidine (His), arginine (Arg), threonine (Thr), alanine (Ala), proline (Pro), tyrosine (Tyr), valine (Val), methionine (Met), cysteine (Cys), isoleucine (Ile), leucine (Leu), phenyl alanine (Phe) and lysine (Lys) were quantified according to Lindroth & Mopper (1979) by high pressure liquid chromatography (Shimadzu Ultrafast LC 2020) equipped with UV&RI detector and octadecayl cilane C18 Columns (25 cm, 2.1mm internal diameter and 5µm particle size diameter).

2.4.8.2 HPLC analysis of amino acids

The instrument is working under room temperature with flow rate 1 mL min^{-1}. Calibration range is 0.0087 µmol mL^{-1} to 0.14 µmol mL^{-1}. The chromatographic condition is as follows. The proposed solvent system consisted of two eluents. Solvent A, an aqueous buffer, was a solution of 50 mM sodium acetate containing tri ethyl ammine (TEA) as a modifier. The solution was degassed before the addition of TEA. The pH was adjusted to the desired value (6.8) using glacial acetic acid, and the solution was filtered through a 0.2 µm membrane filter. Solvent B consists of water, acetonitrile, and methanol. Aqueous solutions containing acetonitrile and methanol are the most common solvents used in the

amino acid analysis in RP-HPLC (Bidlingmeyer et al., 1984; Hariharan et al., 1993). The pH and TEA content of solvent A, the composition of solvent B, the temperature, and the mobile phase flow rate were specified as required for the experimental design. Ultraviolet spectrophotometric detection was carried out at 254 nm. Before starting the gradient for a certain run, the column was equilibrated for 30 min as required with the associated experimental design. Before HPLC analysis, the derivatised forms of the standard amino acid mixture and the individual amino acids were first dissolved separately in 12 µL of 60% solution of acetonitrile and mixed thoroughly (Rotek Cyclo vortex mixer). Then, 113 µL of the corresponding solvent A was added to each sample and mixed well using vortex mixer. 10 µL of the standard amino acid mixture, purchased from Sigma Aldrich (USA) was injected to the HPLC for standardisation. Derivatised samples were injected to the HPLC by the same procedure and identification of individual compounds was achieved by comparison of retention times with those of standard compounds.

2.4.9 Biological activity of identified compounds

2.4.9.1 Anti-bacterial activity of fatty acid fractions

Polyunsaturated fatty acid (PUFA) extracted from *B. spinosa* and *M. trapa* were screened for antimicrobial activity using Kirby-Bauer disc diffusion method (Bauer et al., 1966). Samples, dissolved in dimethyl sulfoxide (DMSO) were used for the experiment. Antimicrobial activity of the extracts was tested against various bacterial pathogens associated with human diseases *viz*., *Escherichia coli, Aeromonas hydrophila, Staphylococcus aureus* and those associated with fish diseases viz., *Vibrio*

parahaemolyticus, Vibrio harveyi, and *Vibrio cholera.* The pathogens were obtained from National Centre for Aquatic Animal Health, CUSAT, Cochin. The screening was carried out on nutrient agar medium and expressed as the diameter (mm) of the inhibition zone. The zone of inhibition was observed after 24 h of incubation at 27 °C. PUFAs were separated from the crude fatty acid fraction of *B. spinosa* and *M. trapa* using thin layer chromatography (TLC). PUFAs were eluted from the TLC plate using ethyl acetate and dried. The ethyl acetate fraction was screened for antimicrobial activity by disc diffusion method using the Kirby-Bauer technique (Bauer et al., 1966). Antimicrobial screening for corresponding blanks was also carried out as a negative control.

2.4.9.2 *In silico* biological activity of steroids

In silico biological activity of steroids identified by GC-MS from molluscs was studied using PASS (Prediction Activity Spectra of Substances). PASS is a software product designed as a tool for evaluating the general biological potential of an organic drug-like molecule. This tool agrees to estimate the probable profile of biological activity of a drug like organic compound whose molecular mass ranges from 50 to 1250 Da. PASS offers simultaneous predictions of many types of biological activity based on the structure of organic compounds (Poroikov et al., 2003; Geronikaki et al., 2008). Thus, PASS can be used to estimate the biological activity profiles for virtual molecules, prior to their biological testing (Lagunin et al., 2010). PASS has been well accepted by the community since 2000 and is now actively used in the field of medicinal chemistry for the prediction of biological activities. The average prediction accuracy calculated is about 95% (Filimonov

et al., 2014). There are more than 200 publications from researchers using PASS (Pass geneXplain). The values obtained after running the software are denoted by Pa and Pi. Pa (Probability to be active) estimates the chance that the studied compound is belonging to the subclass of active compound. Pi (Probability to be inactive) calculates approximately the chance that the studied compound is belonging to the subclass of inactive compounds.

2.4.9.3 *In vitro* antiproliferative effect of fatty acid extracts

The crude PUFA fractions from *B. spinosa*, *M. trapa* and a mixture of eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) separated from crude extracts of *M. trapa* were selected for *in vitro* antiproliferative studies. Human laryngeal carcinoma derived human epithelial type 2 cell lines used in the present study were purchased from National Centre for Cell Science (NCCS) Pune. The cell lines sustained in Dulbecco's modified eagles media (DMEM/F2) supplemented with 10% fetal bovine serum (FBS) and antibiotics such as 100 µg mL^{-1} pencilin G, 100 mg mL^{-1} streptomycin and 250 ng mL^{-1} amphotericin (Invitrogen) in an incubator. It is maintained at humidified 5% CO_2 atmosphere and an optimum temperature of 37 °C (NBS, EPPENDORF, GERMANY). When the cells reached confluence after the four days of incubation, they were treated with 0.5% (W/V) trypsin (0.25% , Invitrogen, USA) Ethylenediaminetetraacetic acid (EDTA) for 3 min. at 37 °C. All the chemicals used for culturing and cell preservation were purchased from Invitrogen and Gibco. Extracts were added to grown cells at a final concentration of 6.25 µg, 12.5 µg, 25µg, 50 µg and 100 µg mL^{-1} from a stock of 1 mg mL^{-1} and incubated for 24 h.

The % difference in viability was determined by standard (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl) tetrazolium (MTT) assay after 24 h of incubation (Arung et al., 2009).

MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent DMSO (Himedia) and the released, solubilised formazan product was measured at 540 nm (Talarico et al., 2004). Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

The cells were washed with 1X PBS and then added 30 µL of MTT solution to the culture (MTT-5 mg/ml dissolved in PBS). It was then incubated at 37 °C for 3 h. MTT was removed by washing with 1X PBS and 200 µl of DMSO was added to the culture. Incubation was done at room temperature for 30 min until the cell got lysed and colour was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2 min to precipitate cell debris. Optical density was read at 540 nm using DMSO as blank in a micro plate reader (ELISASCAN, ERBA).

$$\%\ viability = \frac{optical\ density\ of\ test}{optical\ density\ of\ Control} * 100$$

2.5 Statistical analysis

All the estimations were carried out in triplicates for accuracy and precision. Results were interpreted as average value ± standard deviation. Relevant data were subjected to statistical analysis wherever necessary. Pearson correlations were determined to find out the inter relations between different parameters. The correlation studies were done by using SPSS (22.0) software for windows. Statistical significance of the biochemical components in different molluscs was checked using two way ANOVA (biochemical components x species).

2.6 Quality control

All the bottles and glasswares were washed in acids and thoroughly rinsed with acetone before use. All the glasswares were cleaned by ultra-sonic bath followed by heating at high temperature in oven. Chemicals/solvents used for the analysis of various parameters were purchased from Merck (India/Germany). Calibration standards for fatty acids, alkane, steroids and amino acids were purchased from Sigma-Aldrich (USA).

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Chapter 3

PROXIMATE AND MINERAL COMPOSITION

Contents

3.1 Introduction

Molluscs may be considered as a good source of food, only if they are nutritive. The term nutritive means substances which provide all the basic food requirements that are mandatory for an organism to survive (Margret, 2015). It consists of water, carbohydrate, lipids, proteins and minerals. The knowledge of the biochemical composition of any edible organism is tremendously important since the nutritive value is reflected in its biochemical contents (Nagabhushanam & Mane, 1978; Babu et al., 2012; Margret, 2015). Most of the molluscs reported till date is a good source for delicious and excellent protein rich food (Renitta, 2005; Periyasami et al., 2014; Margret, 2015). In the coastline area, molluscs could form an important source of food, inborn medicine and raw material for village industries. It is also regarded as an under-exploited source of health benefit molecules (Haldar et al., 2014). Even though

large numbers of marine molluscs are suitable for human consumption, our knowledge on their nutritive value is limited.

The awareness of chemical composition of any edible organism is enormously significant because it gives an idea about the nutritive value of that organism (Lakshmanan & Nambisan, 1980; Renitta, 2005; Margret, 2015). Analysis of basic constituents such as water (moisture), proteins, carbohydrates, lipids, ash, crude fiber, calorific value, iodine, phenolics, vitamins, flavonoids, amino acid compositions, saponins, etc. are often referred to as proximate composition. This chapter deals with the proximate compositions and minerals from six molluscs species viz. four gastropods and two bivalves. Proximate composition means percentage composition of five basic constituents such as proteins, carbohydrates, lipids, ashes and moisture. The proximate composition varied extensively depending on several factors like species, size, maturity, sex, season and feeding systems (Orban et al., 2002; Margret et al., 2013). A brief introduction to some facts of these biochemical components is discussed ahead.

The moisture content of food material affects the physical and chemical aspects. It relates with the freshness of food materials storing for a long period. The moisture content of any food material defines the real quality of the food before consumption. It depends on their physical structure. Water is involved in many body processes such as fluid balance, nutrient transport, removal of wastes, nerve impulses, muscle contractions and in chemical reactions. In previous studies, the water content in gastropods and bivalves reported to be ranged 60−83% and

71−86% respectively (Appukuttan & Aravindan, 1995; Mc Lean & Bulling, 2005; Woodcock & Benkendorff, 2008; Margret et al., 2013; Govindarajalu et al., 2016). The ash content is an indication of the presence of inorganic and carbon compounds, which measures the weight left over after the burning of an organism for 2h at 600 °C (Usman, 2006). It does not comprise water, fiber and nutrients that provide calories, but it does include some nutrients, such as minerals (Jatto et al., 2010). The ash content in gastropods and bivalves reported in previous studies were ranging from 0.8−2% and 2−7% respectively (Ahn et al., 2003; Jatto et al., 2010; Padidela & Thummala, 2015; Krishnan & Tharavathy, 2016).

Carbohydrates are biological molecules consisting of carbon, hydrogen and oxygen units, combined in varying configurations. The variation in their compositions develops them into soluble and insoluble, simple and complex and digestible and indigestible carbohydrates. The degree of their complexity defines the magnitude of energy liberated by the compounds. On an average, one gram of carbohydrate is estimated to release 4.2 Kcal of energy (Jequier, 1994; Abhilash, 2015; Anjos et al., 2017). It is a leading source of energy in all human diets. The proportion of carbohydrates in animal tissues, especially in aquatic animals was less compared with other nutrients (Babu et al., 2012; Periyasami et al., 2014). The reported values of carbohydrates in gastropods and bivalves were ranging from 0.5−20% and 2−33% respectively (Hummel et al., 1988; Navarro et al., 1989; Arularasan et al., 2010; Chakravarty et al., 2015; Govindarajalu et al., 2016).

Proteins are the fundamental biomolecules of cell in all the living organisms. It is a good source of energy for the development of muscles and other tissues in the body (Fagbuaro et al., 2006). Proteins also support the nitrogen requirement of an organism. Proteins are nitrogen-containing substances that are made up of amino acid units. They serve as a major role in the production of hormones, enzymes and haemoglobin (King et al., 1990; Ersoy & Sereflisan, 2010; Govindarajalu et al., 2016). One gram of protein is estimated to generate approximately 5.6 Kcal of energy (Ortigues-Marty et al., 2007). In general, proteins from plant sources are less digestible than those from animal sources (Ersoy & Sereflisan, 2010). Heating increases the digestibility, and also processing encourages protein quality. Several studies done on seafood provide high quality protein with all the dietary essential amino acids for the maintenance and growth of human body (Ademolu et al., 2004; Fagbuaro et al., 2006). For this reason, it should be considered as highly proteinaceous food for normal diet. The demand for protein rich food is increasing day by day especially in developing countries with the growth of the human population. So peoples from those countries were searching for unconventional low cost animal protein resources. Among the unconventional sources of animal proteins, molluscs constitute the major and cheapest sources of seafood protein (Haldar et al., 2014). Protein content fluctuates from organisms to organisms (Kunusaki, 2000). Protein contents in gastropods and bivalves reported in previous studies were ranging from 10−78% and 8−61% respectively (Shanmugam et al., 2006; Haldar et al., 2014; Giftson & Patterson, 2016).

Lipids comprise a group of naturally occurring molecules; include long or short, saturated or unsaturated, mono or poly unsaturated fats, sterols, waxes, glycerides, fat-soluble vitamins, phospholipids and others. They consist of a large number of lipid units, so the complete oxidation of lipids provides high caloric content, about 9 Kcal g^{-1} (Subramaniam et al., 2011). The chief biological functions of lipids comprise signaling, storing energy and acting as structural components of cell membranes (Margret, 2015). Lipids have a wide range of applications in the food as well as cosmetic industries and in nanotechnology (Mashaghi et al., 2013). Lipids are acting as a reserve material and are utilized during stress situation (Govindarajalu et al., 2016). They are the major sources of metabolic energy and essential materials for the formation of cell and tissue membranes (Sargent, 1995; Periyasami et al., 2014). The lipid contents in gastropods and bivalves were accounted in the range 1−18% and 1−24% respectively (Hummel et al., 1988; Rees & Hand, 1993; Margret et al., 2013; Shetty et al., 2013; Periyasami et al., 2014; Chakravarty et al., 2015; Giftson & Patterson, 2016).

Calorific value is the collective input of all the leading biochemical constituents. The energy content of an organism, which could be generated through digestion, is usually expressed as its calorific value (Margret, 2015). It is an important parameter in the energy flow studies because it is used for converting the biomass values to energy units (Kalesh, 2003; Singh et al., 2012). The calorific value of flora was much lower than those of fauna. Energy values in gastropods and bivalves vary from 3 Kcal g^{-1} to 5.5 Kcal g^{-1} (Wacasey & Atkinson, 1987; Liu et al., 2011; Singh et al., 2012; Abraham, 2016).

All living organisms required small amounts of essential elements such as calcium, magnesium, iron, manganese, copper, nickel and zinc for their biological metabolism and growth (Yusoff & Long, 2011; Hossen et al., 2015). They are necessary constituents for numerous biological functions such as fluid regulation, muscle movement, nerve functioning and bone structure (Margret et al., 2013). Human body requires somewhat 100 mg essential minerals per day, which include calcium, magnesium, phosphorus, potassium, sodium, and chloride. Sea is a vital source of all minerals and ions include sodium, calcium, zinc, iron, potassium, magnesium etc. Similar to all living organisms, molluscs are a better source of minerals, ions, and metals which could be obtained from the water column or accumulated from the sediment bed, and it exists in free or combined forms. The ash content of an organism is an indication of mineral constituents.

The major biochemical compositions of molluscs were reported in India and abroad, some of them are described in Table 3.1. Commercially important molluscs viz., *P. globosa, B. bengalensis, M. tuberculata, L. marginalis, A. convexiusculus* and *Helix* sp. were investigated for their nutrient compositions (Baby et al., 2010). Molluscan species such as *C. edule* and *Sepia* sp. were analysed for determining the proximate and mineral composition (Abdel-Salam, 2013). Biochemical composition of different body parts of *G. tumidum* collected from Mandapam estuary, south east coast of India was examined by Babu et al. (2012). Proximate composition of baby clam, *K. opima* collected from Ratnagiri coast of Maharashtra gives the knowledge about edible nature of *K. opima* species (Nirmale et al., 2016). Gastropods collected from the sub tidal

habitat at Chauvin (Valdivia, Chile) were examined for its organic constituents such as the carbohydrates, lipids and proteins (Carrasco et al., 2006). Comparative study on biochemical composition and mineral composition of five molluscs viz. *O. edulis, M. galloprovincialis, R. decussatus, R. philippinarum* and *R. venosa* from Canakkale coasts, Turkey gave the edibility of those species (Celik et al., 2014). Beach clam, *D. cuneatus* collected from Veerampattinam beach, Pondicherry, India was analysed for its total protein, carbohydrate and lipid contents (Abirami et al., 2015). Three potamidid snails such as *T. telescopium, C. cingulate* and *C. obtuse* collected from Tekkali creek, a mangrove estuary, Andhra Pradesh, India were analysed for their biochemical composition (Chakravarty et al., 2015). Nutritional quality of eastern oysters, *C. virginica* collected from Chesapeake region was analysed by Chen (2011).

Edible fresh water gastropods collected from Tripura, India were investigated for its biochemical compositions and minerals (Debnath et al., 2016). Jadhav & Gulave (2012) reported seasonal variation in the protein content of the soft body tissues of *L. marginalis* collected from Jayakwadi dam, India. A comparative assessment of metals in the soft tissue of *B. helblingii* collected from Qeshm Island, Persian Gulf gives seasonal changes in the metal concentration (Ansari et al., 2014). Marine neogastropod, *C. melo* collected from Cuddalore, India was analysed for its biochemical compositions including fatty acid content (Palpandi et al., 2010). Karnjanapratum et al. (2013) reported the chemical compositions and nutritional value of Asian hard clam, *M. lusoria* collected from the coast of Andaman Sea. Edible marine clam, *S. diphos* collected from

Bhatye estuary, India was investigated for the seasonal variation in the biochemical constituents and percentage of edibility (Vishwajeet et al., 2015). Nutrional qualities of marine bivalves and gastropods were analysed by many researchers and are shown in Table 3.2 (Tibbetts et al., 2000; Woodcock & Benkendorff, 2008; Zhang et al., 2009; Periyasamy et al., 2011; Zotti et al., 2016).

Malnutrition is a severe issue facing by most of the developing countries. In India 20−30% of the population does not get sufficient nutrition (Malhotra, 2013). About 36% adult women and 34 % adult men in India suffer from chronic energy deficiency, also about 30% of new-borns affect low birth weight (Babu et al, 2012; Sini & Jansi, 2013). Proper exploitation of aquatic organisms will supply the balanced nutritious food and thus malnutrition can be controlled. A balanced diet should provide around 10–12% total calories from proteins, 20–25% from fat and 60−70% from carbohydrates, preferably starch (Lichtenstein et al., 2006). The exploitation of fishery products is increasing with respect to population growth and awareness on the nutritional benefits of marine resources (Jeena et al., 2003). In developing countries, fishes are largely used for animal protein. Fish and fish products are now exported to different countries and hence its freshness or quality is becoming much more important as this reflects on its price. This problem can be overcome by the adequate utilization of nutrient rich molluscan seafood. Generally molluscs comprise 8−10% of protein, 2−3% of minerals 4−5% of carbohydrate, and only 1−2% of fat (Periyasami et al., 2014). But in India, it is not a popular food like bivalves and cephalopods due to the lack of awareness and traditional food habit of the people. In our

country, clams, oysters, mussels and certain gastropods are collected by coastal fishermen for diet. There is a little demand for edible gastropods in India because most of the people do not know the nutritive value of molluscan shell fish and it is fished for food when fish production is insufficient (Sundaram, 1974). In India, the value of the edible gastropods as food is not recognized by most of the people while in other countries, the gastropods are very much delicious. Local people from the coastline area, Tuticorin and Rameswaram consume edible gastropods as boiled or fried products (Margret, 2015).

Biochemical compositions of bivalves have been studied due to the realization of their importance (Appukuttan & Aravindan, 1995; Okumus & Stirling, 1998; Orban et al., 2002; Mc Lean & Bulling, 2005; Fuentes et al., 2009; Sohail et al., 2016). Studies on the nutritional importance of edible gastropods have a significant role in developing countries. Proper consumption of these organisms will supply the balanced nutritious to nullify the malnutrition. Hence, the present study is undertaken to evaluate the nutritive values of both gastropods and bivalves available in plenty such as *T. curta*, *M. trapa, T. telescopium, B. spinosa, P. viridis* and *V. cyprinoids* by estimating the levels of proteins, carbohydrates, lipids, moisture contents, ashes and minerals.

Table 3.1: Reported data of moisture, ash, protein, lipid and carbohydrate of bivalves and gastropods.

Species	Area	Concentration	References
		Moisture content (in %)	
Bivalves			
M. galloprovincialis	Balikesir coast, Turkey	82.0	Celik et al., 2014
R. decussatus	*do*	83.0	*do*
R. philipinarium	*do*	83.0	*do*
O. edulis	*do*	82.0	*do*
M. edulis	Scotland	72.0−85.0	Okumu & Stirling, 1998
M. galloprovincialis	Adriatic sea, Italy	71.0	Orban et al., 2002
U. terminalis	Turkey	80.0	Ersoy & Sereflisan, 2010
P. littoralis	New Zealand	81.0	Mclean & Bulling, 2005
R. philippinarum	Turkey	85.0	Dincer, 2006
M. galloprovincialis	Spain	79.0	Fuentes et al., 2009
T. peruvianus	Costa Rica	80.0	Rodriguez et al., 2011
A. anatina	Chashma Lake, Pakistan	77.0	Sohail et al., 2016
L. elliptica	King George Island, Antarctica	88.0	Ahn et al., 2004
M. lusoria	Andaman Sea	84.0	Karnjanapratum et al., 2013
L. marginalis	West Bengal, India	80.0	Haldar et al., 2014
P. Malabarica	Kerala coast, India	79.0-86.0	Appukuttan & Aravindan, 1995

M. violacea	Thalassery beach, Kerala, India	80.0	Laxmilatha, 2009
Gastropods			
R. venosa	Balikesir coast, Turkey	67.5	Celik et al., 2014
D. orbita	Eyre Peninsulas, South Australia	72.5	Woodcock & Benkendorff, 2008
A. marginata	King's market Ado Ekiti, Nigeria	76.5	Fagbuaro et al., 2006
***Limicolaria* sp.**	*do*	78.9	*do*
A. achatina	*do*	77.5	*do*
H. midae	Hatchery in Hermanus, South Africa	81.3	Knauer et al., 1996
O. strigosa	France	78.0-81.0	Rees & Hand, 1993
P. ampullacea	Fish market in Makurdi, Nigeria	76.3	Obande et al., 2013
H. pomatia	France	81.6	Gomot, 1998
B. areolata	Local fish farm of China	65.4	Zhang et al., 2009
M. virgeineus	Kerala coast, India	76.2	Margret et al., 2013
B. spirata	*do*	72.8	*do*
T. radiatus	*do*	66.7	*do*
C. meto	*do*	76.5	Palpandi et al., 2010
P. glaucum	Gulf of Mannar, India	83.7	Govindarajalu et al., 2016
X. pyrum	*do*	81.2	*do*
H. pugilinus	*do*	71.3	*do*
H. articularis	*do*	72.0	*do*
B. spirata	Parangipettai coast, India	80.0	Shanmugam et al., 2006

	Ash content (in % dwt)		
Bivalves			
M. galloprovincialis	Balikesir coast, Turkey	12.6	Celik et al., 2014
R. philipinarium	*do*	15.1	*do*
O. edulis	*do*	13.9	*do*
R. decussatus	*do*	14.5	*do*
M. galloprovincialis	Adriatic sea, Italy	11.0-17.0	Orban et al., 2002
L. elliptica	Antarctica	1.7	Ahn et al., 2004
M. galloprovincialis	Spain	2.2	Fuentes et al., 2009
U. terminalis	Turkey	1.6	Ersoy & Sereflisan, 2010
E. radiate	Nigeria	3.6	Ehigiator & Akise, 2016
M. edulis	Scotland	4.2	Okumu & Stirling, 1998
A. anatina	Chashma Lake, Pakistan	5.2	Sohail et al., 2016
P. cylindrica	Kaleshwaram lake, India	3.0	Padidela & Thummala, 2015
P. Malabarica	Kerala coast, India	7.0-20.0	Appukuttan & Aravindan, 1995
D. incarnatus	*do*	7.0-17.0	Krishnan & Tharavathy, 2016
D. faba	*do*	11.0-21.0	*do*
L. marginalis	West Bengal, India	2.3	Haldar et al., 2014
K. opima	Ratnagiri estuary, Maharashtra, India	4.2-4.5	Nirmale et al., 2016

Gastropods			
R. venosa	Balikesir coast, Turkey	9.3	Celik et al., 2014
A. archatina	Nigeria	2.0	Jatto et al., 2010
P. ampullacea	*do*	5.5	Obande et al., 2013
H. pomatia	France	1.9	Gomot, 1998
O. strigosa	*do*	10.6	Rees & Hand, 1993
H. articularis	Gulf of Mannar, India	0.8	Govindarajalu et al., 2016
X. pyrum	*do*	0.8	*do*
M. virgeineus	Kerala coast, India	1.1	Margret et al., 2013
B. spirata	*do*	1.1	*do*
B. zeylanica	*do*	0.8	*do*
T. radiatus	*do*	0.9	*do*
	Total protein (in % dwt)		
Bivalves			
M. galloprovincialis	Balikesir Coast, Turkey	54.0	Celik et al., 2014
R. philipinarium	*do*	55.8	*do*
O. edulis	*do*	48.1	*do*
R. decussatus	*do*	56.2	*do*
D. trunculas	Algeria	36.6	Hamdani & Mazouni, 2011
T. peruvianus	Costa Rica	61.0	Rodriguez et al., 2011

P. littoralis	New Zealand	11.9	Mclean & Bulling, 2005
U. terminalis	Turkey	11.8	Ersoy & Sereflisan, 2010
R. philippinarum	*do*	8.7	Dincer, 2006
C. angulata	Portugal	26.0	Anjos et al., 2017
M. lusoria	Andaman	55.5	Karnjanapratum et al., 2013
P. cylindrica	Kaleshwaram lake, India	51.0	Padidela & Thummala, 2015
P. favidens	Tungabhadra river, Karnataka, India	41.2-60.8	Shetty et al., 2013
P. khadakvaslaensis	*do*	40.6-57.2	*do*
L. marginalis	West Bengal, India	8.3	Haldar et al., 2014
P. Malabarica	Kerala coast, India	40.0-71.0	Appukuttan & Aravindan, 1995
D. faba	*do*	47.0-62.0	Krishnan & Tharavathy, 2016
D. incarnatus	*do*	49.0-64.0	*do*
K. opima	Ratnagiri estuary, Maharashtra, India	34.9-40.3	Nirmale et al., 2016
G. tumidum	Mandapam coast, India	61.0	Babu et al., 2012
M. violacea	Thalassery beach, Kerala, India	10.2	Laxmilatha, 2009
Gastropods			
A. achatina	King's market Ado Ekiti, Nigeria	19.2	Fagbuaro et al., 2006
***Limicolaria* sp.**	*do*	18.6	*do*
A. marginata	*do*	20.5	*do*
O. strigosa	France	10.6	Rees & Hand, 1993
C. giganteus	Subtidal habitat at Chaihuin, Chile	8.2	Carrasco et al., 2006

D. orbita	Eyre Peninsulas, South Australia	15.1	Woodcock & Benkendorff, 2008
H. davidis	Kanyakumari, India	38.4	Giftson & Patterson, 2016
P. plicata	Parangipettai coast, India	29.0	Shanmugam et al., 2006
P. glaucum	Gulf of Mannar, India	37.3	Govindarajalu et al., 2016
B. zeylanica	*do*	40.8	*do*
C. ramosus	*do*	41.2	*do*
H. articularis	*do*	31.2	*do*
F. ficus	*do*	37.2	*do*
X. pyrum	*do*	35.2	*do*
B. spirata	*do*	35.1	*do*
T. dolium	*do*	33.6	*do*
B. zeylanica	Puducherry, India	41.5	Jayalakshmi, 2015
C. cingulata	Bhavanapadu Mangroves, Andrapradesh, India	13.0	Chakravarty et al., 2015
B. spirata	Cuddalore, India	56.8	Periyasami et al., 2011
S. canarium **(F)**	Thazhanguda, India	46.0−72.0	*do*
M. casta	India	45.0−39.0	Arularasan et al., 2010
S. canarium **(M)**	Cuddalore, India	45.0−67.0	*do*
T. radiatus	Kerala coast, India	28.5	Margret et al., 2013
M. virgeineus	*do*	39.8	*do*
B. spirata	Mandapam, India	53.0	Babu et al., 2012

	Total lipid (in % dwt)		
Bivalves			
O. edulis	Balikesir coast, Turkey	8.8	Celik et al., 2014
M. galloprovincialis	*do*	10.5	*do*
R. philipinarium	*do*	5.6	*do*
T. peruvianus	Costa Rica	61.0	Rodriguez et al., 2011
V. gallina	France	4.0−9.0	Bodoy, 1983
S. subtruncata	*do*	5.0−9.0	Bodoy, 1980
T. philippinarum	*do*	8.0−12.0	Beninger & Lucas, 1984
R. philippinarum	Turkey	8.7	Dincer, 2006
P. megellanicus	Canada	1.0−15.0	Thompson & Mac Donald, 1990
C. corteziensis	Mexico	10.0−24.0	Paez-Osuna et al, 1993
P. littoralis	New Zealand	11.9	Mclean & Bulling, 2005
C. edule	Netherlands	1.0−25.0	Hummel et al., 1988
A. irradians	USA	4.0−8.0	Epp et al., 1988
B. edule	Spain	7.0−11.0	Navarro et al., 1989
T. tenuis	UK	10.0−18.0	Trevallion, 1971
M. lusoria	Andaman	22.3	Karnjanapratum et al., 2013
K. opima	Ratnagiri coast of Maharashtra, India	2.6−4.2	Nirmale et al., 2016
M. violacea	Thalassery beach, Kerala, India	10.2	Laxmilatha, 2009

D. faba	Kerala coast, India	8.0−13.0	Krishnan & Tharavathy, 2016
D. incarnatus	*do*	8.0−15.0	*do*
P. Malabarica	*do*	0.9−12.0	Appukuttan & Aravindan, 1995
P. cylindrica	Kaleshwaram lake, India	5.0	Padidela & Thummala, 2015
G. tumidum	Mandapam coast, India	14.0	Babu et al., 2012
D. incarnatus	Cuddalore, India	1.3	Periyasami et al., 2014
P. khadakvaslaensis	Tungabhadra river, Karnataka, India	3.2−7.6	Shetty et al., 2013
Gastropods			
B. areolata	China	5.6	Zhang et al., 2009
O. strigosa	France	1.5	Rees & Hand, 1993
A. marginata	King's market Ado Ekiti, Nigeria	1.3	Fagbuaro et al., 2006
C. giganteus	Subtidal habitat at Chaihuin, Chile	0.5	Carrasco et al., 2006
A. achatina	*do*	1.4	*do*
***Limicolaria* sp.**	*do*	1.1	*do*
***R. rapiformis* (M)**	Parangipettai, India	0.8−2.1	Rajakumar, 1995
B. zeylanica	*do*	10.0	*do*
H. pugilinus	Gulf of Mannar, India	4.7	Govindarajalu et al., 2016
B. zeylanica	*do*	6.1	*do*
C. ramosus	*do*	6.6	*do*
P. glaucum	*do*	4.3	*do*

T. dolium	*do*	1.9	*do*
X. pyrum	*do*	2.2	*do*
F. ficus	*do*	3.9	*do*
H. articularis	*do*	1.1	*do*
B. spirata	*do*	2.0	*do*
M. casta	Cuddalore, India	1.3	Arularasan et al., 2010
B. zeylanica	Puducherry, India	7.2	Jayalakshmi, 2015
H. davidis	Kanyakumari, India	3.4	Giftson & Patterson, 2016
***R. rapiformis* (F)**	Parangipettai, India	0.9-3	Rajakumar, 1995
C. cingulata	Bhavanapadu Mangroves, Andrapradesh, India	16.5	Chakravarty et al., 2015
T. telescopium	*do*	18.9	*do*
T. radiatus	Kerala coast, India	1.1	Margret et al., 2013
	Total carbohydrate (in % dwt)		
Bivalves			
R. decussatus	Turkey	23.3	Celik et al., 2014
O. edulis	*do*	29.1	*do*
R. philipinarium	*do*	23.4	*do*
M. galloprovincialis	*do*	22.8	*do*
D. trunculas	Algeria	33.6	Hamdani &Soltani-Mazouni, 2011

p	Sado estuary, Southern Portugal	2.0	Anjos et al., 2017
C. edule	Spain	3.0–27.0	Navarro et al., 1989
V. gallina	France	4.0–17.0	Bodoy, 1983
T. philippinarum	*do*	4.0–27.0	Beninger & Lucas, 1984
S. subtruncata	*do*	3.0–16.0	Bodoy, 1980
T. tenuis	UK	12.0–27.0	Trevallion, 1971
C. gigas	*do*	2.0–28.0	Walne & Mann, 1975
O. edulis	*do*	3.0–24.0	*do*
P. megellanicus	Canada	10.0–24.0	Thompson & Mac Donald, 1990
O. edulis	Netherlands	1.0–37.0	Hummel et al., 1988
C. corteziensis	Mexico	5.0–37.0	Paez-Osuna et al, 1993
A. irradians	USA	9.0–20.0	Epp et al., 1988
D. incarnatus	Kerala coast, India	14.0–25.0	Krishnan & Tharavathy, 2016
P. Malabarica	*do*	9.0–43.0	Appukuttan & Aravindan, 1995
P. cylindrica	Kaleshwaram lake, India	6.0	Padidela & Thummala, 2015
G. tumidum	Tamilnadu, India	32.0	Babu et al., 2012
P. khadakvaslaensis	Tungabhadra river, India	18.3–40.2	Shetty et al., 2013
D. incarnatus	Cuddalore, India	10.1	Periyasami et al., 2014
K. opima	Maharashtra, India	6.0–8.0	Nirmale et al., 2016

Gastropods			
C. giganteus	Subtidal habitat at Chaihuin, Chile	1.2	Carrasco et al., 2006
***Limicolaria* sp.**	King's market Ado Ekiti, Nigeria	0.1	Fagbuaro et al., 2006
A. marginata	*do*	0.3	*do*
A. achatina	*do*	0.4	*do*
B. areolata	China	15.5	Zhang et al., 2009
D. orbita	Eyre Peninsulas, South Australia	5.1	Woodcock & Benkendorff, 2008
B. zeylanica	Puducherry, India	17.3	Jayalakshmi, 2015
M. casta	India	10.0	Arularasan et al., 2010
N. pyramidalis	*do*	4.6	*do*
P. plicata	*do*	0.8	Shanmugam et al., 2006
C. cingulata	Andhra Pradesh, India	0.5	Chakravarty et al., 2015
C. obtusa	*do*	0.8	*do*
T. radiatus	Kerala coast, India	6.7	Margret et al., 2013
B. zeylanica	*do*	19.6	*do*
H. articularis	Gulf of Mannar, India	12.3	Govindarajalu et al., 2016
X. pyrum	*do*	14.3	*do*
B. zeylanica	*do*	16.9	*do*
C. ramosus	*do*	17.5	*do*
H. pugilinus	*do*	16.6	*do*
T. dolium	*do*	13.5	*do*
F. ficus	*do*	14.5	*do*

Table 3.2: Reported data of calorific values of bivalves and gastropods.

Species	Concentration (Kcal g^{-1})	References
Calorific value (in Kcal g^{-1})		
Bivalves		
P. cylindrica	4.5	Abraham, 2016
A. alba	4.6	Dauvin & Joncourt, 1989
C. echinatum	4.5	*do*
C. chione	4.6	*do*
S. elliptica	4.6	*do*
T. fabula	4.6	*do*
T. flexuosa	4.6	*do*
A. prismatica	4.4	*do*
A. islandica	4.7	Steimle & Terranova, 1985
M. modiolus	5.4	*do*
A. undata	5.0	*do*
A. montagui	5.2	Wacasey & Atkinson, 1987
M. moesta	5.4	*do*
M. discors	5.6	*do*
C. islandica	5.2	*do*
D. scortum	4.9	Singh et al., 2012
S. plana	5.2	Wanink & Zwarts, 1993
C. edule	5.2	*do*
M. balthica	5.2	*do*
V. cyprinoids	5.2	Lakshmanan& Nambisan, 1980
T. angulata	4.9	Kumari & Nair, 1988
Gastropods		
B. fabricil	4.6	Wacasey & Atkinson, 1987
B. angulosum	5.1	*do*
C. radiata	5.0	*do*
T. reticulatus	4.0	*do*
V. undata	4.5	*do*
L. pallida	4.7	*do*
T. philippinarum	3.9	Cheung et al., 2008
N. siquijorensis	4.9	Liu et al., 2011
N. conoidalis	5.1	*do*
T. bell	3.1	Mc Clintock et al., 1994
A. kerguelensis	4.8	*do*
D. orbita	4.7	Woodcock & Benkendorff, 2008

3.2 Materials and methods

3.2.1 Species collection

Marine gastropods, *T. curta, M. trapa, T. telescopium, B. spinosa*, and bivalves *P. viridis, V. cyprinoids* were collected and used for nutritional analysis (details of the taxonomical description of the organisms and sampling sites are described in hapter 2).

3.2.2 Proximate composition and mineral analyses

The moisture content of the organism was estimated by the method of Association of Official Analytical Chemists (AOAC, 1994). Total ash content of the samples was determined by the method underlined in American Spice Trade Association manual (ASTA, 1997). Total protein content present in the samples was estimated by Lowry's method (Lowry et al., 1951; Sadasivam & Manickam, 1996). Total carbohydrate was analyzed by the procedure of Sadasivam & Manickam (1996) and lipids of the tissue samples were estimated by the method of Cheng et al. (2011) and Barnes & Blackstock (1973). Energy values were determined by converting the amounts of major biochemical constituents into calorific values using standard calorific equivalents, viz., 5.65 for proteins, 9.45 for lipids and 4.20 for carbohydrates (Dare & Edwards, 1975). Minerals present in the soft tissue were determined by di-acid digestion method using HNO_3 and $HClO_4$ (5:1) (Grasshoff, 1999). All the procedures were detailed in Chapter 2.

3.2.3 Statistical analysis

Species wise variation was assessed by using two-way ANOVA without replication test, which is carried out by means of microsoft

excel. Pearson correlation analysis was performed to find out the degree of relationship within trace metals which is carried out using SPSS 22.0 software for windows. Detailed procedures were explained in Chapter 2.

3.3 Results and discussion

3.3.1 Proximate composition analysis

Biochemical compositions of *T. telescopium, B. spinosa, T. curta, M. trapa, P. viridis* and *V. cyprinoids* were analysed and the pictorial representation of results were illustrated in Figure 3.1. In general, gastropods and bivalves showed considerable differences in their chemical constituents.

Water is an essential component of all the cells which serves as a transport medium for nutrients and excretory products. Also, it is a relevant solvent for electrolytes, vitamins, hormones and enzymes. Moisture content controls the body temperature. The results of the nutritive assessment confessed the dominance of moisture content in all the species followed by total protein. Water content exhibited a range of 65.15 to 82.18% in gastropods and 70.18 to 78.15% in bivalves (Table 3.3). The highest was observed in *T. telescopium* (82.18%) among the gastropods and *V. cyprinoids* (78.15%) among the bivalves (Figure 3.1). Least moisture content was noticed in *T. curta* (65.15%). The earlier reports of Okumus & Stirling (1998) in *M.edulis* (bivalve) collected from Scotland; Carrasco et al. (2006) in *C. giganteus* (gastropod) from subtidal habitat at Chaihuin, Chile; Fuentes et al. (2009) in *M. galloprovincialis* (bivalve) collected from Spain; Zhang et al. (2009) in *B. areolata* (gastropod) collected from local fish farm of China; Celik et al. (2014)

in *R. venosa* (gastropod) Balikesir coast, Turkey; Sohail et al. (2016) in *A. anatine* (bivalve) from Chashma Lake, Pakistan supporting the present observations (Table 3.1).

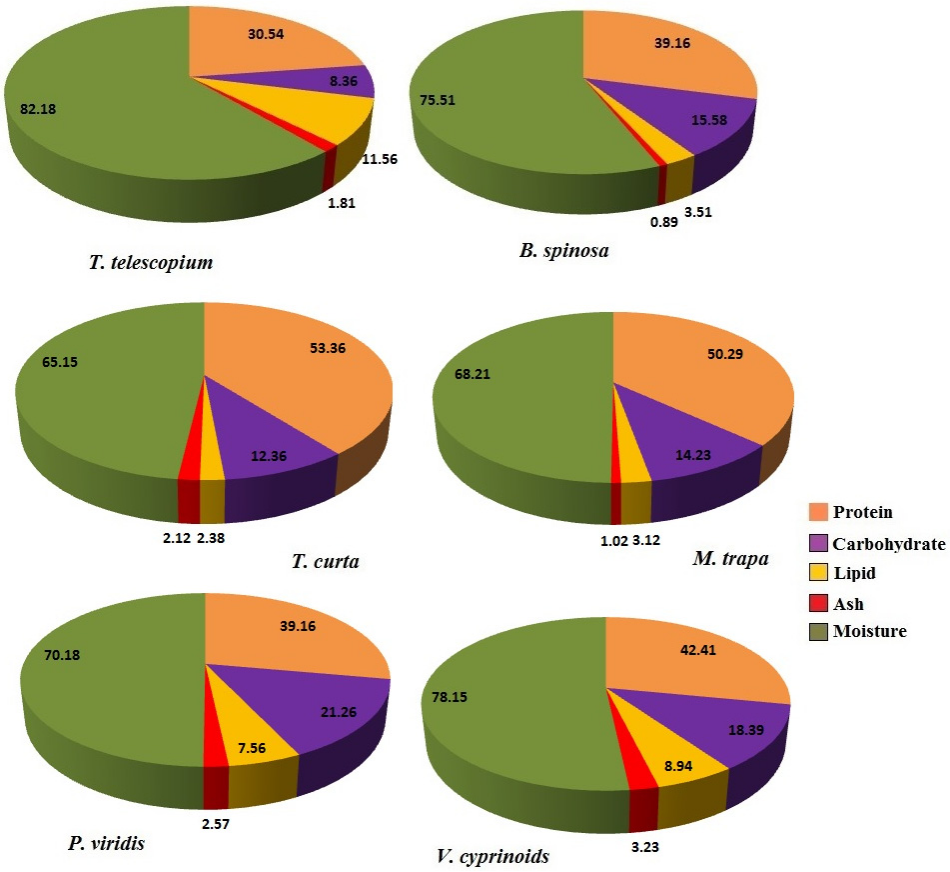

Figure 3.1: Pie diagram representation of biochemical composition of six species.

Laxmilatha (2009) observed that the moisture content of *M. violacea* (bivalve) collected from Thalassery beach, Kerala, India as 80.8%. Moisture content present in foot and columella muscle of fresh *C. ramosus* was 67.4% and 70% respectively (Ramesh et al., 1992). Celik et al. (2014) found 82.2% and 82.4% of moisture content in

the soft tissues of *M. galloprovincialis* and *O. edulis* respectively; both were collected from Balikesir coast, Turkey. Appukuttan & Aravindan (1995) found 79−86% of moisture content in tissues of *P. malabarica*, a bivalve collected from Kerala coast, India, which was more or less similar to the present reports of bivalves.

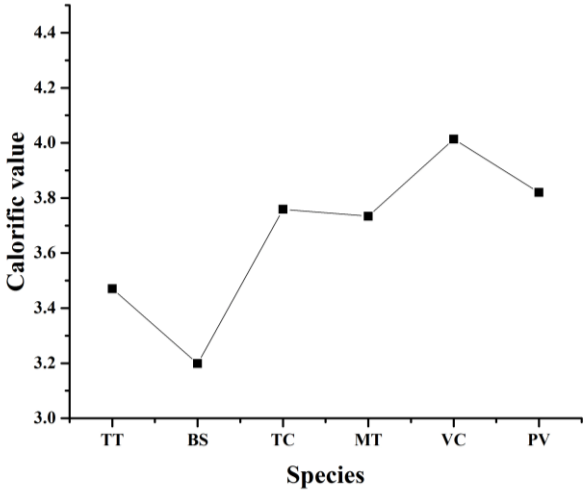

Figure 3.2: Calorific value of molluscs

Ash content of an organism is the non-combustible inorganic matter, which was found to be very low in all the six species. In the present study ash content of molluscs varied from 0.89 to 3.23% dwt. In gastropods, it ranged from 0.89% dwt in *B. spinosa* to 2.12% dwt in *T. curta*. In bivalves, it ranged from 2.57% dwt in *P. viridis* to 3.23% dwt in *V. cyprinoids* (Table 3.3). Ash content of gastropods observed was similar to the reports on *B. spirata* (1.18%) collected from Kerala coast (Margret et al., 2013). Previous reports showed that ash content does not have any kind of relations with sampling location or species variety, but it should be stuck on the structural patterns and size of the

organism (Ortiz et al., 2006; Yaich et al., 2011). Moisture content and ash content were inter-related, because losses of moisture lead to a loss of inorganic water soluble compounds (ash content) (Bochi et al. 2008). Irrespective of sampling locations, similar ash content was reported on the following bivalve species; *P. cylindrica* (3%), *L. marginalis* (2.2%), *M. galloprovincialis* (2.2%) and *E. radiate* (3.64%) (Fuentes et al., 2009; Haldar et al., 2014; Padidela & Thummala, 2015; Ehigiator & Akise, 2016) as well as gastropod species; *X. pyrum* (0.88%) and *A. archatina* (2%) (Jatto et al., 2010; Govindarajalu et al., 2016) (Table 3.1). But some species of gastropods (*O. strigosa, R. venosa*) reported higher values for ash contents (Rees & Hand, 1993; Celik et al., 2014).

Carbohydrates are an assemblage of organic compounds such as starches, sugars, and fibre, which is a chief source of energy for all organisms. In the present study, carbohydrate levels vary from 8.36% to 21.26% (Figure 3.1). Compared to gastropods, bivalve species shows the highest amount of carbohydrates. The percentage composition of carbohydrates in *T. telescopium, B. spinosa, T. curta, M. trapa, P. viridis* and *V. cyprinoids* were observed to be 8.36%, 15.58%, 12.36%, 14.23%, 21.26% and 18.39% respectively (Figure 3.1). The lowest carbohydrate content among the analysed species was in *T. telescopium* (8.36%). Carbohydrate concentration of gastropods varied from 8.36% to 15.58% and those of bivalves varied from 18.39% to 21.26%. Bivalves such as *A. irradians* from USA (Epp et al., 1988), *P. Malabarica* from Ashtamudi, India (Appukuttan & Aravindan, 1995), *P. megellanicus* from Canada (Thompson & Mac Donald, 1990), *R. philipinarium* from Turkey (Celik et al., 2014), *D. incarnates* from Kerala coast (Krishnan & Tharavathy,

2016) (Table 3.1) and gastropods such as *B. areolata* from China (Zhang et al., 2009), *B. zeylanica* from Puducheri, India (Jayalakshmi, 2015), *C. ramosus* from Gulf of Mannar, India (Govindarajalu et al., 2016) were reported to have comparable carbohydrate contents. Carbohydrate content of juvenile *T. telescopium* collected from Tekkali mangroves estuary, Andhra Pradesh, India was 0.80%, whereas in the present study it is 8.36%. This difference in the carbohydrate level may be due to the accumulation of glycogen at different stages like spawning and gametogenesis (Ansari et al., 1981). Carbohydrate content of *B. spirata*, *D. cuneatus* and *L. marginalis* were 16.85%, 14.01% and 14.69% respectively (Periyasamy *et al.,* 2011; Salaskar & Nayak, 2011).

Table 3.3: Proximate composition of bivalves and gastropods

Species	Protein (%)	Carbohydrate (%)	Lipid (%)	Ash (%)	Moisture (%)	Calorific value (Kcal g^{-1})
T. telescopium	30.54 ± 0.15	8.36 ± 0.12	11.56 ± 0.12	1.81 ± 0.08	82.18 ± 1.21	3.17
B. spinosa	39.16 ± 0.25	15.58 ± 0.18	3.51 ± 0.18	0.89 ± 0.15	75.51 ± 0.92	3.20
T. curta	53.36 ± 0.19	12.36 ± 0.21	2.38 ± 0.21	2.12 ± 0.19	65.15 ± 0.95	3.76
M. trapa	50.29 ± 0.51	14.23 ± 0.18	3.12 ± 0.18	1.02 ± 0.19	68.21 ± 0.97	3.73
P. viridis	39.16 ± 0.91	21.26 ± 0.15	7.56 ± 0.15	2.57 ± 0.21	70.18 ± 0.79	3.82
V. cyprinoids	42.41 ± 0.86	18.39 ± 0.12	8.94 ± 0.13	3.23 ± 0.37	78.15 ± 2.17	4.01

The percentage composition of total protein in *T. telescopium, B. spinosa, T. curta, M. trapa, P. viridis* and *V. cyprinoids* were observed to be 30.54%, 39.16%, 53.36%, 50.29%, 39.16% and 42.41% respectively (Figure 3.1). The highest protein content was exhibited by *T. curta* and *M. trapa* (Table 3.3) with respect to the species division, gastropods had a foremost concentration of protein contents. In gastropods, the protein content of *B. spinosa* (39.16%) is similar the least protein containing

bivalve, *P. viridis* (39.16%). Similar to protein contents were observed in previous studies too. Studies done on gastropods by Babu et al. (2012), Periyasamy et al. (2011), Margret et al. (2013), Jayalakshmi (2015), Giftson & Patterson (2016), Govindarajalu et al. (2016) are some of the similar indigenous reports (Table 3.1). Babu et al. (2012) reported 27.9% of total protein in the soft tissue of *B. spinosa* collected from Mandapam coast, India, whereas in the present study 30.54% of the protein was recorded in the same gastropod collected from Ashtamudi estuary, India. The reason for the difference in protein content of same species may be due to the association of the protein content with the gonad development, and it may be used to synthesize carbohydrate or lipid (Mc Lachlan & Lombard, 1980). Previous reports of protein content on *D. cuneatus* and *L. marginalis* were 52.33 to 63.86% and 57.39 to 66.51% respectively (Salaskar & Nayak, 2011). Recent studies reported by Hamdani & Mazouni (2011), Shetty et al. (2013), Krishnan & Tharavathy (2016), Nirmale et al. (2016) (Table 3.1) also had similar protein contents.

Lipids are important cellular constituents and effective source of energy. The lipid content of the molluscs was found to be 11.56% in *T. telescopium*, 3.51% in *B. spinosa*, 2.38% in *T. curta*, 3.12% in *M. trapa*, 7.56% in *P. viridis* and 8.94% in *V. cyprinoids* (Figure 3.1). In the present investigation, the lipid contents of the gastropods ranged from 2.38 to 11.56% and those of bivalves varied from 7.56 to 8.94%. Lipid content of the present study was supported by several previous studies. Notable studies were Fagbuaro et al. (2006), who observed 1.38% of lipid content in *A. marginata* and Padidela & Thummala (2015) in *P. cylindrical* with a value of 5%. Periyasamy et al. (2011) reported

9.3% of lipid in *B. spirata* (Table 3.1). Lipid content of various body tissues of *B. spinosa* from Gulf of Mannar ranging from 2.38 to 4.9% (Babu et al., 2012), which was in agreement with the present lipid content of *B. spinosa* (2.38%). The lipid content of *D. trunculus* (Bivalvia) varied from 6.68 to 48. 45 μg g^{-1} (Hamdani & Soltani-Mazouni, 2011). Lipids are the most important storage form of food, and it contains more than twice the energy of carbohydrate and proteins. The highest lipid content was in *T. telescopium* (11.56%) which was inverse to the observation of both total protein and carbohydrate contents, and this data is supported by Chakravarty et al. (2015) in *T. telescopium* from Tekkali mangrove estuary.

Calorific value, which is the energy released upon combustion of food, exhibited similar trends in both bivalves and gastropods. Calorific value in bivalves varied from 3.82 Kcal g^{-1} to 4.01 Kcal g^{-1} and those of gastropods ranging from 3.17 Kcal g^{-1} to 3.76 Kcal g^{-1} (Figure 3.2). The calorific values of the molluscs were found to be 3.17 Kcal g^{-1} for *T. telescopium*, 3.20 Kcal g^{-1} for *B. spinosa*, 3.76 Kcal g^{-1} for *T. curta*, 3.73 Kcal g^{-1} for *M. trapa*, 3.82 Kcal g^{-1} for *P. viridis* and 4.01 Kcal g^{-1} for *V. cyprinoids* (Table 3.3). Calorific values of bivalves were higher as compared to gastropods. The highest value of calorific value was obtained for *V. cyprinoids*, and the least value was obtained for *T. telescopium*. Calorific values of previous studies on the local gastropods and bivalves were represented in Table 3.2. Abraham (2016) reported a calorific value of 4.5 Kcal g^{-1} for *P. cylindrical* (bivalve). Dauvin & Joncourt (1989) reported a comparable calorific value with 4.61 Kcal g^{-1} for *A. alba* and 4.49 Kcal g^{-1} for *A. prismatica*. Cheung et al. (2008) reported a calorific

value of 3.98 Kcal g^{-1} for *T. philippinarum* (gastropod). Mc Clintock et al. (1994) reported a calorific value of 3.1 Kcal g^{-1} for *T. bell* and 4.8 Kcal g^{-1} for *A. kerguelensis* (Table 3.2).

Table 3.4: Two way ANOVA for the proximate composition of the gastropods

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Biochemical compositions	15567.43	4	3891.857	162.2757	2.46E-10	3.259167
Species	14.97798	3	4.99266	0.208175	0.888784	3.490295
Error	287.7959	12	23.98299			
Total	15870.2	19				

Table 3.5: Two way ANOVA for the proximate composition of the bivalves

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Biochemical compositions	6749.091	4	1687.273	214.0195	6.47E-05	6.388233
Species	10.79521	1	10.79521	1.369301	0.306895	7.708647
Error	31.53494	4	7.883735			
Total	6791.421	9				

Two-way ANOVA result shows that a significant variation is existing between the biochemical constituents, but there is no statistically significant ($P< 0.05$) changes exist between the proximate composition of both gastropods and bivalves (Tables 3.4 and 3.5). It indirectly suggests that there is no alteration in species level composition of the habitat. The proximate composition significantly changes between gastropods and bivalves. However there are no species wise changes

within these classes. Similar results were observed in the two-way ANOVA of biochemical composition of gastropods and bivalves collected from Colachal and Kadipatinam (Margret, 2015). Significant variation in biochemical composition means their level and biosynthetic pathway in body is interrelated.

3.3.2 Mineral composition

Mineral concentrations from six molluscs, namely two bivalves and four gastropods were represented in Table 3.6. Metals in the present study generally follow the order: Mg > Ca > Zn > Fe > Cu > Mn > Cr > Pb > Ni > Co > Cd and their concentrations are expressed in mg kg^{-1} dry wt. Ca and Mg were predominant in all species due to their biological role in living organisms. This result was supported by the previous reports on *T. bufo, C.ramosus* and *B. spirata* (Margret, 2015). Mg was the most abundant metal present in all the species except in *V. cyprinoids* and *T. curta*. Ca was the richest mineral present in *V. cyprinoids* (2219 mg kg^{-1}) and *T. curta* (1855 mg kg^{-1}). Ca varies from 303 mg kg^{-1} to 2219 mg kg^{-1}, of which maximum concentration for *V. cyprinoids* and minimum for *B. spinosa*. The earlier findings were in agreement with the present result. Ca was observed to be maximum for the soft tissue of *M. casta* (Srilatha et al., 2013). Mg ranged from 574 mg kg^{-1} to 2973 mg kg^{-1}, maximum concentration for *P. viridis* and minimum for *V. cyprinoids.* Co is the slightest element present in *P. viridis*, *B. spinosa* and *M. trapa,* while in the remaining species Cd is minimum.

Table 3.6: Mineral concentration (expressed in mg kg^{-1}) in gastropods and bivalves

Metal	*P. viridis*	*V. cyprinoids*	*T. telescopium*	*B. spinosa*	*T. curta*	*M. trapa*
Mg	798.97 ± 2.93	574.99 ± 2.19	2423.61 ± 5.16	1491.94 ± 2.87	1259.46 ± 3.87	2973.76 ± 2.19
Ca	492.70 ± 2.58	2219.85 ± 4.86	605.09 ± 4.44	303.20 ± 3.45	1855.70 ± 3.55	553.03 ± 2.54
Zn	83.06 ± 0.19	76.79 ± 0.67	105.10 ± 2.12	68.16 ± 0.93	113.64 ± 1.42	89.05 ± 0.82
Fe	55.74 ± 2.94	69.23 ± 5.86	63.39 ± 2.94	50.18 ± 1.92	76.85 ± 6.19	55.80 ± 3.19
Mn	4.64 ± 0.03	5.16 ± 0.59	15.08 ± 0.39	2.89 ± 0.91	8.50 ± 0.92	1.30 ± 0.38
Cu	9.54 ± 0.53	13.73 ± 0.59	32.26 ± 0.82	14.19 ± 0.47	37.02 ± 0.81	33.61 ± 0.29
Cr	7.59 ± 0.94	3.57 ± 0.38	2.37 ± 0.39	2.18 ± 0.93	6.47 ± 0.82	2.25 ± 1.18
Ni	0.94 ± 0.27	2.42 ± 0.39	1.97 ± 0.86	2.57 ± 0.48	3.21 ± 0.69	1.60 ± 0.49
Co	0.09 ± 0.17	0.87 ± 0.52	0.44 ± 0.18	0.31 ± 0.11	0.35 ± 0.21	0.09 ± 0.17
Cd	0.14 ± 0.59	0.08 ± 0.39	0.23 ± 0.93	1.47 ± 0.49	0.04 ± 0.29	5.33 ± 0.16
Pb	1.33 ± 0.49	1.46 ± 0.66	2.06 ± 0.83	1.16 ± 0.49	2.64 ± 0.48	2.16 ± 0.38

Fe concentrations (Table 3.6) fluctuated widely from 50 mg kg^{-1} to 76 mg kg^{-1} and 55 mg kg^{-1} to 69 mg kg^{-1} for gastropods and bivalves respectively. The maximum value was detected in *T. curta*, while the minimum value was observed in *B. spinosa*. From the previous reports, Fe contents of *L. littorina* and *P. vulgata* were in the range of 272 mg kg^{-1} to 784 mg kg^{-1} and 891 mg kg^{-1} to 2330 mg kg^{-1} respectively (Bryan & Hummerstone, 1977; Bustamante et al., 2000). Fe is an essential constituent of haeme in haemoglobin, cytochromes, peroxidases, etc. From the testified data of two bivalves (*A. senilis* and *C. gasar*) collected from Nigeria shows that Fe concentration ranged from 62 mg kg^{-1} to 65 mg kg^{-1} (Joiris & Azokwu, 1999). Previous reports on Fe concentrations in different species of bivalves were under the range of 196 mg kg^{-1} to 1540 mg kg^{-1} (Bryan, 1973; Bryan & Hummerstone, 1977; Bustamante et al., 2000). Fe content of some bivalves collected from Ghana (upwelling area) is ranging from 11 mg kg^{-1} to 75 mg kg^{-1} (Joiris & Azokwu, 1999). Zn is the third most abundant element in both bivalves and gastropods.

Zn contents (Table 3.6) in the soft tissue of molluscs showed little fluctuations and ranged from 76 mg kg^{-1} to 83 mg kg^{-1} and 68 mg kg^{-1} to 113 mg kg^{-1} for bivalves and gastropods respectively, with maximum concentration detected for *T. curta* and minimum for *B. spinosa*. Similar type of distribution pattern of Zn has been reported in many marine oysters, bivalves, gastropods and turtles. From the previous reports, wide range of Zn concentrations (37.76 mg kg^{-1} to 343 mg kg^{-1}) was shown by different species of clams collected from diverse locations of Malaysia (Yusof et al., 2004; Yap et al., 2009; Yusoff & Long, 2011; Kamaruzzaman et al., 2011; Lias et al., 2013; Hossen et al., 2015). Zn is essential for insulin structure and functions; co-factor of carbonic anhydrase. Zn concentration in the soft tissue of molluscs from the Basque coast (Northern Spain) reported was in the range of 130 mg kg^{-1} to 260 mg kg^{-1} (Franco et al., 2002). Margret, 2015 reported that mollusc, in general, are excellent sources of iron, calcium, zinc, etc. Different species of pearl shells from the South China Sea were a rich source of Zn (Zhang, 1994). Zn concentrations of two gastropods *L. littorea* and *P. vulgate* ranges 45 mg kg^{-1} to 284 mg kg^{-1} and 83 mg kg^{-1} to 224 mg kg^{-1} respectively (Bryan & Hummerstone, 1977; Bustamante et al., 2000). Rich content of Zn in all the species is due to the upwelling nature of sampling site (Lewis & Luther III, 2000). Productivity is comparably high in upwelling region due to mixing of coastal waters and sea water (Monteiro et al., 2015; Baines et al., 2016). Moreover Zn is an essential element observed in the cell wall of diatoms and bacteria. Due to the death and decay of these micro-organisms, Zn content is comparably high in upwelling region (Rzymski & Poniedziałek, 2014; Grasse et al., 2016).

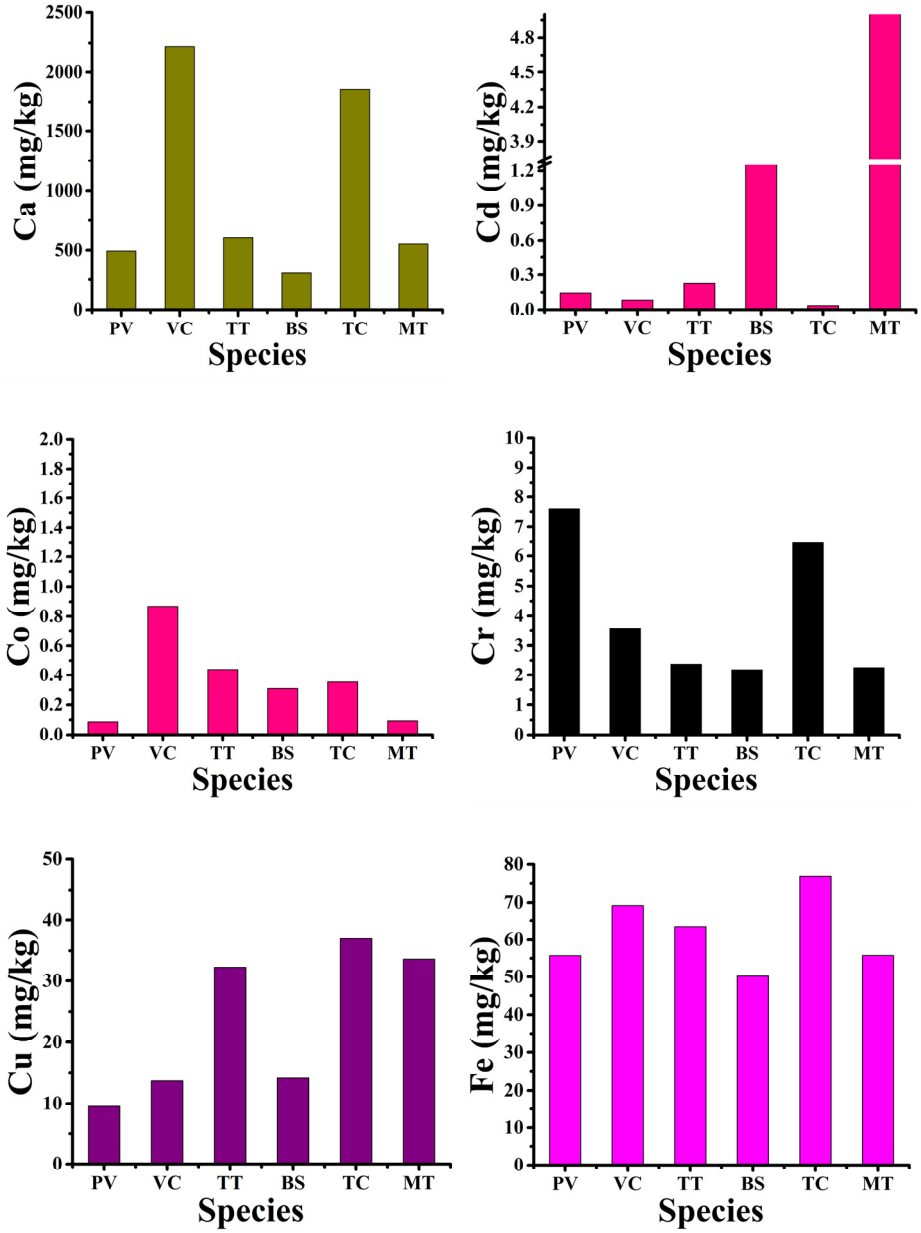

Figure 3.3 continued…

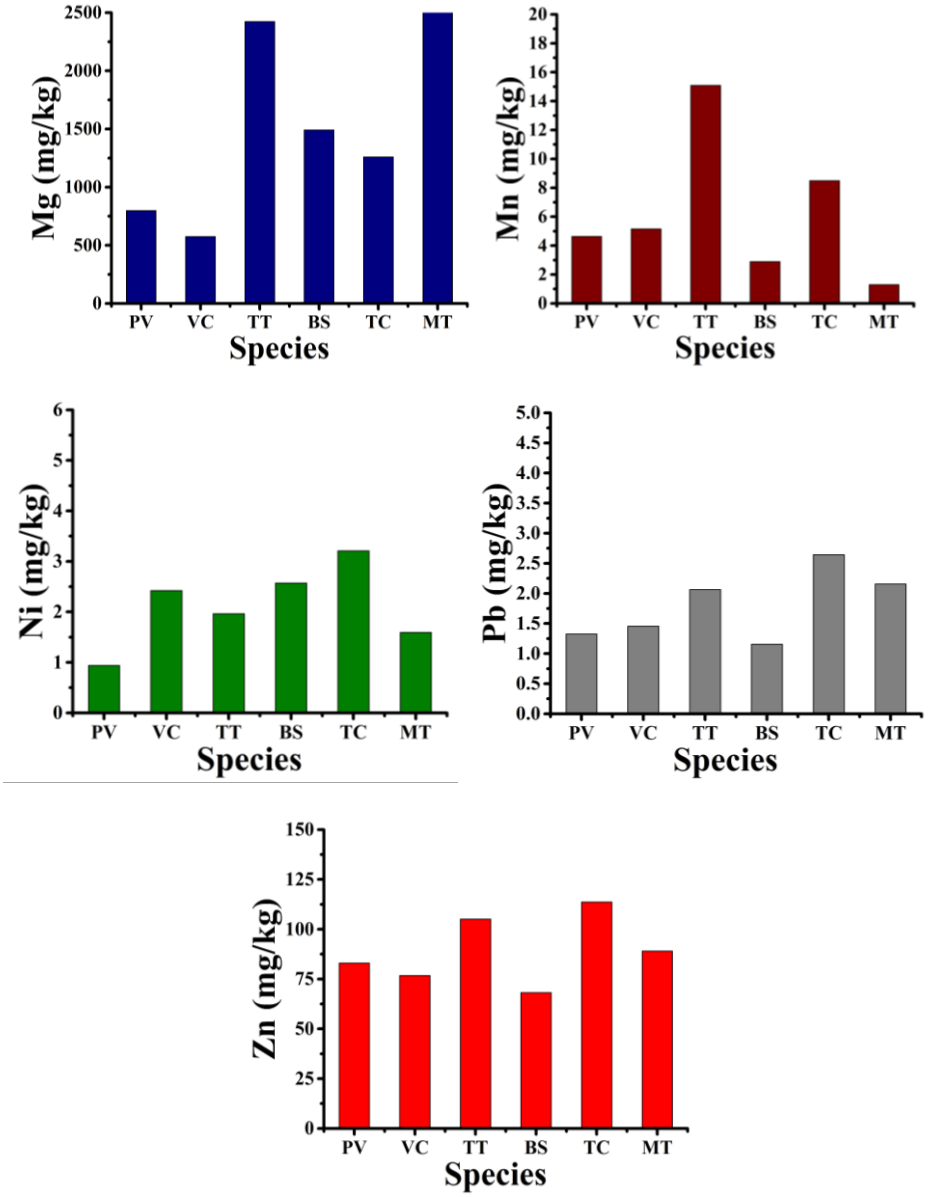

Figure 3.3: Pictorial representation of Metal concentrations in gastropods and bivalves

Like Zn, Cu is also an essential element in living organisms. Cu contents (Table 3.6) in the soft tissues of gastropods ranging from

14 mg kg^{-1} to 37 mg kg^{-1}, whereas that of bivalves varying from 9 mg kg^{-1} to 13 mg kg^{-1} respectively. The concentrations of Cu reported in the present study is similar to those of the values obtained for the bivalve, *L. elliptica* collected from Terra Nova Bay and from Cape Evans (Grotti et al., 2008) and the gastropod (*N. concinna*) collected from King George Island (Ahn et al., 2004). Srilatha et al. (2013) observed minimum copper content in the soft tissues of *M. casta* from Parangipettai and Cuddalore coast. The highest value was observed in *P. viridis*, while the lowest value was observed in *M. trapa*. According to Cheriyan et al. (2015), high Cu contents were observed in the sediment of Neendakara due to the terrestrial discharge containing fertilizers, algaecides, fungicides, molluscicides which originate predominantly from agricultural activities and other economic sectors. Cu is an essential component of haeme in haemocyanin (transportation pigment in gastropods and cephalopods) and also co-factor in tyrosinase and ascorbic acid oxidase. Cu content of bivalves was higher than those of gastropods collected from the similar area. This is because different species accumulate Cu in dissimilar ratio. One comparable study is Cu concentrations of mussels collected from Basque coast, ranged from 13 mg kg^{-1} to 20 mg kg^{-1}, but those of oysters from the same study area ranged from 361 mg kg^{-1} to 524 mg kg^{-1} (Franco et al., 2002).

Mn values of the soft tissues of gastropods and bivalves were ranged from 1.3 mg kg^{-1} to 15 mg kg^{-1} and 4 mg kg^{-1} to 5 mg kg^{-1} respectively (Table 3.6). These values were comparable with the results obtained from *L. littorina* as well as *P. vulgata* (two gastropods) and six different species of bivalves (Bryan, 1973; Bryan & Hummerstone,

1977; Bustamante et al., 2000). But the high concentration of Mn was observed in the soft tissues of oyster (Xu et al., 2016) and bivalves from Mandovy (Al-Usmani et al., 2015). *P. viridis* collected from both Mandovy and Chapora shows higher values for Mn (Al-Usmani et al., 2015) than those of the present study. This is because these regions were polluted by human activity (Qasim & Sengupta, 1980). Mn is a co-factor for arginase and certain other metabolic enzymes and also involved in bone formation and erythrocyte regeneration. Pb belongs to the group of non-essential and toxic metals, and its concentration in the soft tissues of bivalves ranged from 1.33 mg kg^{-1} to 1.46 mg kg^{-1} and those of gastropods widely ranged from 1.16 mg kg^{-1} to 2.64 mg kg^{-1} (Figure 3.3). These results were similar to Pb content of Malacian clams (Hossen et al., 2015). Similar concentrations were in the soft tissues of both oysters and bivalves collected from Northern Spain (Franco et al., 2002).

Ni concentration is usually low in the marine environment and in the present study, concentrations ranging from 0.94 mg kg^{-1} to 2.24 mg kg^{-1} for bivalves and 1.60 mg kg^{-1} to 3.21 mg kg^{-1} for gastropods respectively (Figure 3.3). The highest concentration of Ni was present in *T. curta* and minimum for *P. viridis*. Ni concentrations in two bivalve species *D. trunculus* and *C. gallina* collected from Atlantic Coast were ranging from 0.22 mg kg^{-1} to 2 mg kg^{-1} (Usero et al., 2005), which is analogous with the present study. From the earlier reports, wide ranges of Ni contents (0.9 mg kg^{-1} to 62 mg kg^{-1}) were exhibited by some gastropods and bivalves (Bustamante et al., 2000). Co contents (Figure 3.3) in the soft tissue of gastropods ranged from 0.09 mg kg^{-1} to 0.35 mg kg^{-1}, whereas that of bivalves ranged from 0.09 mg kg^{-1} to 0.87 mg kg^{-1} respectively.

Previous reports of Co contents in some gastropods were ranging from 0.6 mg kg^{-1} to 1 mg kg^{-1} (Bryan & Hummerstone, 1977; Bustamante et al., 2000). Values of Co are very low compared with the values of those in bivalves collected from Fiji (industrial area) (Mason & Reynolds, 1988). Co contents of some bivalves collected from Cochin backwaters (polluted area) varying from 22 mg kg^{-1} to 35 mg kg^{-1} (George et al., 2013), while those of bivalves collected from unpolluted area ranging from 0.02 mg kg^{-1} to 2.3 mg kg^{-1} (Bryan & Hummerstone, 1977; Bustamante et al., 2000).

Cr contents in the soft tissues of bivalves and gastropods extended from 3.57 mg kg^{-1} to 7.59 mg kg^{-1} and 2.18 mg kg^{-1} to 6.47 mg kg^{-1} (Figure 3.3) respectively. These values were similar to Cr content of *M. galloprovincialis* collected from different areas of Mediterranean Sea (Joksimovic et al., 2011; Spada et al., 2012; Spada et al., 2013). Cr is an essential element, which involved in collagen formation and regulation of the rate of glucose metabolism. Reported data of Hamed & Emara (2006), shows that Cr content in the soft tissue of *P. caerulea* (gastropod) ranged from 2.34 mg kg^{-1} to 7 mg kg^{-1} and those of *B. barbatus* (bivalve) ranged from 1 mg kg^{-1} to 4 mg kg^{-1}. From the previous reports, Cr contents of gastropods and bivalves ranged from 0.94 mg kg^{-1} to 303 mg kg^{-1} and 0.13 mg kg^{-1} to 2 mg kg^{-1} respectively (Bryan, 1973; Bryan & Hummerstone, 1977; Bustamante et al., 2000). It was found that Cd concentration in the soft tissue of gastropods and bivalves ranged from 0.04 mg kg^{-1} to 5 mg kg^{-1} and 0.08 mg kg^{-1} to 0.14 mg kg^{-1} respectively (Table 3.6). These values were similar to the results obtained from the soft tissues of mussels and oysters (Franco et al., 2002). From the reported data, Cd concentrations for various gastropods and bivalves

were ranged from 0.21 mg kg^{-1} to 34 mg kg^{-1} and 0.49 mg kg^{-1} to 24 mg kg^{-1} respectively (Bryan, 1973; Bryan & Hummerstone, 1977; Bustamante et al., 2000). From the studies of George et al. (2013), Cd contents of *V. cyprinoids* collected from Cochin backwaters ranged from 1.06 mg kg^{-1} to 1.56 mg kg^{-1}, while those in the present study is 0.08 mg kg^{-1}.

Table 3.7: Maximum permissible limits of certain metals in molluscs.

Organization	Metals					References
	Cd	Cu	Ni	Pb	Zn	
Malaysian Food Regulation	1	30	-	2	100	Malaysian Food Regulation, 1985
International Council for the Exploration of the Sea	1.8	-	-	3	-	ICES, 1988
World Health Organization	0.5	30	-	0.5	40	WHO /FAO, 1993
Food and Agriculture Organization/World Health Organization	0.5	-	-	-	30	WHO /FAO, 1983
Ministry of Public Health Thailand	-	133	-	6.67	667	MPHT, 1986
Australian Legal Requirements for Food Safety	10	350	-	-	750	NHMRC, 1987
Food Safety and Standards Authority of India	1.5	20	1.5	2.5	50	Food Safety and Standards Authority of India, 2011
CODEX STAN	2	-	-	-	-	CODEX STAN, 2016
COMMISSION REGULATION	1	-	-	1.5	-	EC, 2001

Minerals also create important constituents of hormones, enzymes and enzyme activators in human nutrition but it becomes toxic if it exceeds the permissible limits (Khan, 1992). Human body needs all these elements in varying concentrations. All elements in organisms have its own permissible limits of mineral content and were expressed in Table 3.7. All metals in species studied were below the highest maximum permissible limits set by Malaysian Food Regulation, (1985), Ministry of Public Health, Thailand (MPHT, 1986), National Health and Medical Research Council (NHMRC, 1987), Food Safety and Standards Authority of India, (2011) and General Standard For Contaminants and Toxins in Food and Feed (CODEX STAN, 2016) (Table 3.7). According to WHO report (CODEX 2016), the average daily intake of Cu in normal food for adults ranges from 3 mg kg^{-1} to 6 mg kg^{-1} and should not exceed 10 mg kg^{-1}. As stated by Malaysian Food Regulation (1985), the maximum permissible limit of Cu is 30 mg kg^{-1}. Mn is also an essential element for most of the living organisms, but its excess may cause a neurotoxic syndrome called Parkinson-like diseases (Perl & Olanow, 2007). The permissible limit for Mn in molluscs is 100 mg kg^{-1} (WHO, 1993) (Table 3.7). Ni is an essential metal, which becomes toxic at higher concentration but there is no strong evidence about the relationship between the concentration of nickel and its toxicity in the organism (Khan et al., 2012). Cr is a toxic trace metal, and its permissible limit is 3 mg kg^{-1} to 5 mg kg^{-1} (Popa, 2008). Cd is a non-essential toxic metal and may accumulate from food chain magnification and may induce kidney dysfunction, skeletal damage and reproductive deficiencies (European Communities, 2001).

Table 3.8: Correlation matrix indicating the inter-relationship among various metals.

	Zn	Cu	Fe	Mn	Pb	Ca	Ni	Co	Cr	Cd	Mg
Zn	1										
Cu	0.78	1									
Fe	0.66	0.43	1								
Mn	0.63	0.23	0.61	1							
Pb	**0.92****	**0.92****	0.63	0.32	1						
Ca	0.39	0.22	**0.93****	0.35	0.43	1					
Ni	0.20	0.42	0.54	0.27	0.29	0.48	1				
Co	−0.06	−0.06	0.58	0.45	−0.09	0.65	0.59	1			
Cr	0.28	−0.28	0.40	0.25	0.10	0.39	−0.19	−0.16	1		
Cd	−0.27	0.27	−0.61	−0.80	0.05	−0.51	−0.19	−0.52	−0.62	1	
Mg	0.36	0.72	−0.30	−0.11	0.49	−0.49	−0.02	−0.47	−0.61	0.67	1

**Correlation significant at the 0.01 level (n=27).

Correlation analysis was done and the metal-metal correlation coefficients in the soft tissues of both gastropods and bivalves are expressed in Table 3.8. It shows that strong positive correlations exist between element pairs Pb-Cu, Pb-Zn and Ca-Fe ($R = 0.92–0.93$, $p < 0.01$). Ca and Fe are essential for the electrical activity needed to support muscle contractions and neuron activation in living organisms. Strong positive correlation of Cu in this study is due to large Cu content on gastropods and bivalves. This is because hemocyanin is the pigment, which transports oxygen in the hemolymph of many in both gastropods and bivalves (Rzymski & Poniedziałek, 2014). Their exists strong positive correlations between Pb and Zn in the soft tissues of molluscs ($R = 0.92$, each $p< 0.01$), which suggested common metal contamination origin, from marine transport (mainly from ships) as well as agricultural, industrial, and domestic sewage discharge, including fuel burning (Zhou et al., 2007; Zhang et al., 2013; Zhang et al., 2016). The correlation

between the concentrations of metals such as Co, Ni, Ca and Co (above 0.5) supports these observations

3.4 Summary

This chapter stated the determination of the proximate and mineral composition of selected gastropods and bivalves from Kerala coast, India. Out of the six species, three of them are already reported as edible, of which two are bivalves, and one is gastropod. *P. viridis* and *V. cyprinoids* are the edible bivalves and *T. telescopium* is the edibile gastropod. *P. viridis* and *V. cyprinoids* are major dish in Kerala. Peoples from Alapuzha, Cochin and Kannur districts largely cultivated these two bivalves. *T. telescopium* is not a common food in India, but it is largely used in South Malaya, Philippines, Australia and Madagascar. Gastropods and bivalves in this study had almost comparable biochemical constituent and minerals. The protein content of *T. curta* and *M. trapa* were found to be high as compared to other species. In this study, carbohydrate content exceeds lipids in all the species except *T. telescopium*. Bivalves were found to possess higher protein, lipid, calorific value and water holding capacity. From the two-way ANOVA result, significant variation is existing between the biochemical constituents. But there is little variations exist between the proximate composition of both gastropods and bivalves. All the species were fit for human consumption on the basis of low-fat content. Energy contribution was found to be highest in *P. viridis* and *V. cyprinoids*, so these organisms could act as food and energy alternatives. Ash content of bivalves were high indicates the presence of minerals. Mg, Ca, Zn, Fe, Cu, Mn, Cr, Pb, Ni, Co, Cd was the minerals present in

both gastropods and bivalves. Ca and Mg were the most abundant and these are essential for biological metabolism and growth of organisms. Toxic elements such as Pb and Cd contents were very minute in these organisms. Metal present in all the species were below the permissible limit approved by World Health Organizations, Malaysian Food Regulation, Ministry of Public Health, Thailand, National Health and Medical Research Council, Food Safety and Standards Authority of India, and General Standard for Contaminants and Toxins in Food and Feed. The overall results under consideration with total ash, mineral, protein, carbohydrate, lipid and water content suggest all the six molluscs studied would be considered as nutritional supplements.

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Chapter 4

CHARACTERIZATION OF AMINO ACIDS

Contents

4.1 Introduction

The demand for proteinaceous food is increasing gradually with the continuous development of world population. According to United Nations, global population is estimated to reach 7.50 billion on April 2017 and it will further rise to 10.90 billion in the year 2050 (World Population Clock, 2017). According to FAO, there are 795 and 194 million undernourished people in the world and in India respectively (FAO, 2015). This major issue can be controlled by proper exploitation of aquatic organisms, which will supply the balanced, nutritious food. Seafood provides high-quality protein, containing all the dietary essential amino acids for the growth and maintenance of human body (Ademolu et al., 2004; Fagbuaro et al., 2006; Domingo, 2016; Wells et al., 2017). Proteinaceous food from aquatic resources is entirely trusted on fishery products. According to Jeena et al. (2003), fisheries occupy an important part in the world protein supply systems, which accounts for 10% of the total protein supplies. But, recently fish and fish products are now transferred to different places, and hence its

quality is becoming much more important, which reflects on its price (Hassoun & Karoui, 2017). So various other sources such as crustaceans, molluscs and phytoplanktons were used for balancing protein supply. In this context, marine molluscs, which contain a considerable amount of proteins were investigated to fulfill these demands (Pandey et al., 2017). At the time of consumption, proteins from molluscs degraded to form amino acids units. They contain both the essential and non-essential amino acids, which are required by the human body to meet the daily requirements. These amino acids are accessible in its free form, and they play a vital role in the reconstruction process of proteins in human body (Dean et al., 2001).

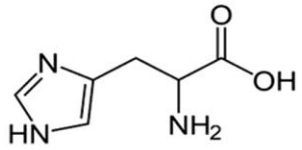
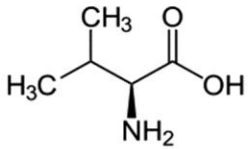
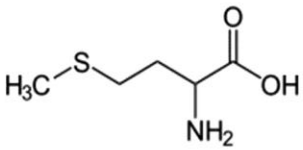
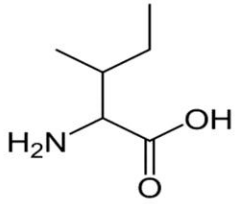
Amino acids are the building blocks of proteins, and they occupy a prominent position among all other nutrients. They are essentially organic compounds consisting of amino as well as acidic groups and contribute a considerable role in the health of the human nervous system, hormone production, and muscular structure (Wells et al., 2017). Also, they are needed for vital organs and cellular structure. There are 20 naturally occurring amino acids of which asparagine was the first discovered amino acid in the year 1806 followed by cysteine, leucine, and glycine (Furst & Stehle, 2004). Out of the 20 amino acids, nine are essential, six are conditionally essential, and five are non-essential amino acids. Essential amino acid means, they cannot be synthesized by the organism, and thus must be obtained from normal diet. This includes phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine. Six other amino acids are considered conditionally essential in the human diet, meaning their synthesis can be limited under special pathophysiological conditions, such as prematurity

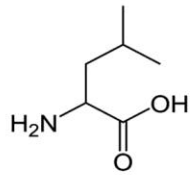
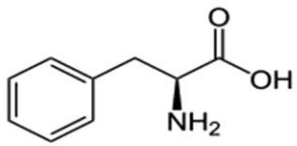
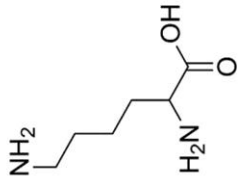
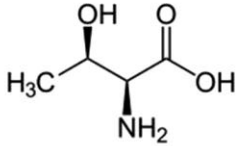
in the infant or individuals in severe catabolic distress. They are arginine, cysteine, glycine, glutamine, proline and tyrosine (Furst & Stehle, 2004). Five amino acids are non-essential to humans, meaning they can be synthesized in the body. These are alanine, aspartic acid, asparagine, glutamic acid and serine (Reeds, 2000; Furst & Stehle, 2004). They are listed in Table 4.1.

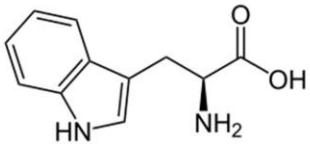
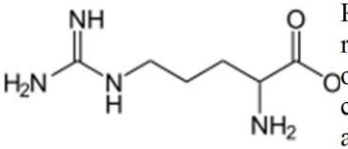
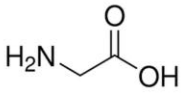
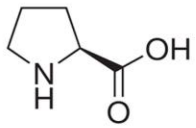
Amino acids are micro molecules and play a vital role in the metabolism of all living organisms (Christensen, 1990). They function as the chief structural module of muscle and other tissues in the body. Moreover, they are involved in the production of enzymes, hemoglobin and hormones (Leuchtenberger et al., 2005; Meister, 2012). Amino acids are naturally available and safe, having an essential role in pharmacological sectors, which promote it widely in biotechnological applications widely. They are used for clinical diagnosis and treatments for diseases associated with liver, hereditary, neuropathy, acute coronary syndromes, diabetics, etc. (Li et al., 2011; Bifari & Nisoli, 2017; Maynard & Manzini, 2017). The quality and quantity of amino acids and its availability determines the nutritional quality of the proteins (Gressler et al., 2010). Animals don't have the ability to synthesize all these amino acids of primary metabolisms by themselves, and it will earn through their diet. Threonine and methionine were usually absent in grains, and it is balanced by the consumption of legumes, meat or fish (Joshi et al., 2010). Isoleucine present in organisms is synthesized from threonine and methionine which are acquired from the enzymatic actions on aspartate. This statement proves that amino acids in all living organisms are observed through many interdependent biochemical pathways (Joshi et al., 2010).

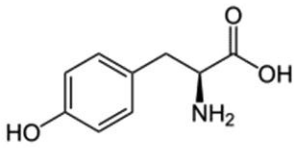
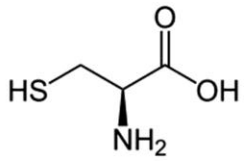
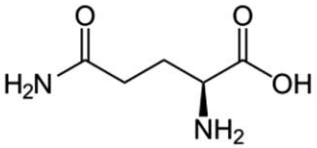
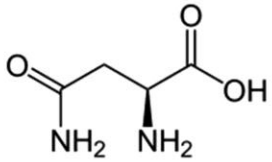
Alanine metabolism helps in ATP production by glycolysis and its accumulation in organisms related to oxygen availability (Rocha et al., 2010). Glycine is observed largely in mammals as an extracellular structural protein. It doesn't have D or L form, and it is synthesized from choline, serine, hydroxyl-proline and threonine (Wang et al., 2013). L-amino acids are widely observed in nature and are stereospecific. Unusual D-amino acids were also observed, which is the mirror images of L-amino acids. D-alanine, D-glutamic acid and D-aspartic acid were seen in pea seed (*P. sativum*), hops blossom (*H. lupulus*) and barley (*H. vulgare*) (Ogawa et al., 1977; Erbe & Brückner, 2000). Glycine, proline, alanine, glutamic acid, taurine, etc., exhibit osmolytic properties and are known as osmolytes (Di Martino et al., 2003; Lopez-Fontanals et al., 2003; Shellhammer et al., 2017). Any change in the osmotic pressure of external seawater will affect the concentration of amino acids and quaternary amine compounds (Kinne & Zeidel, 2008; Yuan et al., 2017). They also act as stabilizers of proteins in a frozen state by increasing the melting points of proteins (Chang et al., 2005; Tang & Pikal, 2005; Amorini et al., 2017). Previous reports show that amino acids such as leucine, valine, and isoleucine were used for maintaining nitrogen balance in conditions such as sepsis, trauma and burns, and they also supports protein synthesis after injury and decrease muscle proteolysis (Williams & Barbul, 2003). The deficiency of essential amino acids may cause hormonal imbalances, irritability, lack of concentration, and even depression (Furst & Stehle, 2004; Concolino et al., 2017).

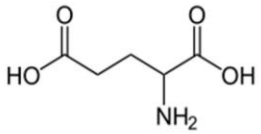
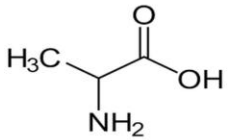
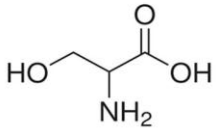
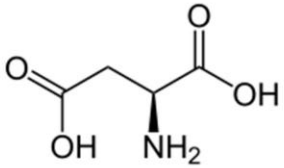
Table 4.1: Structure, abbreviation, and use of twenty amino acids.

Amino acids	Abbreviation	Structure	Important use	References
		Essential amino acids		
Histidine	His		Precursor to histamine, local mediator of inflammation and the immune response to certain pathogens	Fahey et., 2001
Valine	Val		Used in treating liver and gall bladder damages, tissue repairs.	Mitrega et al., 2011
Methionine	Met		Prevents liver damage in acetaminophen poisoning, regulates urine pH. Supplementation for those suffering from Parkinson's diseases, copper poisoning, asthma, allergies, alcoholism, or depression	Bellamy et al., 1998
Isoleucine	Ile		Muscular repairs, muscle rebuilding	Casperson et al., 2012

Leucine	Leu		Used in biosynthesis of protein	Takeshita et al., 2012
Phenylalanine	Phe		Used for depression, chronic pain, Parkinson's disease, osteoarthritis, rheumatoid arthritis, alcohol withdrawal symptoms and a skin disease called vitiligo.	Antoniou et al., 1989
Lysine	Lys		Used for preventing and treating cold sores.	Di Giovanna & Blank, 1984
Threonine	Thr		It promotes normal growth by helping to maintain the proper protein balance in the body and also supports cardiovascular, liver, central nervous, and immune system function.	Yaffe & Elia, 2001

Tryptophan	Trp		Used for insomnia, depression, anxiety, sleep apnea, a severe form of premenstrual syndrome called premenstrual dysphoric disorder (PMDD), grinding teeth during sleep (bruxism), facial pain, smoking cessation, attention deficit-hyperactivity disorder (ADHD), Tourette's syndrome, and to improve athletic performance.	Richard et al., 2009
Conditionally essential amino acids				
Arginine	Arg		Precursor of creatine (an energy reservoir in muscles), nitrogen oxide (a mediator of blood vessel constriction), citrulline (an antioxidant).	Stechmiller et al., 2005
Glycine	Gly		Used for treating schizophrenia, benign prostatic hyperplasia (BPH), stroke, it is also used to protect kidneys from the harmful side effects of certain drugs.	Coyle & Tsai, 2004
Proline	Pro		It helps heal cartilage and cushion joints.	Siebert, 2010

Tyrosine	Tyr		Used in protein supplements to treat an inherited disorder called phenylketonuria.	Rasmussen et al., 1983
Cystine	Cys		Helps in metabolism and an important precursor of glutathione, good antioxidant that prevents the cells from toxins.	Waite & Tanzer 1981
Glutamine	Gln		It is a preferred fuel source rather than glucose and also reduces severe complications of sickle cell anaemia disease.	Newsholme et al., 2003
		Non-essential amino acids		
Asparagine	Asn		It serves as an amino donor in liver transamination processes and also maintains equilibrium of the central nervous system and metabolism of toxic ammonia in the body. It also participates in metabolic control of the brain and nervous system having some therapeutic uses in these areas.	Ruzzo et al., 2013

Glutamic acid	Glu		Major inhibitory brain neurotransmitter, flavor enhancer (accelerates plant growth).	Newsholme et al., 2003
Alanine	Ala		Plays a key role in maintaining glucose levels and thus energy supplies in the body.	Antoniou et al., 1989
Serine	Ser		Used to build phospholipids, precursor of ceramide, and as neurotransmitter.	Mothet et al., 2000
Aspartic acid	Asp		Aspartic acid plays an important role in the citric acid cycle, during which other amino acids and biochemical and also it is used to treat fatigue and depression.	Topo et al., 2009

Marine molluscs contain a substantial amount of proteins which are potential for human and animal nutrition and also help to lighten the increasing shortage of world's protein supply. Many of the mollusc proteins have high nutrient quality due to the presence of eight essential amino acids needed for the maintenance of good health (Fagbuaro et al., 2006). Several studies have been carried out on the protein quality and amino acid profiles of marine molluscs from different parts of the world. Earlier studies include those of Ouchi (1959), Anderson & Bedford (1973), Bamford & Mc Crea (1975), Stewart & Bamford (1975), and Bamford & Campbell (1976). Some of the recent studies include those of Saito (2004), Chan et al. (2004), Gokoglu et al. (2006), Su et al. (2006), Woodcock & Benkendorff (2008), Vasconcelos et al. (2009), Freiji & Awadh (2010), Saito & Hashimoto (2010), Periyasamy et al. (2011), Saito & Aono (2014), Ab Lah et al. (2016), Shellhammer et al. (2017) and Bonnefille et al. (2018).

The amino acid concentration of organisms is interrelated with the size of that organism (Koehn, 2016). Babarro et al. (2006) studied the variability of amino acids with a body size of the organisms. Allen & Garret (1972) experimentally proved that taurine varies with the size of *M. arenaria* (bivalve). Arularasan et al. (2010) determined the essential and non-essential amino acids in the gastropod, *S. canarium* from Tamil Nadu coast. Badiu et al. (2010) studied the amino acid profile of marine bivalves like *M. galloprovincialis* and *R. venosa*. Padidela & Thummala studied seasonal variation of amino acids in bivalves such as *P. cylindrica* from Waddepally and Kaleshwaram Lake in the year 2015. Essential amino acids in *P. viridis*, collected from Tamil Nadu coast were higher

compared to non-essential amino acids (Saritha et al., 2015). Emoto et al. (2008) analyzed the sequence of amino acids from four gastropods and seven bivalves collected from Tokyo market. Fuentes et al. (2009) determined the taste active values of amino acids in *M. galloprovincialis* proteins collected from different areas of Spain. The action of glutamic acid and other naturally occurring amino acids in gastropod nervous system was studied by Gerschenfeld & Lasansky (1964). Periyasamy et al. (2011) made observations on the amino acid composition and nutritional importance of *B. spirata* from Cudallore. The role of amino acids on osmotic regulation in intertidal molluscs was studied by Hoyaux et al. (1976). Seasonal variability of free amino acids in two marine bivalves, *M. balthica* and *Mytilus* sp. was studied by Kube et al. (2007). Salman & Nasar (2013) investigated the amino acid contents of some marine molluscs from Euphrates River.

Some studies have been carried out on the protein quality and amino acid profiles of marine molluscs from Tamilnadu coast. Ajayabhaskar (2002) observed the amino acid content and nutritional importance of three bivalve molluscs *P. viridis*, *C. madrasensis*, and *M. casta* collected from Cudallore. Seasonal variation of amino acid content in herbivorous gastropods, abalone (*Haliotidae*), was carried out by Chiou et al. (2001). Mason & co-authors (2014) conducted a detailed study on the amino acid concentration of one gastropod *C. sulcata*. Margret et al. (2013) reported different types of amino acids from muricidae *C. ramosus* and its variation with salinity. Nutritional importance and amino acids from *H. trunculus*, a gastropod was analyzed by Zarai et al. (2011). Comparative study of amino acids of some edible marine molluscs and two gastropods (*H. trunculus* and *R. venosa*) were reported by Celik et al. (2014) and

Zarai et al. (2011) respectively. Seasonal variations of the amino acid content of marine gastropods such as *T. dolium* and *P. glaucum* from the Gulf of Mannar were observed by Babu et al. (2012). Belisle & Stickle (1978) determined the amino acid composition of *T. haemastoma*, a gastropod. Babu & co-authors (2010) studied amino acid composition in mesogastropod, *B. spinosa* from Tamil Nadu coast.

Although there are some studies on the amino acid composition of the world's marine molluscs, information on the amino acid profiles of molluscs from the southwest coast of India, especially Kerala, is rather scanty. In India, amino acid studies were concentrated only in molluscs collected from Tamil Nadu coast. Though a few studies were reported about the nutritional quality of amino acids, but these studies were focused only on bivalve variety. Few studies were conducted for the amino acid concentration of gastropod variety. No detailed studies have been carried out so far on the amino acid content of marine molluscs from Kerala coast. The present study provides information on the total amino acid pool and nutritional quality of selected species of marine molluscs (both gastropods and bivalves) from six locations on the southern and Northern coasts of Kerala.

4.2 Materials and methods

4.2.1 Species collection

Marine gastropods, *T. curta, M. trapa, T. telescopium* and *B. spinosa*, and bivalves *P. viridis* and *V. cyprinoids* were selected for amino acid analysis (details of taxonomical description of the organisms and sampling sites were described in Chapter 2).

4.2.2 Amino acid analyses

Amino acids were extracted by using the method Sánchez-Machado et al. (2004) and analyzed using HPLC equipped with UV detection at 254 nm. Detailed procedures were given in Chapter 2.

4.2.3 Statistical analysis

The correlation studies were done on the amino acid contents derived from the gastropods and bivalves using the SPSS (22.0) software for windows by utilizing the bivariate Pearson correlation. Detailed procedures were explained in Chapter 2.

4.3 Results and Discussion

4.3.1 Amino acid composition

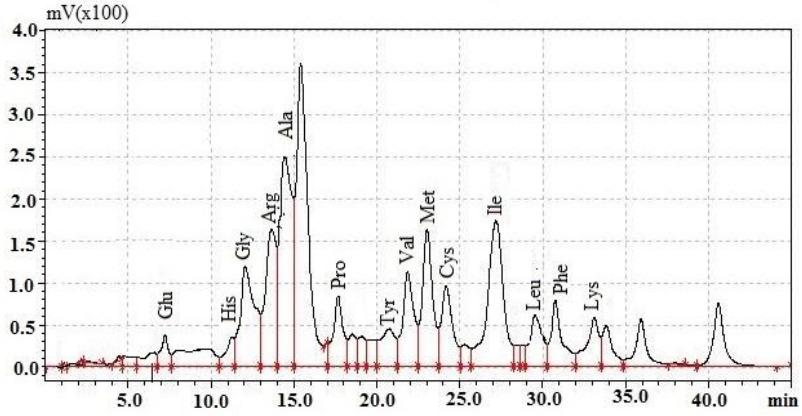
The total amino acid pool symbolizes both the amino acids occurring in free-state and those bounded in peptides. So the total amino acids from different species of molluscs samples collected from six different locations in the south-west coast of India were determined by the acid hydrolysis of proteins. Out of twenty amino acids, fifteen were identified from the soft tissue of molluscs. The chromatograms of amino acid profiles of six species, selected for the current analysis, are presented in Figure 4.1. Detected amino acids (AA) include glutamic acid (Glu), serine (Ser), glycine (Gly), arginine (Arg), alanine (Ala), proline (Pro), tyrosine (Tyr), cysteine (Cys), histidine (His), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), phenylalanine (Phen) and lysine (Lys). Concentrations of all the identified amino acids in mg g^{-1} are presented in Table 4.2. Total amino acid content in the protein of *M. trapa* was 14.99 mg g^{-1} (Figure 4.2), out of which, 8.57 mg g−1 were essential, and 6.41 mg g^{-1} were non-

essential (Figure 4.3). Comparable amino acid contents were observed in the soft tissue of *P. viridis* (15.69 mg g^{-1}) and *V. cyprinoids* (15.43 mg g^{-1}) (Figure 4.2), but the essential and non-essential compositions varied between species. Essential amino acids present in *P. viridis* and *V. cyprinoids* were 10.51 mg g^{-1} and 8.30 mg g^{-1} (Figure 4.3) respectively and the corresponding non-essential amino acids observed in those species were 5.18 mg g^{-1} and 7.13 mg g^{-1}. Amino acid content was highest in *T. curta* (24.73 mg g^{-1}) followed by *T. telescopium* (17.13 mg g^{-1}). Essential amino acid contents of *T. curta* and *T. telescopium* were 12.55 mg g^{-1} and 10.75 mg g^{-1} respectively and the corresponding non-essential amino acids of those species were 12.18 mg g^{-1} and 6.38 mg g^{-1}. Total amino acid content in *B. spinosa* was 16.40 mg g^{-1}, of which 11.10 mg g^{-1} were essential and 5.29 mg g^{-1}were non-essential.

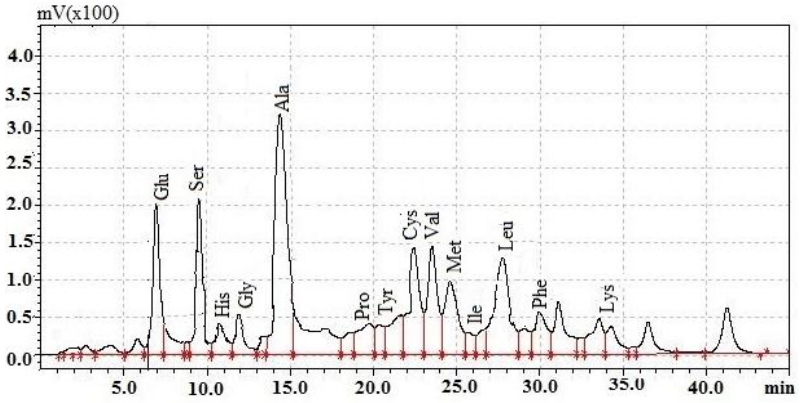
Table 4.2: Concentrations of individual amino acids in six species (in mg g^{-1})

Amino acids	MT (mg g^{-1})	TC (mg g^{-1})	BS (mg g^{-1})	TT (mg g^{-1})	PV (mg g^{-1})	VC (mg g^{-1})
Glutamic acid	4.15 ± 1.52	0.50 ± 0.15	1.06 ± 0.24	0.97 ± 0.23	1.57 ± 0.11	1.41 ± 0.94
Serine	0.28 ± 0.42	0.68 ± 0.55	0.00	2.06 ± 0.57	1.58 ± 0.84	1.37 ± 0.18
Glycine	0.26 ± 0.21	1.72 ± 0.83	0.88 ± 0.48	0.50 ± 0.36	1.71 ± 0.15	1.07 ± 0.37
Arginine	0.00	2.85 ± 0.71	2.73 ± 1.15	0.62 ± 0.15	0.00	0.22 ± 0.15
Alanine	0.17 ± 0.12	0.00	0.13 ± 0.21	0.16 ± 0.12	0.72 ± 0.46	1.09 ± 0.93
Proline	1.08 ± 0.52	5.98 ± 0.95	0.12 ± 0.15	1.68 ± 0.05	0.17 ± 0.03	0.58 ± 0.19
Tyrosine	0.46 ± 0.38	0.44 ± 0.38	0.35 ± 0.16	0.44 ± 0.18	0.17 ± 0.15	0.58 ±0.29
Cystine	0.00	0.00	0.02 ± 0.03	0.00	0.39 ± 0.36	0.25 ± 0.05
Histidine	0.09 ± 0.27	0.70 ± 0.48	0.12 ± 0.11	2.53 ± 0.14	0.11 ± 0.12	0.93 ± 0.06
Valine	0.00	0.19 ± 0.15	0.22 ± 0.03	0.00	0.15 ± 0.21	0.00
Methionine	2.85 ± 1.38	3.67 ± 1.52	4.17 ± 1.45	2.01 ± 0.19	3.94 ± 1.15	1.82 ± 0.95
Isoleusine	3.19 ± 0.92	0.02 ± 0.09	0.15 ± 0.12	0.05 ± 0.25	0.08 ± 0.01	0.00
Leusine	0.00	5.90 ± 0.35	2.98 ± 1.11	2.86 ± 0.67	4.17 ± 1.18	1.10 ± 1.05
Phenyl alanine	0.05 ± 0.12	0.13 ± 0.37	0.04 ± 0.12	0.22 ± 0.11	0.06 ± 0.12	0.12 ± 0.05
Lysine	2.41 ± 0.15	1.94 ± 0.82	3.42 ± 1.35	3.08 ± 0.11	2.01 ± 1.15	4.34 ± 1.83

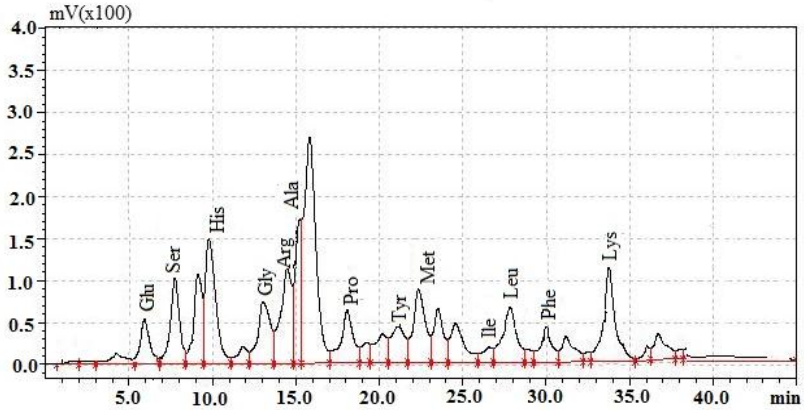
MT- *M. trapa*, TC- *T. curta,* BS- *B. spinosa,* TT- *T. telescopium*, PV- *P. viridis*, VC- *V. cyprinoids*



B. spinosa



P. viridis



T. telescopium

Figure 4.1 continued…

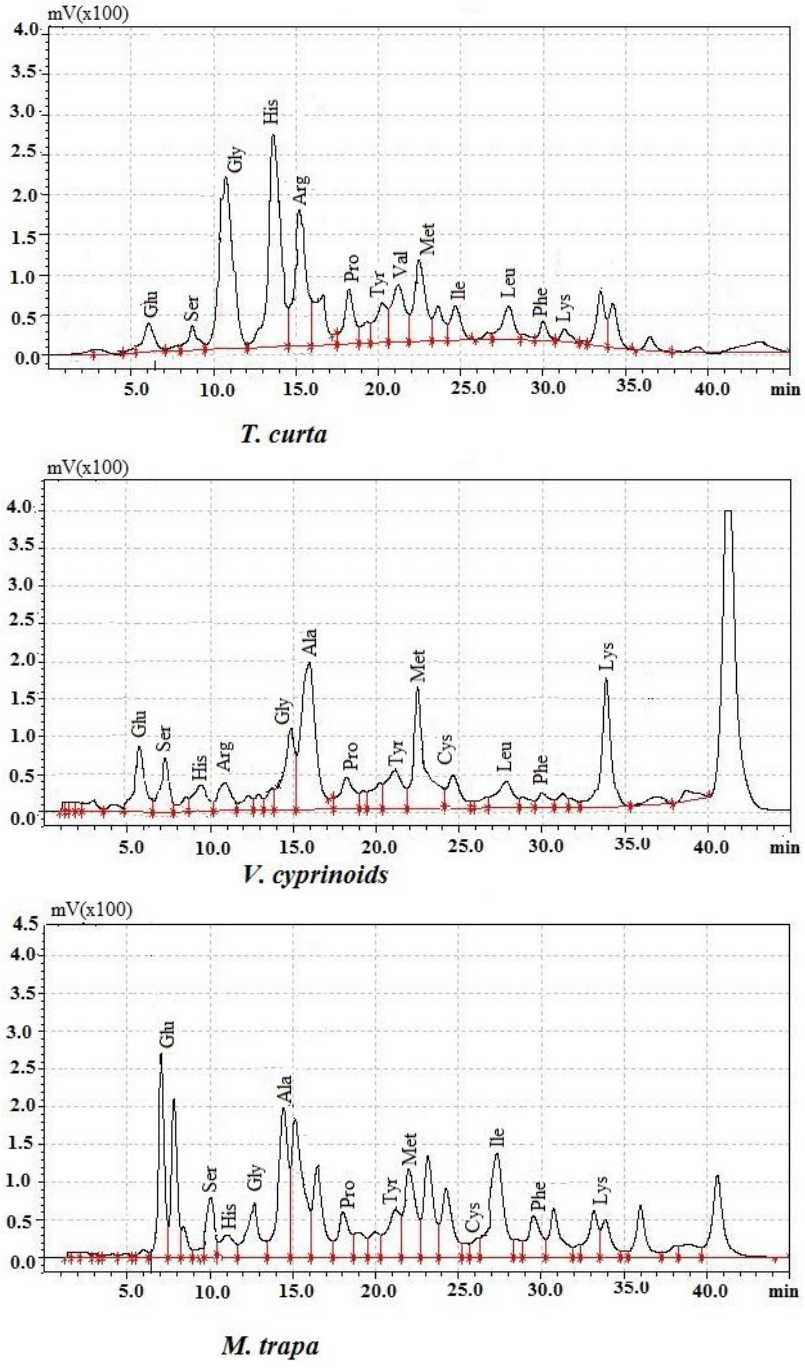
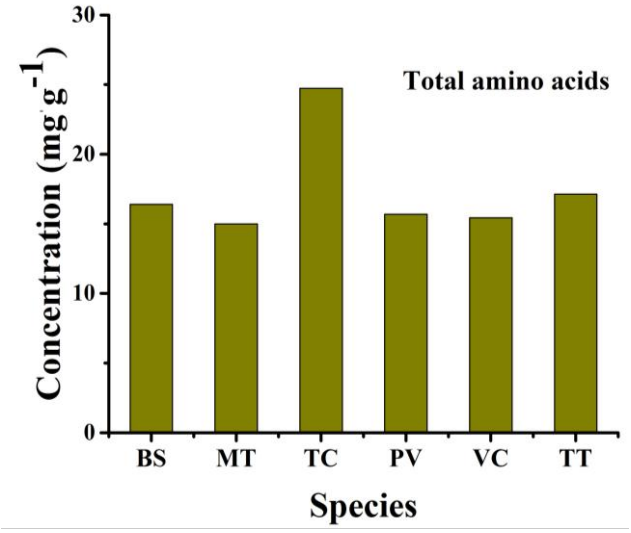
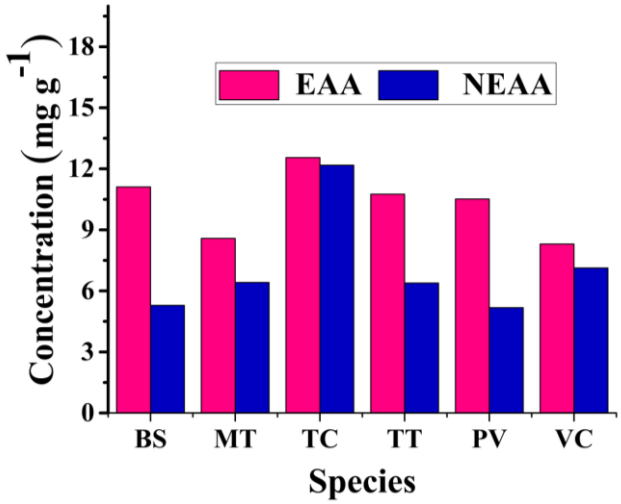


Figure 4.1: Total ion chromatogram of amino acid compositions of six molluscs



BS- *B. spinosa*, MT- *M. trapa*, TC- *T. curta*,
PV- *P. viridis*, VC- *V. cyprinoids*, TT- *T. telescopium*

Figure 4.2: Graphical representation of total amino acids (mg g^{-1})



BS- *B. spinosa*; MT- *M. trapa*; TC- *T. curta*; PV- *P. viridis*; VC- *V. cyprinoids*;
TT- *T. telescopium*; EAA- essential amino acids; NEAA- non-essential amino acids

Figure 4.3: Graphical representation of essential and non-essential amino acids (mg g^{-1})

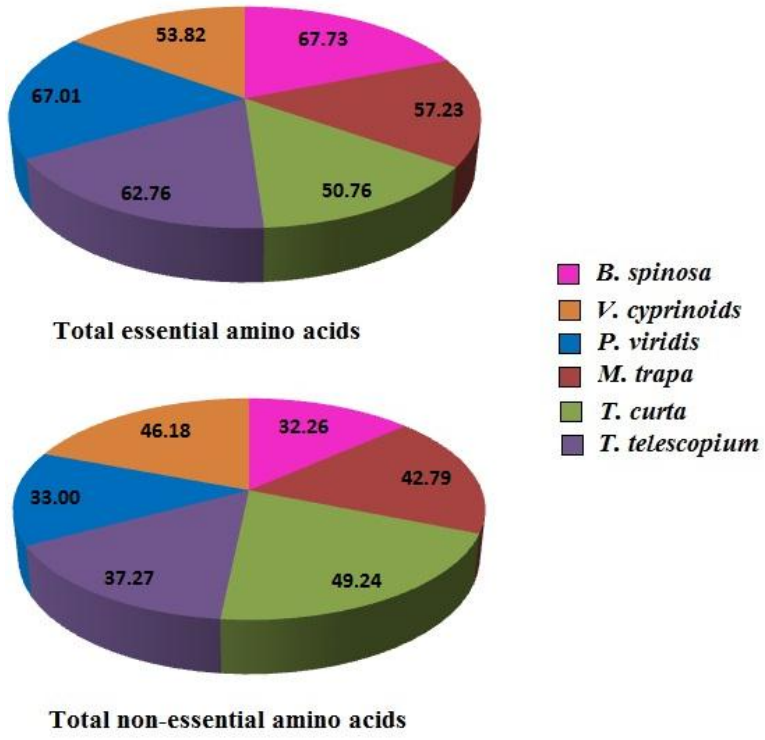


Figure 4.4: Percentage composition of essential and non-essential amino acids

Amino acids are the building blocks of proteins. So the hydrolysis of proteins yields total amino acids in the species. From the previous reports, it is clear that amino acids produced between 18 upto 20 indicate the effective hydrolysis (Takarina et al., 2016). The present study revealed that percentage composition of total EAA was found to be higher than the total NEAA in all the samples. The total essential amino acids recorded were 57.23 % and 50.76%, and non-essential amino acids recorded were 42.79% and 49.24% for the soft tissue of *M. trapa* and *T. curta* respectively (Figure 4.4). Total EAA contents in the soft tissues of two bivalve species, *P. viridis* and *V. cyprinoids* were 67.01% and 53.82% respectively. Similarly, total EAA contents in the soft tissue of

B. spinosa and *T. telescopium* were in the order of 67.73% and 62.76 %. Total NEAA present in the soft tissues of *P. viridis, V. cyprinoids, B. spinosa* and *T. telescopium* were in the order 33.0%, 46.18%, 32.26% and 37.27%. These results are in close resemblance with the observations in the soft tissue of dog conch *S. canarium* (gastropoda) from Gulf of Mannar (Arularasan, 2009) and amino acids from the soft tissue of sea snail *N. crepidularia* collected from mangroves of Vellar estuary (Palpandi, 2010). The percentage of essential amino acids was more (80.97%) than those of non-essential amino acids (15.07%) from the gastropod *S. canarium*. Total percentage of amino acids from the common marine sandy beach clam, *D. incarnates* collected from the Cuddalore, southeast coast of India, constituted 93.61%, of which 58.21% were essential, and 35.4% were non-essential (Periyasami et al., 2014). Total amino acid content in the protein of *B. spinosa* collected from Mudasalodai, near Cuddalor coast was 96.8%. Among them, the essential amino acids (EAA) 50.1%, non-essential amino acids (NEAA) 46.79% and unidentified amino acids 3.2% were found (Babu et al., 2010). As previously reported by many researchers, the present study also found that the total content of EAA dominated the NEAA. The biological value of protein is apparently reflected upon its essential amino acid concentration (Delgado et al., 2017).

Highest methionine content was observed in *B. spinosa* (4.17 ± 1.45 mg g^{-1}) followed by *P. viridis* (3.94 ± 1.15 mg g^{-1}) (Table 4.2). 25.42% of total amino acid in *B. spinosa* and 25.13% of total amino acids in *P. viridis* were methionine (Figure 4.5). Methionine content of *M. trapa*, *T. curta* and *T. telescopium* followed the order; 3.67 ± 1.52 mg g^{-1}, 2.85 ±

1.38 mg g^{-1} and 2.01 ± 0.19 mg g^{-1} respectively (Table 4.2). Least content of methionine was observed in *V. cyprinoids* (1.82 ± 0.95 mg g^{-1}). It was found that; methionine was the most dominant EAA in most of the species studies. This was in accordance with earlier reports. Mercer et al. (1993) obtained methionine as the dominant compound in the protein hydrolysates of two species of abalone, *H. tuberculata* & *H. discushannai*. Idayachandiran et al. (2014) showed that methionine was one of the predominant amino acids in marine bivalve, *D. cuneatus*. Leusine content was observed highest in *T. curta* (5.90 ± 0.35 mg g^{-1}), followed by *P. viridis* (4.17 ± 1.18 mg g^{-1}). The comparable leucine content was seen between *B. spinosa* (2.98 ± 1.11 mg g^{-1}) and *T. telescopium* (2.86 ± 0.67 mg g^{-1}) (Table 4.2). Leusine was absent in *M. trapa*. The least leucine content was observed in *V. cyprinoids* (1.10 ± 1.05 mg g^{-1}). The results of the present study revealed that methionine and leucine form the major EAAs. These findings were in good agreement with the former studies on *B. spinosa* collected from Cuddalore coast (Babu et al., 2010). Sulfur-containing amino acids such as methionine and cysteine were comparatively abundant in *C. cochleata* and *P. alba* (Pujol et al., 1970). But in the present study, cystine content was very low. The highest amount of cysteine was present in *P. viridis* (0.39 ± 0.36 mg g^{-1}) and lowest was in *B. spinosa* (0.02 ± 0.03 mg g^{-1}). Cystine was absent in *T. curta, M. trapa* and *T. telescopium*. From the previous studies, it was clear that cysteine has the lowest concentration in both small *A. indica* at Cibungur and large *A.indica* at Garapan rivermouth (Noverita et al., 2016). Its concentration in *V. cyprinoids* was 0.25 ± 0.05mg g^{-1} (Table 4.2).

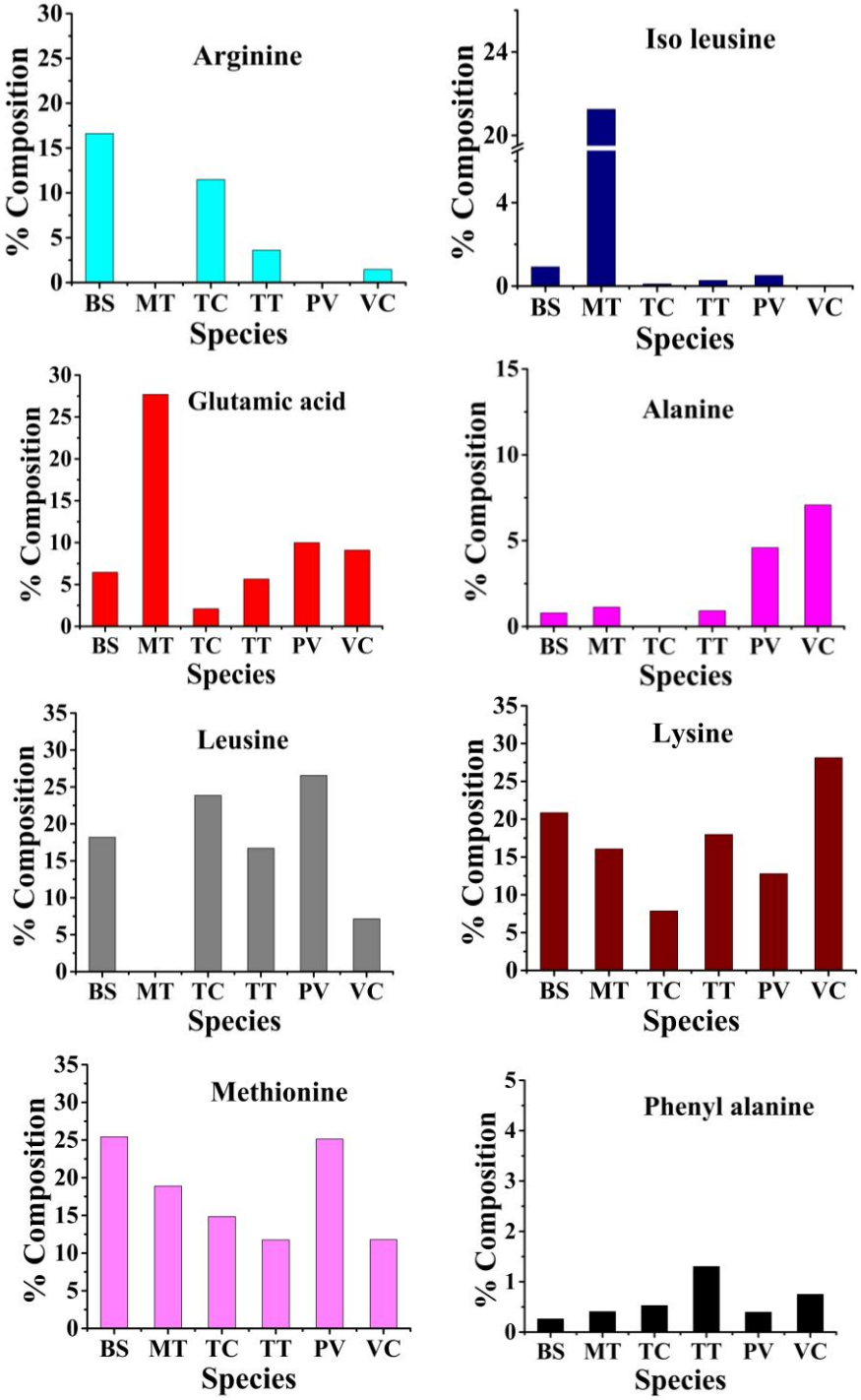


Figure 4.5 continued…

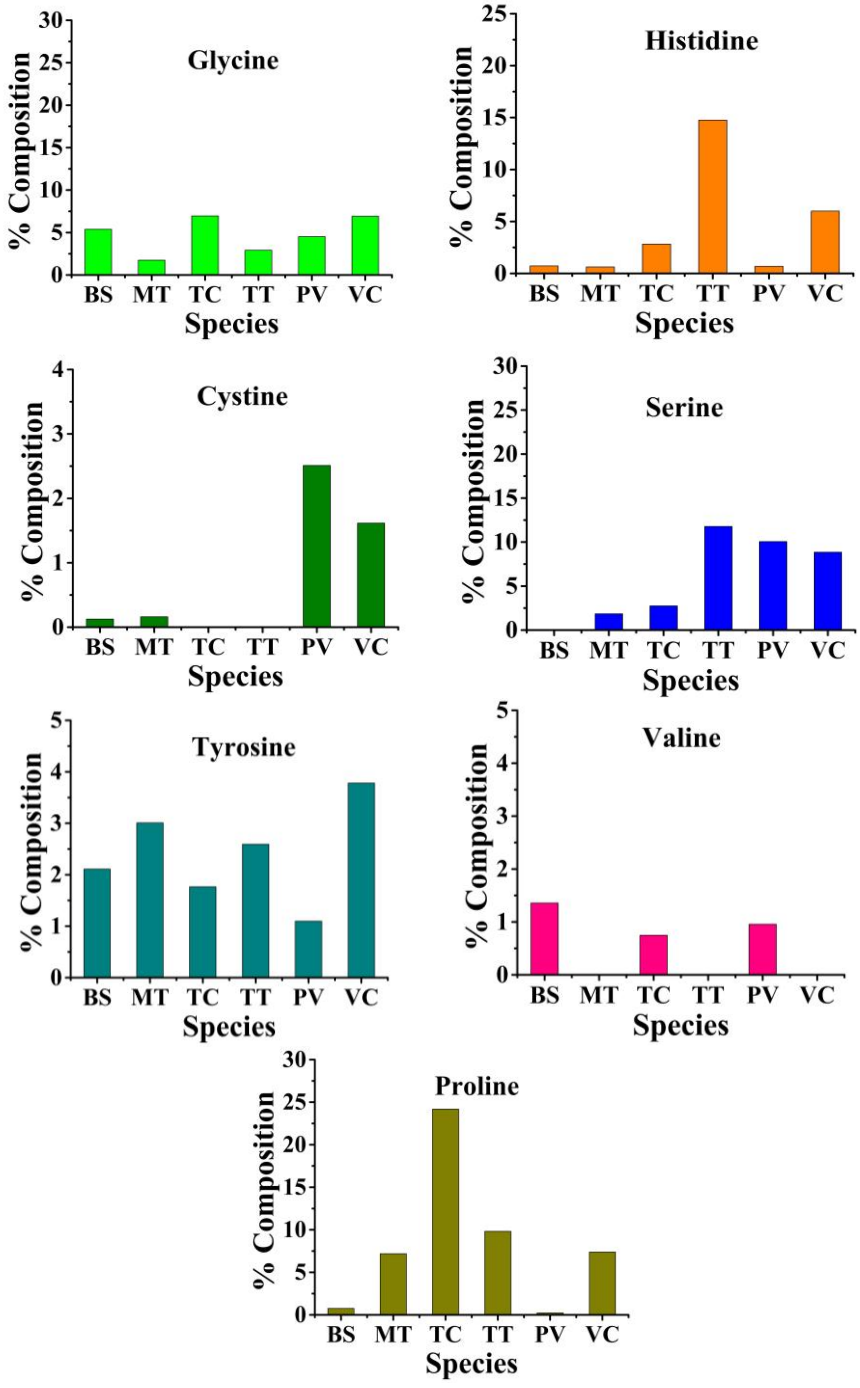


Figure 4.5: Percentage composition of individual amino acids

Lysine content was seen highest in *V. cyprinoids* (4.34 ± 1.83 mg g^{-1}) (Table 4.2) followed by *B. spinosa* (3.42 ± 1.35 mg g^{-1}). Lysine content of *M. trapa* was 2.41 ± 0.15 mg g^{-1} and those of *T. telescopium* was 3.08 ± 0.11 mg g^{-1}. Comparable lysine concentrations were seen *T. curta* (1.94 ± 0.82 mg g^{-1}) and *P. viridis* (2.01 ± 1.15 mg g^{-1}). Padidela & Thummala (2015) also obtained the similar result for bivalve tissue, *P. cylindrica*. Amino acid analysis of clam, *M. casta* from Cuddalore and Parangipettai coast showed that lysine predominated over other amino acids (Srilatha et al., 2013). Lysine is an essential amino acid, and its deficiency causing the disease pellagra. Normally, human diets required 1−1.5 g lysine per day, and its deficiency leads to the deficiency of niacin and vitamin B (Ozden & Erkan, 2011; Voronezhskaya & Croll, 2016). Reported data showed that leucine and lysine were highly found in sea molluscs (Villanuela et al., 2004). Relatively high value of lysine was measured in *J. Dory* (fish) (Ozden & Erkan, 2011). Isoleucine content was observed highest in *M. trapa* (3.19 ± 0.92 mg g^{-1}). Its content in *B. spinosa* was 0.15 ± 0.12 mg g^{-1}. *T. curta* (0.02 ± 0.09 mg g^{-1}), *T. telescopium* (0.05 ± 0.25 mg g^{-1}) and *P. viridis* (0.08 ± 0.01 mg g^{-1}) exhibited comparable isoleucine contents. Isoleucine was absent in *V. cyprinoids*. Histidine concentrations were seen highest in *T. telescopium* (2.53 ± 0.14 mg g^{-1}), which was 14.75% of total amino acid. Comparable histidine concentration was observed *V. cyprinoids* (0.93 ± 0.06 mg g^{-1}) and *T. curta* (0.70 ± 0.48 mg g^{-1}). Also, *M. trapa* (0.09 ± 0.27 mg g^{-1}), *P. viridis* (0.11 ± 0.12 mg g^{-1}) and *B. spinosa* (0.12 ± 0.11 mg g^{-1}) displayed matching concentrations. Histidine is one of the major amino acids present in the soft tissue of *P. cylindrical* (Swapna et al., 2015). Relatively

high value of histidine was found in the soft tissue of *L. peali* (cephalopod), while only trace amounts were recorded in *M. solidissima* (pelycypod) and *B. canaliculatum* (gastropod) (Allen 1961). *T. telescopium* (0.22 ± 0.11 mg g^{-1}) exhibited maximum phenylalanine content, which was 1.30% of total amino acid (Figure 4.5). Comparable phenylalanine contents were seen in *T. curta* (0.13 ± 0.37 mg g^{-1}) and *V. cyprinoids* (0.12 ± 0.05 mg g^{-1}). *M. trapa* (0.05 ± 0.12 mg g^{-1}), *P. viridis* (0.06 ± 0.12 mg g^{-1}) and *B. spinosa* (0.04 ± 0.12 mg g^{-1}) were exhibited comparable phenylalanine content.

Similar arginine content was seen in *T. curta* (2.85 ± 0.71 mg g^{-1}) and *B. spinosa* (2.73 ± 1.15 mg g^{-1}). Least arginine content was observed in *V. cyprinoids* (0.22 ± 0.15 mg g^{-1}). Arginine was absent in both *M. trapa* and *P. viridis*. Arginine concentration of *T. telescopium* was 0.62 ± 0.15 mg g^{-1}. In the soft tissue of *T. curta* and *B. spinosa*, arginine was present in large quantity (16.62% and 11.48% respectively) (Figure 4.5), while in others they present as minor constituents. Similar results were observed in the species of vertebrates and invertebrates (Allen, 1961; Sokolova & Portner, 2003; Solorzano et al., 2009). Arginine was the major amino acid present in both small *A. indica* at Cibungur and large *A. indica* at Garapan rivermouth (Noverita et al., 2016). Villanueva et al. (2004) showed that arginine was needed for metabolism process in cephalopod. Previous studies indicate that this amino acid was highly found in mollusc muscle (Pereira et al., 2000). Comparable arginine content was observed in *L. elliptica* collected from Antarctica and *P. viridis* (present study) (Rodrigues et al., 2009). Large quantity of arginine was found to be present in the soft tissues of both *M. edulis*

(bivalve) and *E. moschata* (cephalopod) (Papov et al., 1995; Kim et al., 2012). Arginine was richly found in the flesh of red scorpion fish, spiny lobster (crustacean) and sea snail (mollusc) (Ozden & Erkan, 2011). Valine concentration was seen highest in *B. spinosa* (0.22 ± 0.03 mg g^{-1}), which is 1.36% of the total amino acid (Figure 4.5). Comparable valine content was observed in *T. curta* (0.19 ± 0.15 mg g^{-1}) and *P. viridis* (0.15 ± 0.21 mg g^{-1}). Valine was absent in *M. trapa*, *T. telescopium* and *V. cyprinoids*.

Glutamic acid level was seen highest in *M. trapa* (4.15 ± 1.52 mg g^{-1}) and least in *T. curta* (0.50 ± 0.15 mg g^{-1}). The comparable glutamic acid content was observed in *P. viridis* (1.57 ± 0.11 mg g^{-1}) and *V. cyprinoids* (1.41 ± 0.94 mg g^{-1}). *B. spinosa* (1.06 ± 0.24 mg g^{-1}) and *T. telescopium* (0.97 ± 0.23 mg g^{-1}) exhibited almost similar glutamic acid concentrations. Comparatively large quantity of glutamic acids was present in the soft tissue of euryhaline gastropods, for example, *E. chlorotica*, *A. crenata*, and *N. obsoletus* (Shumway et al., 1977; Amende & Pierce, 1980; Pierce, 1982; Kinne & Zeidel, 2008). In the present study, amino acids such as leucine, glutamic acid and lysine were also found to be the major constituents in all the species. These findings were in accordance with the earlier reports of Litaay et al. (2001). According to Pujol et al. (1970), high level of glutamic acid was found in the soft tissue of bivalve species *V. pullastra*. In the same year, Pujol & co-authors extracted a large quantity of glutamic acid from the soft tissue of *A. ephippium*. These findings were similar to the results of amino acid content in *A. indica* collected from Cibungur and Garapan (Takarina et al.,

2016). According to Derby et al. (2007), glutamic acid is an important source of nitrogen in sea molluscs.

Highest glycine content was seen in *T. curta* (1.72 ± 0.83 mg g^{-1}) followed by *P. viridis* (1.71 ± 0.15 mg g^{-1}). Glycine content of *V. cyprinoids* was 1.07 ± 0.37 mg g^{-1}, which is 6.91% of total amino acid. *B. spinosa* (0.88 ± 0.48 mg g^{-1}) and *T. telescopium* (0.50 ± 0.36 mg g^{-1}), exhibited comparable glycine content. Least glycine content was seen in the soft tissue *of M. trapa* (0.26 ± 0.21 mg g^{-1}). Highest alanine concentration was detected in the soft tissue of *V. cyprinoids* (1.09 ± 0.93 mg g^{-1}) followed by *P. viridis* (0.72 ± 0.46 mg g^{-1}). Soft tissues of *M. trapa* (0.17 ± 0.12 mg g^{-1}), *B. spinosa* (0.13 ± 0.21 mg g^{-1}) and *T. telescopium* (0.16 ± 0.12 mg g^{-1}) exhibited similar alanine concentration. Alanine content was absent in the soft tissue of *T. curta*. Euryhaline gastropods such as *P. ebenius, P. lapillus, B. perversum* and *O. sayana* contain large quantity of alanine, while the concentration of glycine was relatively high in *H. ulvae, F. distans* and *A. crenata,* coming under the same family (Shumway et al., 1977; Ivanovici et al., 1981; Kinne & Zeidel, 2008; Gharbi et al., 2016; Parker et al., 2017). Higher quantity of alanine content was present in the soft tissues of *M. balthica* and *Mytilus* sp. (Kube et al., 2006). Studies on various abalone tissues indicated that they are a rich source of alanine, phosphor arginine and taurine (Webber, 1970; Gade & Grieshaber, 1986; Tjeerdema et al., 1991; Gao et al., 2017). Shumway & co-authors (1977) experimentally proved that glycine and alanine concentrations increase at the time of salinity variation in some bivalves such as *P. plana*, *C. edule, M. mercenaria, M. arenaria, C. gigas,* and *C. opercularis*. Amino acid

concentration is higher in euryhaline species than stenohaline species (Kinne & Zeidel, 2008; Muraeva et al., 2016).

Highest serine content was observed in the soft tissue of *T. telescopium* (2.02 ± 0.57 mg g^{-1}) and least was seen in *M. trapa* (0.28 ± 0.42 mg g^{-1}). Serine content was not detected in the soft tissue of *B. spinosa*. Comparable serine contents were observed in between *P. viridis* (1.58 ± 0.84 mg g^{-1}) and *V. cyprinoids* (1.37 ± 0.18 mg g^{-1}). 0.68 ± 0.55 mg g^{-1} serine content was present in the soft tissue of *T. curta*. Highest proline content was observed in the soft tissue of *T. curta* 5.98 ± 0.95 mg g^{-1}, which is 24.18% of total amino acid. It was followed by *T. telescopium* (1.70 ± 0.05 mg g^{-1}) and least was observed in the soft tissue of *B. spinosa* 0.12 ± 0.15 mg g^{-1}. Proline contents of *P. viridis*, *M. trapa* and *V .cyprinoids* were 0.17 ± 0.03 mg g^{-1}, 1.08 ± 0.52 mg g^{-1} and 0.58 ± 0.19 mg g^{-1} respectively. Highest tyrosine content was detected in the soft tissue of *V. cyprinoids* (0.58 ± 0.29 mg g^{-1}), and least was observed in the soft tissue of *P. viridis* (0.17 ± 0.15 mg g^{-1}). Soft tissues of *M. trapa* (0.46 ± 0.38 mg g^{-1}), *T. curta* (0.44 ± 0.38 mg g^{-1}), *B. spinosa* (0.35 ± 0.16 mg g^{-1}) and *T. telescopium* (0.44 ± 0.18 mg g^{-1}) exhibited comparable tyrosine contents. In the current study, comparatively rich amounts of glutamic acid, glycine, alanine, proline and serine were present in all the six species. This is because all the species for the present analyses were estuarine molluscs. Marine molluscs such as cephalopods, solenogasters, monoplacophorans, polyplacophorans and apalcophorans were stenohaline species, while bivalves and gastropods were euryhaline species (Kinne & Zeidel, 2008; Muraeva et al., 2016). In Euryhaline animals, any change in the external osmotic concentration

will result in a change in osmotic concentration of their extra-cellular fluids (Di Martino et al., 2003; Long et al., 2017). The cytoplasm of animals is in osmotic equilibrium with extracellular fluids that has the same osmotic pressure of the seawater (Hoyaux et al., 1976; Lopez-Fontanals et al., 2003; Yuan et al., 2017). Any change in the osmotic pressure of external seawater will affect the concentration of amino acids and quaternary amine compounds (Kinne & Zeidel, 2008). Usually, changes occurred for only a few of the amino acids such as glutamic acid, taurine, glycine, alanine, proline and serine of which the concentration of glycine, glutamic acid and proline were increased (Ginguay et al., 2016; Shellhammer et al., 2017).

In the current investigation, some of the mollusc species were observed to contain alanine, serine, and histidine in large amounts, while in others they present as minor constituents. Reported data on the amino acids content in *D. cuneatus* indicated that serine and alanine were found in higher and lower levels respectively (Idayachandiran et al., 2014). Britz & Hecht (1997) extracted significant amount of alanine from the tissue of South African abalone *H. midae*. A trace amount of alanine, histidine, and glycine were present in the bivalve, *C. cochleata* (Pujol et al., 1970). Proline and glycine were found to present in fairly good quantity. These findings were similar to the observations of Kube et al. (2007). Previous studies on molluscs discovered that glycine was present in large quantity (Derby, 2007). According to Pila et al. (2016), several species of invertebrates contain a large amount of glycine. Yu et al. (2017) extracted pure glycine from the muscle of *A. irradians*. Proline was the most dominant non-essential AA in the soft tissue of blacklip

abalone (*H. rubra*) (Litaay et al., 2001). Previous studies indicated that glycine and alanine were richly present in the soft tissue of *V. pullastra* (Pujol et al., 1970). Earlier reports demonstrated that average quantity of glycine was present in *P. nobilis* (Pujol et al., 1970).

Amino acids are the building block of proteins and which significantly influence the taste of foods. Owing to their biological significance they are important in nutrition and are commonly used in food technology, nutritional supplements and fertilizers. Although some amino acids were present in small amounts, their taste impacts were high because of their low threshold values (Chen & Zhang, 2007; Gunlu & Gunlu, 2014). Each amino acid usually contributes a sour, bitter or sweet taste, which significantly influences the taste of foods (Shallenberger, 1993; Chen & Zhang, 2007; Gunlu & Gunlu, 2014). Glycine, serine, arginine and alanine have a pleasant sweet taste while lysine and methionine have both sour and sweet taste. Taste values of each amino acid were summarized in Table 4.3. Glycine and alanine are usually present in large quantities in sea foods (Fuke & Konosu, 1991; Wu & Shiau, 2002; Chen & Zhang, 2007). Histidine, leucine, aspartic acid and glutamic acid have a sour taste. Though aspartic acid and glutamic acid have a sour taste, in the presence of sodium salts (their sodium salts were monosodium glutamate like components) which gave the umami (together with sweetness, sourness, bitterness, and saltiness) taste (Yamaguchi et al., 1971; Gunlu & Gunlu, 2014). There is also a symbiotic interaction of umami taste between sweet amino acids, and inosine monophosphate (IMP), is a nucleoside monophosphate and sweetness would increase in the presence of IMP (Yamaguchi & Ninomiya, 2000; Chen & Zhang,

2007). From Table 4.4, it is clear that taste active values (TAV) of glutamic acid and methionine were high in *M. trapa*. Intermediate TAV of leucine and lysine was also observed in *M. trapa*. TAV of methionine shows high value in the soft tissue of *B. spinosa, T. curta* and *V. cyprinoids*. Comparatively high amounts of lysine and arginine were observed in *T. curta* and *B. spinosa*. The bitter taste of histidine in *T. telescopium* was balanced by the high content of lysine and methionine (sweet taste). Sweet amino acids such as methionine and lysine were predominating in the soft tissue of *P. viridis*.

Table 4.3: Taste threshold values of amino acids

Amino acids	Taste threshold value
Glutamic acid	0.3
Serine	1.5
Glycine	1.3
Arginine	0.5
Alanine	0.6
Proline	3
Tyrosine	–
Cystine	–
Histidine	0.2
Valine	0.4
Methionine	0.33
Isoleucine	0.9
Leucine	1.9
Phenylalanine	0.9
Lysine	0.5

(Shallenberger, 1993)

Table 4.4: Taste active value of identified amino acids

Amino acids (mg g^{-1})	MT	TC	BS	TT	PV	VC
Glutamic acid	13.86	1.68	3.54	3.36	5.27	4.74
Serine	0.19	0.46	0.00	1.37	1.09	0.91
Glycine	0.20	1.33	0.68	0.39	0.55	0.82
Arginine	0.00	5.71	5.46	1.25	0.00	0.46
Alanine	0.29	0.00	0.23	0.27	1.17	1.83
Proline	0.36	0.29	0.04	0.57	0.01	0.38
Tyrosine	0.00	0.00	0.00	0.00	0.00	0.00
Cystine	0.00	0.00	0.00	0.00	0.00	0.00
Histidine	0.50	3.41	0.54	12.68	0.57	4.68
Valine	0.00	0.49	0.57	0.00	0.39	0.00
Methionine	8.65	11.14	12.50	6.31	11.97	5.49
Isoleusine	3.55	0.03	0.16	0.06	0.09	0.00
Leusine	0.00	1.00	1.55	1.53	2.20	0.59
Phenyl alanine	0.07	0.14	0.04	0.08	0.06	0.15
Lysine	4.83	3.96	6.72	5.93	4.04	8.87

BS- *B. spinosa*; MT- *M. trapa*; TC- *T. curta*; PV- *P. viridis*; VC- *V. cyprinoids*; TT- *T. telescopium*;

Total sweet amino acids (lysine, methionine, and alanine) were dominated over sour amino acids such as histidine in the soft tissue of *V. cyprinoids*. Due to the presence of large quantity of EAA and high TAV in both *P. viridis* and *V. cyprinoids*, they are widely used as a common nutritional food stuff in Kerala. It is suggested that in muscle, glutamic acid and arginine were major amino acids and thus may contribute significantly prompting the taste (Heu et al., 2003). In molluscs, amino acid concentration increases with mature development. This is because accumulation of EAA during development could be

increased by absorption from the external medium (Manahan, 1983; Litaay et al., 2001). This was experimentally proved by Litaay & co-authors (2001) from blacklip abalones. Higher levels of alanine, glycine and arginine were present in the soft tissue of molluscs, which are used in energy metabolism by maintaining glycolysis through the formation of opines under hypoxic conditions (Gade & Grieshaber, 1986; Litaay et al., 2001). Barbarro et al. (2006) reported that the concentrations of aspartic acid and alanine showed a significant drop with increasing size of the individuals. In the current study, aspartic acid was absent due to the large size of all species. Alanine concentration was comparatively high in *V. cyprinoids* and *P. viridis*

4.3.2 Statistical analysis

All the amino acids obtained from both gastropods and bivalves were subjected to inter compositional correlation study (Table 4.5). The results have a significant correlation between amino acids (R^2 ranges from 0.782 to 0.973; $p < 0.001$). Non polar aliphatic amino acids show strong positive correlation. Leusine shows significant positive correlation with glycine ($R^2 = 0.847$) and proline ($R^2 = 0.943$). Moreover, isoleusine exhibits highly positive correlation with glutamic acid ($R^2 = 0.960$). Methionine shows positive correlation with histidine ($R^2 = 0.922$). Proline and glycine shows positive correlation ($R^2 = 0.782$), since both of them have important role in osmoregulation (Di Martino et al., 2003; Long et al., 2017). Moreover, cysteine and alanine displayed a positive correlation with $R^2 = 0.843$. The correlation study revealed that histidine and serine recorded a significant positive correlation. Moreover, arginine shows positive correlation with valine ($R^2 = 0.763$).

Table 4.5: Pearson's bivariate correlation analysis data of amino acids (n=27)

	Glu	Ser	Gly	Arg	Ala	Pro	Tyr	Cys	His	Val	Meth	Ile	Leu	Phe	Lys
Glu	1														
Ser	-0.33	1													
Gly	-0.69	-0.121	1												
Arg	-0.578	-0.533	0.686	1											
Ala	0	0.443	-0.028	-0.596	1										
Pro	-0.384	-0.108	**0.782***	0.577	-0.438	1									
Tyr	0.071	-0.026	0.161	-0.011	0.109	0.249	1								
Cys	-0.017	0.439	-0.046	-0.553	**.843***	-0.412	-0.416	1							
His	-0.395	**0.705***	-0.073	-0.111	-0.086	0.124	0.395	-0.299	1						
Val	-0.49	-0.455	0.541	**0.763***	-0.329	0.273	-0.593	-0.003	-0.496	1					
Meth	-0.148	-0.54	0.264	0.552	-0.375	0.12	-0.759	0.054	-0.681	**.922****	1				
Ile	**.960****	-0.46	-0.584	-0.373	-0.253	-0.176	0.137	-0.27	-0.356	-0.411	-0.089	1			
Leu	-0.537	-0.11	**.847***	0.675	-0.422	**.943****	-0.045	-0.257	-0.004	0.544	0.387	-0.354	1		
Phe	-0.419	0.7	0.076	-0.061	-0.127	0.328	0.413	-0.308	**.973****	-0.46	-0.648	-0.351	0.189	1	
Lys	-0.144	0.09	-0.064	-0.103	0.51	-0.425	0.608	0.038	0.271	-0.363	-0.567	-0.246	-0.527	0.128	1

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

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

4.4 Summary

A total of fifteen amino acids were detected from the acid hydrolysis of proteins. In the present study, all the constituent amino acids were not determined (e.g: glutamine, asparagine, aspartic acid, threonine, and tryptophan). It may be due to the partial hydrolysis of some proteins during the preparation of acid. Regarding the amino acid content, all the species of molluscs contain a large quantity of EAA than NEAA. So they have high nutritional quality. Among the 15 amino acids, lysine, methionine and leucine were the major EAA forms, and glutamic acid and proline were the major NEAA forms. Gastropods such as *B. spinosa*, *T. curta* and *T. telescopium* were observed to be rich in amino acids, especially essential amino acids.

Glutamic acid content was high in *M. trapa* and comparable concentration was observed in bivalves. Minute quantity of glutamic acid was observed in *T. curta*, since it is an important source of nitrogen in sea molluscs. Comparable serine was observed in bivalves and it was absent in *B. spinosa*. Significant proline, glycine and alanine were observed in both gastropods and bivalves. Glutamic acid, serine, proline, glycine and alanine were higher in marine gastropods and bivalves, because they are euryhaline species. In these species, changes in osmotic pressure of seawater affect the osmotic concentration of their extra-cellular fluids and leads to a hike in certain amino acids such as glutamic acid, serine, proline, glycine and alanine. From this study, it is clear that all the species contain significant amount of amino acids. *T. telescopium* is used as an edible food in South Malaya, Philippines, Australia and Madagascar. Since *P. viridis* and *V. cyprinoids* were already edible food

in India and other countries, the amino acids content observed in this study supports their enhanced utilization in food.

Glutamic acid is a well-known flavor enhancer, which is richly present in almost all the species. Glycine, serine, arginine, and alanine have a pleasant sweet taste. Lysine and methionine have both sour and sweet taste. All species contain a large quantity of glycine, serine, arginine, methionine, lysine and alanine. Results from the present analysis conclude that all the species selected for the current study are recommended as delicious food for humans based on the presence of high taste activity AAs values and a large quantity of EAA.

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Chapter 5

CHARACTERIZATION OF n-ALKANES

Contents

5.1 Introduction

Alkanes are the least polar natural compounds composed only of carbon and hydrogen. They form a significant class of diverse organic compounds. Alkanes are saturated hydrocarbons, and they may be n-alkanes, cycloalkanes, and iso-alkanes. Alkanes and alkenes were found mainly in petroleum compounds, but living organisms such as eukaryotes and prokaryotes normally derived hydrocarbons from fatty acids (Beaudoin et al., 2016; Bhattacharya et al., 2017; Huang, 2017; Khmelevtsova et al., 2017). They are usually straight chain molecules, but some methyl-branched alkanes found in insect species (Ginzel & Blomquist, 2016; Sakata et al., 2017). Odd chain alkanes from C_{15} to C_{33} were predominant in the lipid fraction of plants (Foo et al., 2017; Li et al., 2017; Machado et al., 2017). Most of the species in the marine world contain alkanes ranging from C_{15} to C_{20}, of which C_{17} predominant (Nesvacil et al., 2016). Most of the alkanes present in the molluscs are mainly derived

from its-own environment, and others are formed as products of fatty acid cleavage during peroxidation processes (Herman & Zhang, 2016; Nesvacil et al., 2016; Foo et al., 2017). Due to the filter feeding nature of these organisms, alkanes are accumulated to their body through the diet. Mangrove forests, as well as estuaries along coastal regions, are rich sources of alkanes (Yamamoto et al., 2003; Killops & Killops, 2005; Silva et al., 2012). Alkanes are derived in the environment from biogenic as well as anthropogenic sources (Head et al., 2006; Hu et al., 2009). Biogenic hydrocarbons are coincidental by-products of biochemical synthesis. They are derived from phytoplankton bacteria as well as plants (Blumer et al., 1971; Smith et al., 2013). Anthropogenic hydrocarbons were produced from petroleum reservoirs (Hornafius et al., 1999; Seewald, 2003; Kostka et al., 2011).

The major sources from which alkanes entered to the marine environment are discussed in Table 5.1. The central sources of natural hydrocarbons conveyed to aquatic environments include autochthonous sources such as algal and bacterial inputs as well as allochthonous inputs from terrigenous plants (Cranwell, 1982; Meyers & Ishiwatari, 1993; Meyers, 1997; Bianchi & Elizabeth, 2011; Apostolopoulou et al., 2016; Berg et al., 2016). Aquatic macrophytes are generally characterized by a dominance of mid-chain length n-alkanes with odd chain preference (Bianchi & Elizabeth, 2011). Alkanes were produced in the marine environment by the bacterial reduction of some fatty acids under anaerobic conditions (Blumer et al., 1971; Elias et al., 1997; Kim et al., 2016; Haushalter et al., 2017; Jimenez-Diaz et al., 2017; Kang & Nielsen, 2017) A wide range of isomeric hydrocarbons was present in

crude oils, which include many homologous series such as isoparaffins, cycloalkanes and aromatics. From the reports of National Research Council (USA), approximately 1.3 million tons of hydrocarbons were entered to the marine environment per year (NRC, 2003; Halpern et al., 2008). Oil entered to the marine environment through runoff, industrial and sewage effluents, storm-water drainage, shipping activities, spillages, etc (Eganhouse & Kaplan, 1982; Burns & Saliot, 1986; Farid et al., 2008; Farid et al., 2010; Al-Saad et al., 2011; Fingas, 2016; Mapelli et al., 2017). Phytane was produced to the marine environment from oil, but it is not a common component of oil. Phytane is synthesized from oil by methanogenic and photosynthetic bacteria (Steinhauer & Boehm, 1992; Sakata et al., 1997; Peters et al., 2017).

Aliphatic hydrocarbons are the predominant group occur in several species of plants and animals (Youngblood & Blumer, 1973; Pena-Mendez et al., 1999; Carro et al., 2006). Olefins of phytoplankton and zooplankton are markedly different. Several microalgae contain highly unsaturated alkenes from C_{19} to C_{38} with one or four double bonds (Lee et al., 1972; Volkman et al., 1998; Nesvacil et al., 2016). Terrestrial plants contain n-alkanes with 25–31 carbon atoms with a strong odd over even carbon number predominance (Nikolic et al., 2009; Foo et al., 2017; Li et al., 2017; Machado & Froehner, 2017). So alkanes present in plants and diatoms are used as chemotaxonomic markers. Long-chain n-alkanes were present in the outer surface of insects, plants and several marine organisms, and they may vary with environmental parameters such as temperature and humidity. Thus, abundance and distribution of long-chain n-alkanes in sediments have been suggested as chemical

indicators or biomarkers in the marine environment, which reflecting climate change of that environment (Singer et al., 2000; Li et al., 2017). Since the tissues of molluscs are an important hydrocarbon storage site, they are widely used as sentinel organisms in monitoring marine pollution (Farid et al., 2008; Farid et al., 2010; Al-Saad et al., 2011). Alkane content of molluscs gives an idea about the food source of that species since they are filter feeders. From the reports of Cobb & Jallon (1990), it was clear that hydrocarbons act as sexual pheromones. According to Petereson et al. (2007), n-alkanes were used as cuticular waxes. Cuticular hydrocarbons have proved to be useful for identifying insect species and differentiating populations. Undecane, tridecane, pentadecane, 2-tridecanone, and 2-pentadecanone were used as alarm pheromones (Regnier & Wilson, 1968; Quarrell et al., 2016). Numerous aroma alkanes such as octane, nonane, dodecane, hexadecane, etc. were found in environmental food systems (Li et al., 2016). A series of long-chain methylated alkanes (both saturated and unsaturated) are produced by planktonic bacteria and they are used as molecular markers (Fukushima et al., 2004). Several saturated isoprenoids such as pristane, phytane and squalene were used as molecular biomarkers (Meyers, 2003; Gireeshkumar, 2013). Marine sources of these compounds include zooplankton, lobster, fish, sharks, sperm whale, etc (Beaudoin et al., 2016; Bhattacharya et al., 2017; Tessin et al., 2017).

Table 5.1: Sources of major hydrocarbons in the marine environment

Compound	Source	Reference
n-Alkanes with C_{15}, C_{17} or C_{19} predominance	Algae	Sikes et al., 2009
n-alkanes C_{21}, C_{23} or C_{25} predominance	Aquatic macrophytes	Mead et al., 2005
n-alkanes with C_{27}, C_{29} or C_{31} predominance	Terrestrial input	Eglinton & Hamilton, 1967
n-alkanes with C_{20}-C_{40} without odd over even predominance	Petrogenic input	Peters et al., 2005
Shorter chain n-alkanes from C_{14} to C_{24}	Phytoplankton	Meyers & Ishiwatari, 1993
Very long chain n-alkanes	Micro algae Zooplankton processing	Volkman et al., 2008
Pristane	Chlorophyll a	Risatti et al., 1984
Phytane	Erosion of sedimentary rocks and methnogenic bacteria	Meyers, 2003

Hydrocarbons are richly present in edible oils which include vegetable oils and fish oils. Hydrocarbon present in vegetable oils is not confined to n-alkanes, but includes branched alkanes and terpenoids (Peinado et al., 2016; Kraiem et al., 2017). Odd carbon number molecules predominate over even number carbon molecules in crude sunflower oil (Itoh et al., 1973; Orsomando et al., 2016). Long chain alkanes dominated over short chain ones in both sunflower oil and rice bran oil. Saturated alkanes such as C_{14}, C_{17}, C_{19}, C_{21} and C_{29} were richly present in crude rapeseed oil, crude and refined sunflower oil and rice bran oil (Moffat et al., 1995). Less pronounced variation of odd-even

and short-long chain alkanes were observed in seed oils and nut oils (Itoh et al., 1973; Kamzolova et al., 2016). n-Alkanes ranging from C_{14} to C_{33} were observed in fish oil, of which shorter chain predominates (Moffat et al., 1995; Kraiem et al., 2017). Phytoplankton and detritus matters are the major food source of molluscs. Alkanes present in molluscs are mainly coming from their food source (Farid et al., 2008; Maioli et al., 2010; Al-Saad et al., 2011; Azab et al., 2016). In some anaerobic conditions, fatty acids undergo bacterial reduction to produce alkanes (Herman & Zhang, 2016; Nesvacil et al., 2016; Foo et al., 2017). Petroleum hydrocarbons also play a role in alkanes of molluscs, but the contribution is very little in the uncontaminated marine environment. Edible burrowing bivalve mollusc shows odd chain n-alkane predominance, while n-alkanes from the gastropod *C. nemoralis*, which has no predominance of odd chain n-alkane over even chains (Van der horst & Oudejans, 1972). According to Yuan et al. (2012), even numbered higher chain n-alkanes were dominantly present in gastropods as compared with bivalve species.

Limited studies were carried out on the alkane profiles of various species of molluscs from different parts of the world. n-Alkane contents in marine gastropod *L. japonica* and sea snail, *H. discushannai* were mainly focussed on bio-monitoring of n-alkanes (Joseph, 1982). Distribution and seasonal variation of n-alkanes in some species of molluscs from Shatt Al-Arab River was studied by Al-Saad et al. (2011). Farid (2007), Maioli et al. (2010) and Veerasingam et al. (2011) have reported the presence of petroleum hydrocarbon in bivalve collected from Shatt Al-Arab River. Shaw & Wiggs (1980), analysed the variation

of alkane concentration in four gastropods (*C. fenestrata, C. pelta, N. lima* and *C. scutum*) and two bivalves (*M. edulis* and *M. balthica*). Verlecar et al. (2006) determined the source of marine pollution along the Indian coast using bivalves as biomarkers. The relationship between tissue concentrations of polycyclic hydrocarbons and anti-oxidative responses of marine mussels *P. viridis* was studied by Cheung et al. (2001). Alkane content of gastropod, *L. lambis* from the Mediterranean Sea and three bivalve species, *C. opercularis, O. bartrami* and *S. oualaniensis* from the Atlantic Ocean were used for monitoring study (Mironov et al., 1981). Mackie et al. (1974) observed the alkane profile of sea snail (*B. undatum*) and in the same year Mayo & co-authors investigated alkane content from the soft tissue of *L. vulgaris* (bivalve).

n-Alkanes in sandeels (*A. tobianus*) and capelin (*M. villosus*) collected from Norway were used as fish oil (Moffat et al., 1993). Clarke & Law (1981) studied the alkane concentration from two sea snail *C. denseculpta* and *N. eatoni*. Lee et al. (1972) observed the alkane content from marine bivalve, *M. edulis*. Petroleum monitoring role of mussels *M. edulis* collected from the Atlantic Ocean near Sable Island were analyzed by Zhou et al. (1996). Origin and distribution of polycyclic aromatic hydrocarbons in molluscs from Lagoon ecosystems of Morocco were studied by Semlali et al. (2012). Clarke & Finley (1973) as well as Shaw & Wiggs (1980) analysed the same species for finding the seasonal variation of alkanes. n-Alkanes in horse mackerel (*T. murphyl*), sardine (*Sardinops* sp.) and anchoveta (*Engraulis* sp.) collected from Chile were used as fish oil (Moffat & Mc Grill, 1993). Bouzid et al. (2012) determined the hydrocarbon levels in bivalves from

Mediterranean Sea. Presence of polycyclic aromatic hydrocarbons from petroleum fractions was determined by Pampanin & Sydnes (2013). Blumer et al. (1970a,b) analysed the alkane content of one edible clam, *A. irradians*. Farrington & Medeiros (1975) studied the alkane concentration of a marine bivalve, *T. squamosa*. Mironov et al. (1981) observed the alkane concentration of a marine bivalve *L. vulgaris*. Bioaccumulation of petroleum hydrocarbons by *M. nodosa* (gastropod) in Tigris River was analysed by Shaker & Almukhtar (2016). Avelar et al. (2000) analysed *P. viridis* from the river of Brazil as indicator organisms for monitoring of oil pollution. Evaluations of total hydrocarbon levels in some aquatic molluscs from oil polluted mangrove wetland were studied by Clinton et al. (2009).

Hydrocarbons are notably stable in the marine food chain. From the reports, it is clear that this stability and the great variability of the hydrocarbon composition of different sources, suggest the possibility of using hydrocarbon as a tool in the investigation of dynamic processes in the marine food chain (Blumer, 1967; Blumer et al., 1970a, b; Peinado et al., 2016). Thus, a wide-range characterization and separation of complex hydrocarbons to individual compounds is essential for differentiating its origin, whether these are coming from the biogenic source or fossil fuels. Estuaries are highly productive area and receive large amounts of pollutants from the terrestrial input. So the study of hydrocarbons in these environments is immensely significant. Alkane content in molluscs species collected from the estuarine system is enormously important since they are used as bio-indicators. From the previous reports, saturated alkanes are widely used as pollution indicator (Avelar et al.,

2000; Shaker & Almukhtar, 2016). Long chain saturated hydrocarbons influence the rate of induction of skin tumors by polycyclic aromatic hydrocarbons (Yang et al., 2017). Alkanes are ranging from C_{14} to C_{24}, which have biological activity as accelerators of carcinogenesis affecting model membrane systems (Moffat et al., 1995; Hajlaoui,et al., 2016; de Araujo et al., 2017; Sharopov et al., 2017; Xiang et al., 2017). From the previous reports, it was clear that saturated alkanes have massive significance in both commercial and pharmaceutical industry. In this context, present study aims to determine the distribution of n-alkanes in some species of molluscs collected from different estuaries along the Southwest coast of India. Molluscs analysed for the present study includes gastropods as well as bivalves. *B. spinosa*, *T. telescopium*, *T. curta*, *M. trapa* were the gastropods and *V. cyprinoids*, *P. viridis* were the bivalves selected for the present study.

5.2 Materials and methods

5.2.1 Species collection

Marine gastropods, *T. curta, M. trapa, T. telescopium, B. spinosa*, and bivalves *P. viridis, V. cyprinoids* were selected for alkane analysis (Details of the taxonomical description of the organisms and sampling sites are described in Chapter 2).

5.2.2 Analyses of alkanes

Neutral lipids were extracted from the species using 2:1 $CHCl_3$ and CH_3OH (Harvey, 1994). Alkanes were separated from crude lipid through column chromatography using n-hexane (Medeiros & Simoneit,

2007) and analyzed using GC-FID. Detailed procedures were given in Chapter 2.

5.2.3 Statistical analysis

The correlation studies were done on alkane contents derived from the gastropods and bivalves using the SPSS (22.0) software for windows by utilizing the bivariate Pearson correlation. Detailed procedures were given in Chapter 2.

5.3 Results and discussion

5.3.1 n-Alkanes

In this study, aliphatic hydrocarbons ranging from C_{11} to C_{28} were identified, and their distributions in μg g^{-1} are presented in Table 5.2. The chromatograms of alkanes of six species, selected for the current analysis are presented in Figure 5.1. There is a general uniformity of hydrocarbon compositions within the major gastropods and bivalves groups. The values of ∑n-alkane concentrations ranged from 4.23 μg g^{-1} to 19.55 μg g^{-1} (Figure 5.2) with the maximum value observed in *T. telescopium* and minimum concentration in *P. viridis*. Reported data of n-alkanes in vegetable oils and fish oils were comparable with the present study. Total alkane content of soya bean, coconut, peanut, palm, palm kernel and fish oils were ranging from 3.18 μg g^{-1} to 20 μg g^{-1} (Moffat et al., 1995). Total odd chain n-alkanes ranged from 1.46 μg g^{-1} to 5.06 μg g^{-1} (Figure 5.3) and even chain n-alkanes ranged from 1.46 μg g^{-1} to 14.48 μg g^{-1} (Figure 5.4).

Table 5.2: Concentration of n-alkanes in molluscs in μg g^{-1}

Alkane	MT	TC	TT	BS	VC	PV
C_{11}	0.30 ± 0.08	0.55 ± 0.16	0.22 ± 0.17	0.19 ± 0.04	0.30 ± 0.11	0.10 ± 0.08
C_{12}	0.77 ± 0.12	1.53 ± 0.34	0.86 ± 0.44	0.45 ± 0.12	0.96 ± 0.39	0.65 ± 0.29
C_{13}	0.14 ± 0.06	1.10 ± 0.39	0.02 ± 0.01	0.12 ± 0.09	0.01 ± 0.01	0.01 ± 0.01
C_{14}	0.16 ± 0.02	0.33 ± 0.22	0.69 ± 0.15	0.09 ± 0.01	0.08 ± 0.01	0.81 ± 0.22
C_{15}	0.03 ± 0.01	0.02 ± 0.01	0.05 ± 0.03	0.03 ± 0.02	0.04 ± 0.02	0.28 ± 0.14
C_{16}	0.11 ± 0.05	0.51 ± 0.29	0.07 ± 0.02	0.72 ± 0.32	0	0.22 ± 0.16
C_{17}	0.83 ± 0.32	0.90 ± 0.18	0.02 ± 0.01	0.30 ± 0.07	0.97 ± 0.02	0.75 ± 0.41
C_{18}	0.11 ± 0.07	0.51 ± 0.15	0.13 ± 0.09	0.42 ± 0.26	0.49 ± 0.29	0.75 ± 0.41
C_{19}	0.13 ± 0.02	0.47 ± 0.07	2.76 ± 1.03	0.18 ± 0.11	0.03 ± 0.02	0.03 ± 0.01
C_{20}	0.13 ± 0.03	0.45 ± 0.09	1.04 ± 0.16	0.05 ± 0.01	0.30 ± 0.09	0.04 ± 0.02
C_{21}	0.13 ± 0.02	0.56 ± 0.16	0.04 ± 0.01	0.02 ± 0.01	0.09 ± 0.05	0.02 ± 0.01
C_{22}	0.11 ± 0.05	0.55 ± 0.21	0.07 ± 0.01	0.06 ± 0.03	0.01 ± 0.01	0.01 ± 0.01
C_{23}	0.93 ± 0.15	0.16 ± 0.07	0.90 ± 0.19	0.47 ± 0.29	0.40 ± 0.16	0.61 ± 0.32
C_{24}	0.03 ± 0.01	0.10 ± 0.02	0.37 ± 0.05	2.50 ± 0.85	0.08 ± 0.04	0.02 ± 0.02
C_{25}	0.04 ± 0.01	0.08 ± 0.01	0.01 ± 0.01	0.09 ± 0.04	0.01 ± 0.01	0.06 ± 0.04
C_{26}	0.01 ± 0.02	0.02 ± 0.02	3.57 ± 0.94	0.02 ± 0.01	0.90 ± 0.06	0.03 ± 0.01
C_{27}	0.64 ± 0.08	1.16 ± 0.81	1.05 ± 0.14	0.61 ± 0.43	0.58 ± 0.18	1.01 ± 0.86
C_{28}	0	0	7.65 ± 1.24	0	0	1.96 ± 0.21

MT- *M. trapa*, TC- *T. curta*, TT- *T. telescopium*, BS- *B. spinosa*, PV- *P. viridis*, VC- *V. cyprinoids*

Alkanes ranging from C_{11} to C_{27} were present in *M. trapa* species, of which C_{23} (0.93 ± 0.15 μg g^{-1}) was dominated (Table 5.2). C_{17} (0.83 ± 0.32 μg g^{-1}), C_{12} (0.77 ± 0.12 μg g^{-1}) and C_{27} (0.64 ± 0.08 μg g^{-1}) were also present in large quantity. The concentrations of C_{15}, C_{24}, C_{25}, and C_{26}, were comparatively low in *M. trapa* and C_{26} (0.01 ± 0.02 μg g^{-1}) was minimum (Table 5.2). n-Alkanes from C_{11} to C_{27} were present in *T. curta* species of which C_{12} (1.53 ± 0.34 μg g^{-1}) dominated, while C_{15}

(0.02 ± 0.01 μg g^{-1}) and C_{26} (0.02 ± 0.02 μg g^{-1}) were minimum (Table 5.2). Comparable, but low content of C_{11} was observed in *M. trapa* (0.30 ± 0.08 μg g^{-1}) and *V. cyprinoids* (0.30 ± 0.11 μg g^{-1}). C_{12} content observed in *T. telescopium* (0.86 ± 0.44 μg g^{-1}) and *V. cyprinoids* (0.96 ± 0.39 μg g^{-1}) were analogues (Table 5.2). In *B. spinosa* species, C_{24} dominated over n-alkanes having the concentration 2.50 ± 0.85 μg g^{-1} and it was followed by C_{16} showing 0.72 ± 0.32 μg g^{-1} alkane content (Table 5.2). C_{21} (0.02 ± 0.01 μg g^{-1}) and C_{26} (0.02 ± 0.01 μg g^{-1}) were minimum in *B. spinosa*. C_{28} (1.96 ± 0.21μg g^{-1}) was the dominant n-alkane present in the soft tissue of *P. viridis*. C_{13} and C_{22} were the least n-alkane present in both *P. viridis* and *V. cyprinoids*. C_{13} content in *P. viridis* (0.01 ± 0.01 μg g^{-1}) and *V. cyprinoids* (0.01 ± 0.01 μg g^{-1}) as well as C_{22} in *P. viridis* (0.01 ± 0.01 μg g^{-1}) and *V. cyprinoids* (0.01 ± 0.01 μg g^{-1}) were comparable. C_{28} was the highest and C_{25} was the lowest n-alkane present in *T. telescopium* (Table 5.2). The corresponding concentration was 7.65 ± 1.24 μg g^{-1} and 0.01 ± 0.01 μg g^{-1} respectively. In the present study, the amount of C_{26} was least of all the species except *V. cyprinoids*. In *V. cyprinoids* C_{26} was the third most abundant n-alkane (0.90 ± 0.06 μg g^{-1}) and C_{17} was the dominant n-alkane (0.97 ± 0.02 μg g^{-1}). The C_{27} content of *T. telescopium* (1.05 ± 0.14 μg g^{-1}) and *P. viridis* (1.01 ± 8.76 μg g^{-1}) were analogous. Similar, but the low content of C_{26} was observed *T. curta* (0.02 ± 0.02 μg g^{-1}) and *B. spinosa* (0.02 ± 0.01 μg g^{-1}).

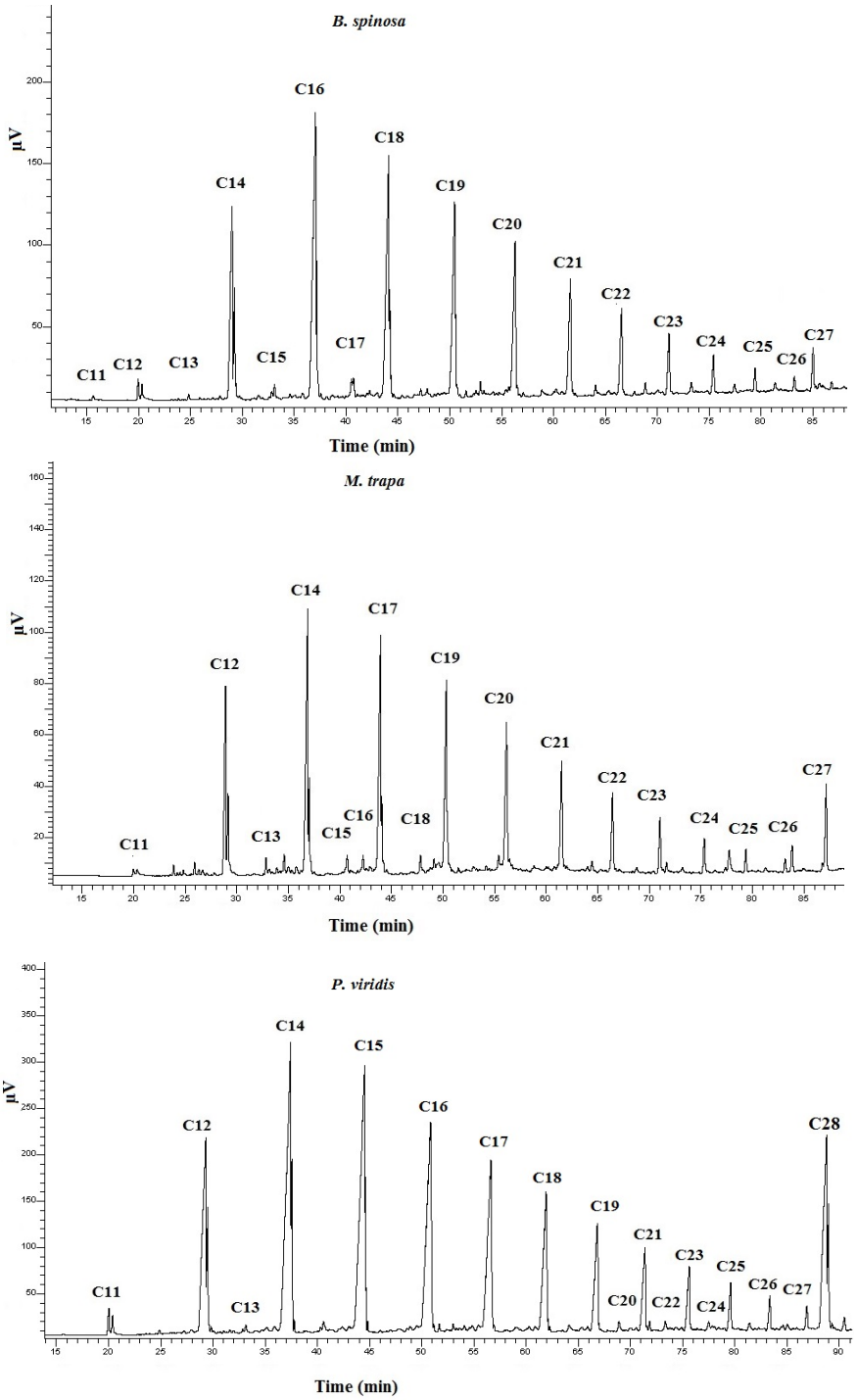

Figure 5.1 continued…

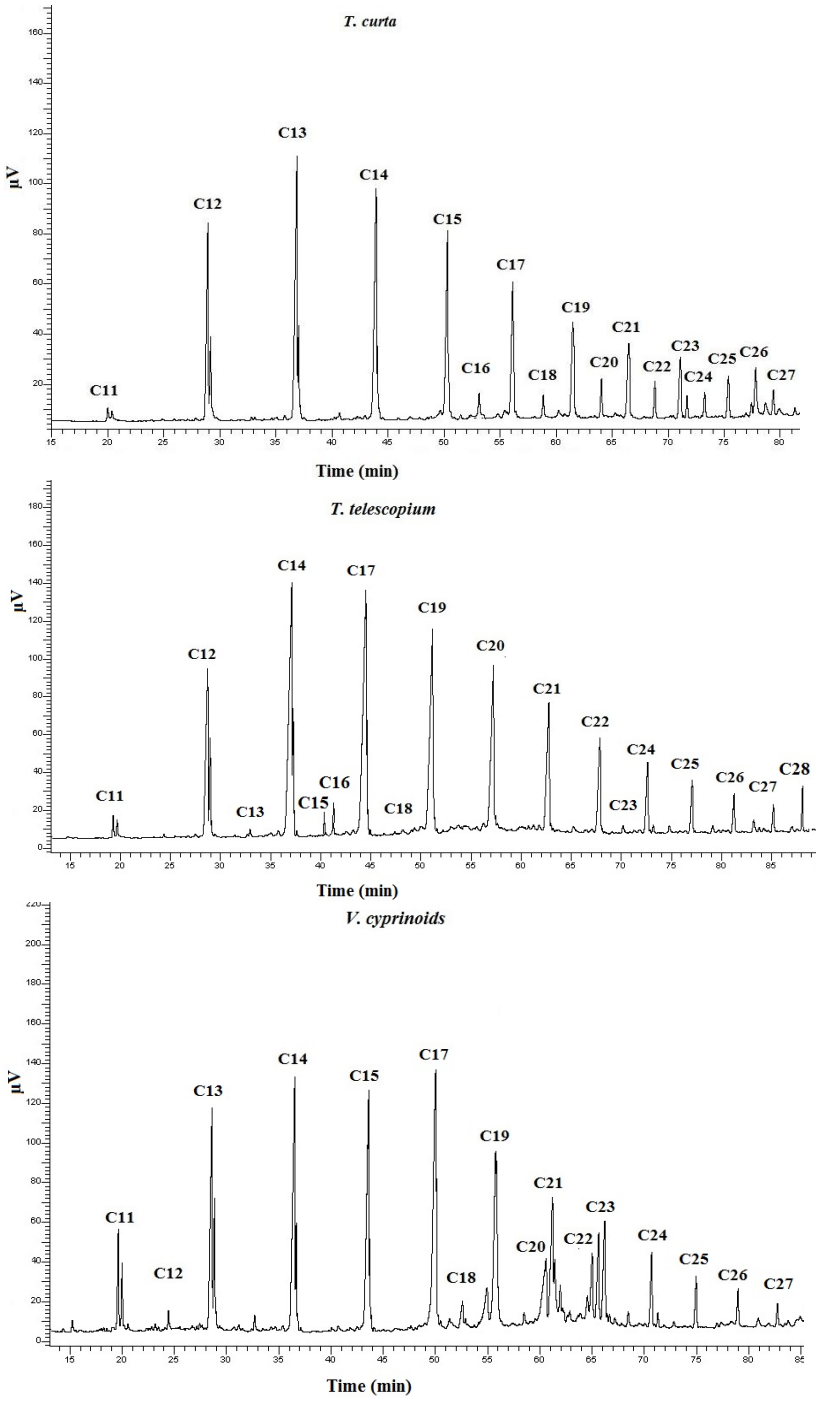

Figure 5.1: Total ion chromatogram of alkanes in molluscs species

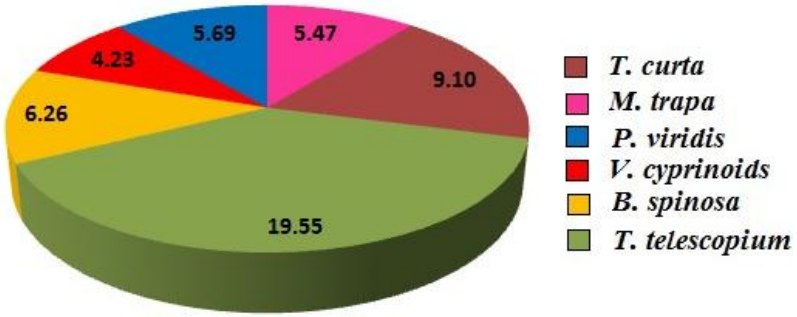

Figure 5.2: Total concentration of n-alkanes in molluscs species (µg g^{-1})

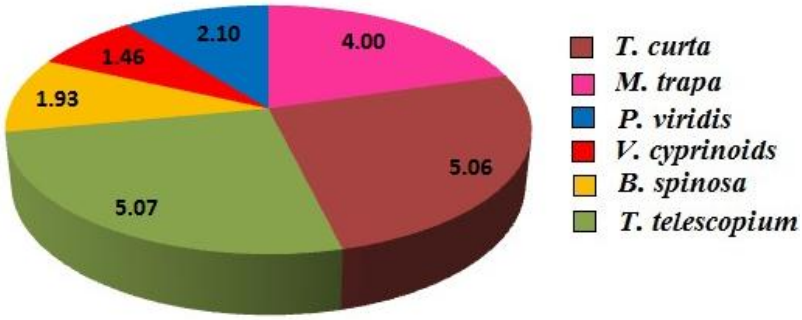

Figure 5.3: Total concentration of odd chain n-alkanes in molluscs species (µg g^{-1})

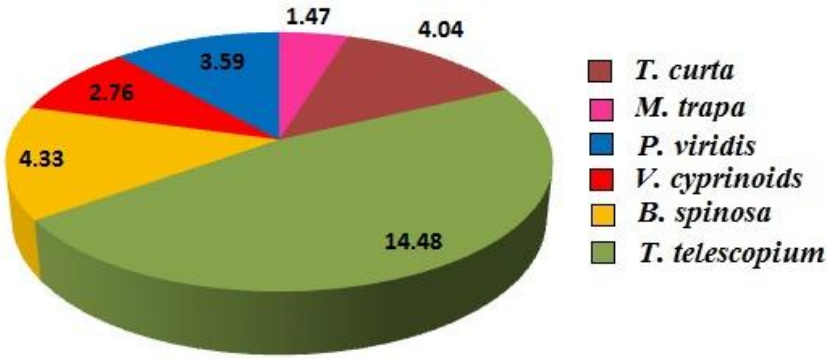

Figure 5.4: Total concentration of even chain n-alkanes in molluscs species (µg g^{-1})

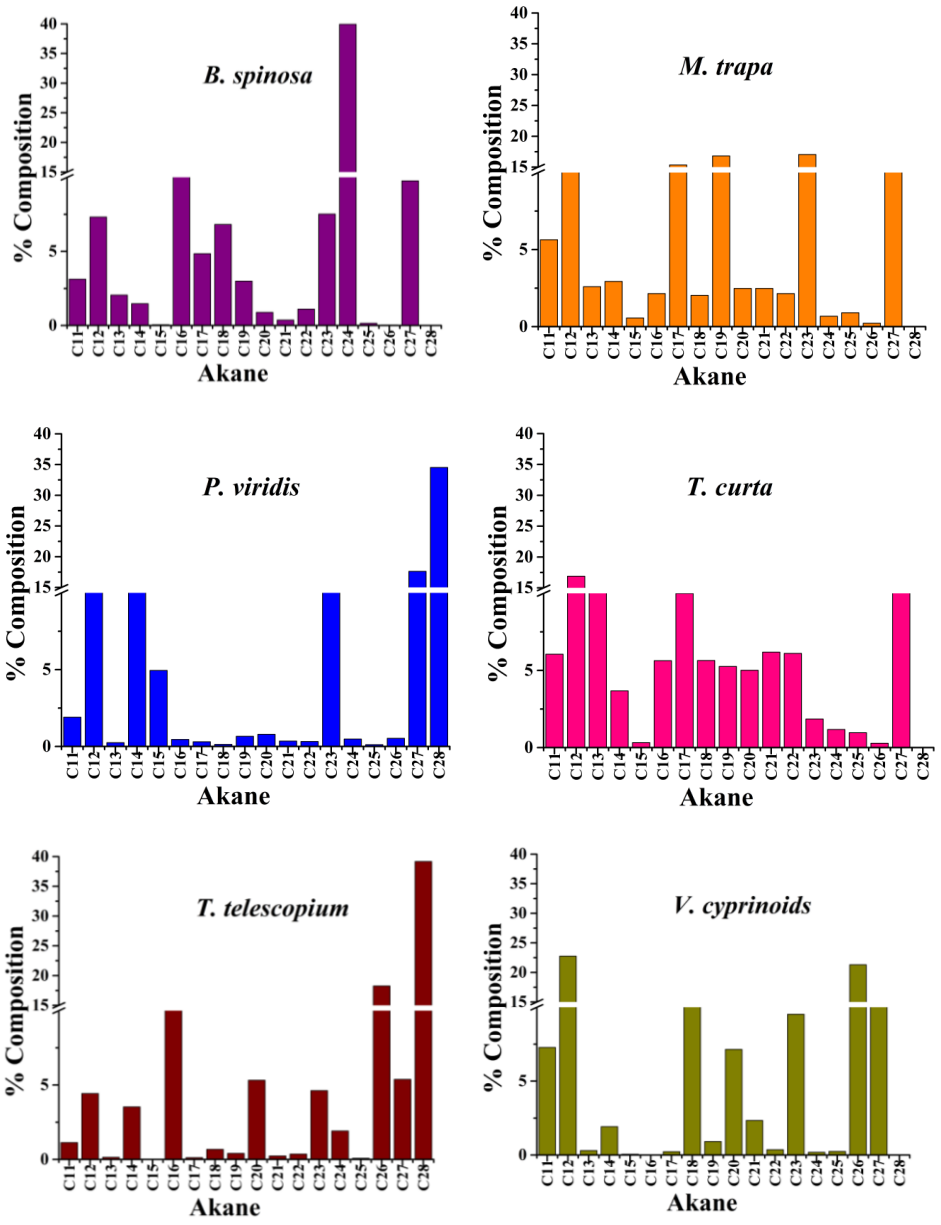

Figure 5.5: Percentage compositions of n-alkanes from six molluscs

In addition to the odd carbon predominance at C_{19} and C_{23}, there was a minor predominance in $C_{11,}$ C_{12} and C_{27} in the soft tissue of *M. trapa*. C_{27} (1.16 ± 0.81 μg g^{-1}) exhibited maximum contribution to the n-alkane pool of *T. curta*. In the present study, soft tissue of *M. trapa* and *T. curta* contains a large quantity of C_{12}, C_{17} and $C_{27.}$ These findings were in close resemblance with the results of alkanes from gastropods and bivalves collected from Indian Ocean (Mironov et al., 1981, Lehahn et al., 2016). C_{23} was the dominant alkane present in *M. trapa,* which is 17.04% of the total alkane (Figure 5.5). The concentration of C_{28} in *P. viridis* was 1.96 ± 0.21 μg $g^{-1,}$ and it was followed by C_{27} with concentration 1.01 ± 0.86 μg g^{-1} (Table 5.2). *V. cyprinoids* contained large concentrations of C_{12} and C_{26}. In addition to the even carbon predominance at C_{12} and C_{26}, there was a minor predominance in C_{11} and C_{27} were observed in this species. In *V. cyprinoids,* C_{12} (0.96 ± 0.39 μg g^{-1}) dominated other n- alkanes, which is 22.76% of the total alkane (Figure 5.5). From the current study, it is clear that soft tissues of two bivalve species *V. cyprinoids* and *P. viridis* contain a large quantity of C_{12}, C_{23} and C_{27} n-alkanes. These results have similarities with the result of alkane from marine clam (Bochem et al., 1982; Sun et al., 2016; Joy & Chakraborthy, 2017; Wang et al., 2017). Also, C_{14} and, C_{28} were highest in *P. viridis*, and respective percentage compositions were 14.35% and 34.52% (Figure 5.5). Generally, shorter chain n-alkanes (C_{14} to C_{24}) are largely derived from algal or phytoplankton sources (Meyers & Ishiwatari, 1993; Sorigue et al., 2017) while, freshwater, aquatic and marine mactophytes have a dominant mid chain length C_{23} to C_{27} hydrocarbons. On the other hand, slightly longer mid chain n-alkanes predominant in mangrove leaves (Mead et al., 2005; Krishnan et al., 2014; Sumesh et al., 2014).

Gastropods and bivalves are not capable of biosynthesizing n-alkanes, and the n-alkanes were assimilated from their food (Bhandari et al., 2016; Sun et al., 2017). Therefore, the n-alkane content in tissues probably gives an account of the nature of food source in an environment where the organism thrives. Due to the filter feeding nature of mollusc, all the alkanes detected in the present study were already reported in the marine environment. Alkanes isolated from marine environment typically fall into two categories, *viz*., short chained n-alkanes (C_{10} - C_{21}) with no distinct odd-over-even predominance, indicative of algal or bacterial input (Table 5.1), and long chained n-alkanes (C_{22} - C_{33}) derived from higher plant sources (Han et al., 1968; Volkman et al., 1998; Ficken et al., 2000; Joseph, 2009). n- Alkanes ranging from C_{11} to C_{27} were found to be present in the soft tissue of *M. trapa, T. curta, B. spinos* and *V. cyprinoids* of which C_{16} was absent in *V. cyprinoids* (Figure 5.5). n-Alkanes ranging from C_{11} to C_{28} were found in the soft tissue of *T. telescopium* and *P. viridis*. Long chain n-alkane C28 was observed in both *T. telescopium* and *P. viridis* (Figure 5.5) compared to other organisms which may be attributed to the nature of organic matter sources in the ecosystem where these species collected. *T. telescopium* was sampled from Pappinissery mangrove eco-system situated near Kannur, while *P. viridis* from Dalavapuram situated near to Neendakara. Pappinisheri estuary is an extensive mangrove area, where species of *Avicennia, Rhizophora, Kandelia candel* and *Acanthus* are common (Resmi, 2015). Furthermore, Dalavapuram area comprises patches of *Rhizophora, Sonneratia alba*, *Acanthus ilicifolius, Acrostichum aureum, Avicennia officinalis* and *Brugiera gymnorrhiza* mangroves (Krishnan et al.,

2014; Sumesh et al., 2014). Reported data on the C_{28} n-alkane in *Avicennia* and *Rhizophora* from French Guiana (Rafii et al., 1996; Dodd et al., 1998) and Vypin, Kerala (Murukesh, 2016) were 5 μg g^{-1} to 85 μg g^{-1}. Also, from the studies of Resmi (2015) and Manju (2015), C_{28} content of sediments collected from pappinissery was ranging from 5 μg g^{-1} to 25 μg g^{-1}. These data support the dominance of C_{28} in *T. telescopium* and *P. viridis,* since they are collected from mangrove area.

From the present study, it is clear that total alkane content in the soft tissue of *T. telescopium* collected from Pappinissery mangrove estuary was higher than total alkane content of other species. This is because of large deposition of alkanes in sediments of this area. Gastropods as well as bivalves were filter feeders, and these organisms acquire organic compounds from sediments through diet. Large deposition of organic compounds in sediment depends on both the texture and oxygen content of the sediment (Mays et al., 2017). Resmi (2015) has reported the high concentration of sedimentary n-alkanes (ranging from 5 μg g^{-1} to 25 μg g^{-1}) in Pappinissery. So the high content of alkanes in soft tissue (*T. telescopium*) may be attributed to the assimilation of organic detritus enriched with n-alkanes. According to Resmi (2015) and Manju (2015), the clay content in sediments of Pappinissery mangrove ecosystem was found to be higher. Strong positive inter relationship exists between organic matter and clay content of sediment (Liu et al., 2008; Chaudhari et al., 2013). From the study of Resmi (2015), there is a depletion of dissolved oxygen in the Pappinissery sediments. According to Elias et al. (1997), alkanes were produced by the decarboxylation of fatty acids using bacteria under

anaerobic conditions. The high content of C_{21} and C_{19} alkanes in *T. telescopium* may arise as a result of decarboxylation of fatty acids which is further confirmed by the data of fatty acids in Chapter 7. Another reason for the high content of organic matter in Pappinissery mangrove ecosystem is poultry and slotted waste by human activities.

Compared to *T. telescopium,* low content of alkanes was observed in all the remaining five species. From the previous reports, sandy nature of sediments was observed in Neendakara, Sakthikulangara, Chavara, and Dalavapuram (Sujatha et al., 2009). Organic compounds and sand content were negatively correlated. n-Alkanes in molluscs have been successfully used to distinguish among algal, bacterial, and terrestrial sources of organic carbon in these species (Canuel et al., 1997; Killops & Killops, 2005; Silva et al., 2012; Nesvacil et al., 2016; Li et al., 2017; Machado & Froehner, 2017). From the previous reports, it is clear that n-alkanes ranging from C_{15} to C_{19} are synthesized by aquatic bacteria and algae (Cranwell et al., 1987; Kang & Nielsen, 2017; Mays et al., 2017) and mid- chain alkanes from C_{21} to C_{25} compounds are produced from submerged or floating plants (Baas et al., 2000; Ficken et al., 2000). Long chain alkanes coming from plant leaves (Eglinton & Hamilton, 1967; Li et al., 2017), and their concentration may vary with species as well as with environmental conditions (Dodd et al., 1999; Mays et al., 2017). Concentrations of C_{17} alkanes were higher in *M. trapa, T. currta, P. viridis* and *V. cyprinoids*. Similar results were observed in n-alkanes of horse mackerel (*T. murphyl*), sardine (*Sardinops* sp.) and anchoveta (*Engraulis* sp.) collected from Chile, which was used to make fish oil (Moffat et al., 1993). Mid chain

alkanes were richly present in all the six species, which is similar to hydrocarbon levels in bivalves from Mediterranean Sea (Bouzid et al., 2012). A similar observation was reported in some fishes such as sandeels (*A. tobianus*) and capelin (*M. villosus*) collected from Norway (Moffat et al., 1995).

∑Alkane content of bivalves, *P. viridis* and *V. cyprinoids* was 5.68 μg g^{-1} and 4.22 μg g^{-1} respectively (Figure 5.2). These results were comparable with the total concentrations of n-alkanes in edible mussel and cockle from the Atlantic Ocean, ranged between 0.3 μg g^{-1} – 17.57 μg g^{-1} and 0.08 μg g^{-1} – 5.09 μg g^{-1} respectively (Carro et al., 2006). The total concentrations of n-alkanes in the molluscs varied from 1.50 μg g^{-1} dry weight in the *T. jordani* to 8.78 μg g^{-1} dry weights in the *C. fluminea* (Al-Saad et al., 2011). The concentration of C_{27} is highest in all the species indicating terrestrial biogenic input and maximum C_{17} content indicating phytoplankton input (Bouzid et al., 2012). Mean concentration of n-alkanes in crab *(C. sapidus*) ranging from 0.28 μg g^{-1} to 1.24 μg g^{-1} (Yang et al., 2016) also supports the present study. Sources of n-alkanes in molluscs were determined using different indices such as carbon preference index (CPI), average chain length (ACL), low molecular weight n-alkanes to high molecular weight n-alkanes (LMW/HMW), terrestrial alkane (TAlk), algal alkane (AAlk) and terrigenous to aquatic ratio (TAR), are described in Table 5.3. In the present study, the CPI values were calculated according to Harji et al. (2008).

$$\mathbf{CPI=}\ \frac{\sum \boldsymbol{Odd\ Chain\ n-alaknes}}{\sum \boldsymbol{Even\ Chain\ n-alkanes}}$$

Table 5.3: Characteristics of n-alkanes from different species

Parameters	MT	TC	TT	BS	VC	PV
CPI	2.72	1.25	0.35	0.45	0.53	0.59
ACL	26.86	26.86	26.97	26.97	26.96	26.99
TAR	0.36	0.82	0.38	1.24	11.43	2.98
AAlk	1.79	1.42	2.80	0.49	0.05	0.34
TAlk	0.64	1.17	1.05	0.61	0.58	1.00
LMW/HMW	1.65	1.91	0.33	0.67	0.82	0.53

MT- *M. trapa*, TC- *T. curta*, TT- *T. telescopium*, BS- *B. spinosa*,
VC- *V. cyprinoids*, PV- *P. viridis*

Table 5.4: Characteristics of n-alkanes from different sources

Parameters	Oil	Aquatic	Terrestrial
CPI	≈1	≤1	>1
ACL	Wide range	Narrow range	Narrow range and above 28
LMW/HMW	>2	≈1	<1
TAR		<1	>1

Ref:- (Mille et al., 2006; Mille et al., 2007; Asia et al., 2009; Azis et al., 2016).

In *M. trapa,* the odd chain alkanes show a greater abundance than the even chain *n*-alkanes, while the reverse was observed in case of *B. spinosa*. Carbon preferred index (CPI) value of *M. trapa,* is approximately equal to 2.72 (Table 5.3) and those of *B. spinosa* is 0.45. In soft tissue of *T. curta,* there is a slighter prevalence of odd chain n-alkanes over even chain n-alkanes and it shows CPI approximately equal to 1.25. Even chain n-alkane predominance was observed in the case of *P. viridis*, *V. cyprinoids* and *T. telescopium* (CPI: 0.59, 0.53 and 0.34 respectively) (Table 5.3). Generally, CPI values have been reported to range between 2 to 5.5 in the case of odd to even predominance (Evans et al., 1990). In the present

study, CPI values of *M. trapa* and *T. curta* were greater than one while those of others show lower values. CPI values of *A. atra* (1.21) and *T. petitiana* (8.85) from Bahía Nueva were greater than 1. The former one was mainly a filter and suspension-feeder mussel while, the later was mainly a suspension and detritus-feeder clam (Paletto et al., 2008). CPI values in all oils were very close to unity and seem to be no odd even preference. If the terrestrial input is negligible a significant proportion of long chain alkanes may be derived from planktonic algae and bacteria which do not generate a predominance of the odd numbered long chain molecules, (Pandey, 2012) and the corresponding CPI value is near 1.0 (Table 5.4). In this study, C_{15}, C_{17} and C_{19} alkanes were richly present in all species. Planktonic organisms generally produce a simple mixture of odd chain n- alkanes preferably C_{15}, C_{17} and C_{19} (Gogou et al., 2000).

Variation in CPI values of molluscs may be due to the differences in organic matter sources in food. *M. trapa* and *T. curta* were collected from Neendakara barmouth region and old Neendakara fishing harbour. *B. spinosa* and *V. cyprinoids* were collected from Sakthikulangara and Chavara, while *P. viridis* was collected from Dalavapuram. This may be the reason for a high content of odd chain alkanes in *M. trapa* as well as *T. curta*. Previous works on cockle, an edible burrowing bivalve mollusc with a strong ribbed shell sample showed odd chain alkane predominance (CPI > 3), suggesting the presence of terrestrial plant input (Carro et al., 2006). Reported data of n-alkanes from gastropod *C. nemoralis*, which have no predominance of odd chain n-alkane over even chains (Van der horst & Oudejans, 1972), indicating a strong planktonic input. The studies on benthic bivalves show CPI around 4 which usually indicate

the presence of biogenic hydrocarbon pollution (Carro et al., 2006). Even chain n-alkanes predominance was observed in *T. telescopium*, *P. viridis*, *V. cyprinoids* and *B. spinosa* (Figure 5.5). In Sakthikulangara, both terrestrial and petrogenic input were observed, of which petrogenic input diluted the terrestrial input. So, lower CPI values were observed for *B. spinosa* collected from Sakthikulangara. In Chavara and Dalavapuram, phytoplankton input prevalence and CPI values were minimum. CPI value of *T. telescopium* was very low, which may be due to planktonic input. *T. telescopium* was collected from Pappinissery mangrove estuary. Detritus organic matters are the food of *T. telescopium.*

The average chain length (ACL) is a parameter which describes the weight average number of atoms per molecule based on the abundance of the odd-numbered higher plant-derived alkanes (Poynter & Eglinton, 1990; Boot et al., 2006; Jeng, 2006; Manju, 2015) that can be used to identify different n-alkane source (Ternois et al., 2001; Boot et al., 2006; Jeng, 2006). ACL which is the weighted average of the various carbon chain lengths can be expressed as,

$$ACL = \frac{\Sigma\,(CnXn)}{\Sigma Cn}$$

where, *Cn* is the concentration of each n-alkane with n carbon atoms and *Xn* is the number of carbon atoms

In the present study, the ACL values ranged from 26.86 to 26.99 (Table 5.3). Very narrow range indicates the phytoplankton input (Cranwell, 1982; Seki et al., 2010). ACL value depends only on the source from which the hydrocarbons originates, not the process. Consistent ACL values indicate minute petrogenic input of n-alkanes (Vaezzadeh

et al., 2015). Higher ACL values are a sign of terrestrial input of hydrocarbons (Schefuss et al., 2003; Jeng, 2006; Wang et al., 2015).

LMW/HMW index, $C_{16,}$ and C_{26} have indicated the presence of light and heavy compounds. Long chain n-alkane predominance was observed in soft tissues of *T. telescopium*, *B. spinosa*, *P. viridis* and *V. cyprinoids* showing a LMW/HMW ratio 0.33, 0.67, 0.82 and 0.53 respectively (Table 5.3). Short chain n-alkanes dominated over long chain n-alkanes in soft tissues of *T. curta* and *M. trapa*, corresponding LMW/HMW values were 1.91 and 1.65 respectively. Gastropods have higher chain n-alkanes as compared with bivalve species, but in some cases variation may occur due to petrogenic input (Yuan et al., 2012). The high content of LMW alkanes in *T. curta* and *M. trapa* may be due to the petroleum content in Neendakara collection location associated with fishing boat traffic. Noteworthy, CPI, ACL and LMW/HMW values have some similarities (Table 5.3). Highest CPI and ACL values as well as lowest LMW/HMW n-alkane values of *T. curta* and *M. trapa* study indicate minor impacts on anthropogenic inputs (Vaezzadeh et al., 2015).

The total content of $C_{15}+C_{17}+C_{19}$ n-alkanes is normally considered as marine organic matter indicator, while the total content of $C_{27}+C_{29}+C_{31}$ n-alkanes have been used as terrestrial organic matter indicator (Xing et al., 2011). So it is revealed that n-alkanes in all the molluscs were arising from both terrestrial and planktonic inputs (Table 5.3). Terrigenous to aquatic ratio (TAR) (Meyers, 1997) values were calculated using the equation

$$TAR = \frac{C27 + C29 + C31}{C15 + C17 + C19}$$

Higher TAR values are the indication of terrestrial input (Table 5.4). In the present study, a minute amount of TAR values of all the species except *V. cyprinoids* were observed (Table 5.3), which indicates a terrestrial input of alkane. Table 5.3 shows that in all the species except *M. trapa* and *T. curta* even chain n-alkanes predominate.

5.3.2 Statistical analysis

Principal component analysis (PCA), Factor analysis (FA) and Hierarchical Cluster analysis (HCA) were used for the careful treatment of data set (even a small one). PCA employs mathematical transformation to the original data with no assumptions about the form of the covariance matrix. The aim of this procedure is to find out a few linear combinations of the original variables that can be used to summarize the data set without losing much of the information in the data (Massart et al., 1980; Massart et al., 1988; Grimalt et al., 1993; Pena-Mendez et al., 1999). PCA was carried out, in order to identify different source of n-alkanes in marine molluscs. Principal components were considered significant if the eigen values were greater than 1.

Table 5.5: Total cumulative variance

Eigen values	% of variance
7.56	42.67
3.89	20.65
2.58	17.83

Factor analysis provided three components for the alkanes in mollusc with a total cumulative variance of 80.15 %. The component1 accounted for a total variance of 42.67% (Table 5.5) and exhibited

highly significant loading of n-alkanes such as C_{11}, C_{12}, C_{13}, C_{17}, C_{20}, C_{22} and C_{25} (Figure 5.6) indicating the biogenic contribution of marine derived organic matter and microbial derived organic matter. Alkanes such as C_{17}, C_{20}, C_{22} and C_{25} were coming from aquatic bacteria and algae (Mironov et al., 1981; Joseph, 2009; Gireeshkumar et al., 2015) and that of C_{11}, C_{12} and C_{13} were coming from diatoms (Mironov et al., 1981; Manju, 2015). PC1 demonstrate that these alkanes are entered to the molluscs through food.

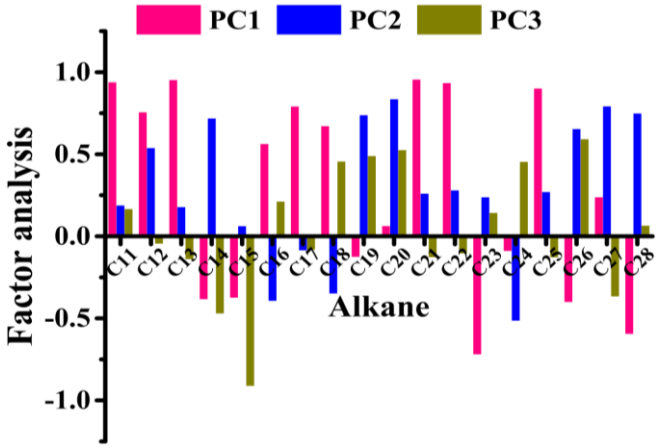

Figure 5.6: Principal component analysis representing source of n-alkanes in molluscs

The second component accounted for a total variance of 20.67% (Table 5.5) and exhibited highly significant loading of n-alkanes such as C_{19}, C_{21}, C_{26} and C_{28} (Figure 5.6). PC2 explaining the biodegradation of fatty acids as well as alkane from mangrove source. In molluscs, slight amount of alkanes are produced from the decomposition of fatty acids (Kim et al., 2016; Nesvacil et al., 2016; Foo et al., 2017; Kang & Nielsen,

2017). C_{19} and C_{21} are coming from the decarboxylation of fatty acids. C_{26} and C_{28} were common in mangrove leaf. The third component accounted for a total variance of 17.83% (Table 5.5) and exhibited highly significant loading of n-alkanes such as C_{14}, C_{18} and C_{27}. PC3 is explaining bacterial input of normal hydrocarbons (Figure 5.6).

5.4 Summary

Alkanes ranging from C_{11} to C_{27} were observed in *M. trapa*, *T. curta*, *B. spinosa* and *V. cyprinoids* and alkanes ranging from C_{11} to C_{28} were observed in *T. telescopium* and *P. viridis.* C_{28} was observed in species collected from mangrove area. From the reported data, alkanes ranging from C_{15} to C_{27} were present in edible oils, which were also present in all the six species. The present study revealed that source of n-alkanes in molluscs varies with the sampling locations. Molluscs acquire alkanes from the surrounding environment through diet, since they are filter feeders. *T. curta* and *M. trapa* showed similarities in the values of CPI, TAR and LMW/HMW, due to the resemblance in sampling site. *T. curta* and *M. trapa* were collected from Neendakara barmouth and Neendakara fishing harbour respectively. Ashtamudi estuary opens to the Arabian Sea at this point, were terrestrial input as well as petrogenic input from fishing boat are observed. This would lead to the high content of CPI and LMW/HMW values.

Even chain n-alkanes predominance was observed in *T. telescopium*, *P. viridis*, *V. cyprinoids* and *B. spinosa*, may also due to the similarities in sampling locations. *B. spinosa* was collected from Sakthikulangara, where both terrestrial and petrogenic inputs were observed. Similar

observations occurred in *P. viridis* and *V. cyprinoids* collected from Dalavapuram and Chavara. In these three places, petrogenic input diluted the terrestrial input. So, lower CPI values were observed for *B. spinosa*, *P. viridis*, and *V. cyprinoids.* CPI value of *T. telescopium* was very low, which may be due to planktonic input. *T. telescopium* was collected from Pappinissery mangrove estuary. Detritus organic matters are the food of *T. telescopium.* All the parameters confirmed that n-alkanes in molluscs are originated from the surrounding environment through diet.

Even chained n-alkanes were richly present in *T. telescopuim*. Total alkane content of *T. telescopium* is higher than those of other species; this is because of the peculiar nature of sampling location. Pappinissery mangrove ecosystem is contaminated by human activities such as poultry and slotted waste. Alkanes from petroleum and grease were released to the Pappinissery mangrove estuary from motor vehicle service station, which is situated near to the sampling area. Unusual presence of C_{19} and C_{21} n-alkanes compared to other species may be from the biochemical degradation processes. In *T. telescopium*, these alkanes are coming from the decarboxylation of fatty acids under anaerobic condition. Thus, all the observations from this study conclude that n-alkanes in molluscs are either accumulated from the surrounding environment or formed as a by-product of chemical reactions occurred inside the body.

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Chapter 6

CHARACTERIZATION OF STEROLS AND *IN SILICO* BIOLOGICAL ACTIVITY STUDIES

Contents

6.1 Introduction

Marine organisms are excellent sources of biologically active secondary metabolites. Lots of this metabolites exhibit structural and chemical features which is not observed in compounds isolated from terrestrial organisms (Gavagnin & Fontana, 2000; Murphy et al., 2003). Based on the species number, molluscs are the second largest phylum in the marine environment and there are more than 1,00,000 molluscs species all over the world (Myers et al., 2017). They are segregated into seven classes, out of which the main four classes includes gastropoda, bivalvia, polyplacophora and cephalopoda, all of which include snails, clams, scallops, oysters, cuttlefish, and octopus. Gastropods are the most dominant, possess more than 80% of the total living organisms, and bivalves are the second most dominant class in this phylum (Venkatesan & Mohamed, 2015a,b). In the marine environment, secondary metabolites

produced by the defensive mechanism of organisms against consumers and competitors, many of which show allelopathic activities and predator deterrence (Benkendorff, 2010). The phylum molluscs possess an array of secondary metabolites, moreover they are regarded as excellent sources of nutritionally important sterols (Kanazawa, 2001; Kandyuk, 2006; Zhukova, 2007; Pereira et al., 2013).

Steroids are prevalent lipid components with saturated tetracyclic hydrocarbon group and they are highly diverse group of metabolically active compounds (Goad, 1978; Byju, 2015; Liu et al., 2015). The term sterols also known as steroid alcohols refer to an important group among the steroids with a fused cyclopentanophenanthrene ring with a 3-hydroxyl moiety (Napolitano et al., 1993). Researchers have keen interest on sterols and steroid compounds isolated from marine organisms ever since the earliest studies of Henze & Hoppe Seylor (1904) on marine organisms (Goad, 1978). The renaissance of interest in marine sterols since 1960 stems in part from the search for new marine natural products with useful pharmacological properties (Goad, 1978). Majority of secondary metabolites were isolated from marine molluscs, include steroids, terpenoids, isoprenoids, nonisoprenoids, quinones, brominated compounds, nitrogen heterocyclics, and nitrogen sulphur heterocyclics, often exhibiting antiviral, antimicrobial, antiprotozoal, antifungal, antihelminthic and anticancer activities (Zapata & Amemiya, 2000; Datta et al., 2015; Pereira et al., 2016). The "usual" sterols observed in marine organisms have a 3β-hydroxy-Δ5- (or Δ0-) cholestane nucleus and a C8-C10 side chain. There are over 200 such sterols, occurring in marine organisms as complex inseparable mixtures (Blunt et al., 2014).

Cholesterol is usually the predominant sterol present in mollusc species with exception of some bivalve species (Bergmann, 1949; Idler & Wiseman, 1972; Benkendorff et al., 2005). Bivalves contain large variety of sterols like brassicasterol, crinosterol, 24-methylenecholesterol, sitosterol, stigmasterol, etc. (Sun et al., 2014). Cholesterol is the only sterol occurring in cephalopoda and pelecypoda, while numerous other sterols were detected in the gastropod, but in such small quantities that still remain unidentified. Variations in sterol content of gastropods, bivalves and cephalopods were reported by many researchers, although they represent the same phylum, molluscs (Idler & Wiseman, 1972; Benkendorff et al., 2005). This is because sterol profiles can be characteristic of a particular family, genus, class, or even species, and so are often used as chemotaxonomic markers. Several studies have been carried out on the protein quality and amino acid profiles of marine molluscs from different parts of the world. But isolation and purification of sterol compounds from marine organism are too difficult until the introduction of refined techniques such as HPLC, GLC, capillary GC/MS, etc. So, many of the sterols were inseparable and thus not detected at the earlier period. In recent years, isolation and characterization of sterols from different species of molluscs were studied by many researches (Sima & Vetvicka, 2011; Rahman, 2012; 2014; Garcia, & Monzote, 2014).

Estrogens and their derivatives isolated from marine mollusc have been shown to exert a hypocholesterolemic effect (Teshima et al., 1993). 7- Cholestenol and 24-methylenecholesterol from molluscs were found to significantly decrease the cholesterol level in both serum and liver of

rat (Teshima, 1991; Kanazawa, 2001). The higher levels of sterols (especially cholesterol) with highly unsaturated fatty acids are important components of biological membranes (Le Nechet et al., 2007). Further, they are the mediators in the biosynthesis of sex hormones (ecdysteroids) (Teshima, 1991). Higher level sterols have importance in reproductive biochemistry (Allen, 2000). Kaur et al. (2011) reported that stigmasterol has various pharmacological properties, including antiosteoarthritic, hypo-glycemic, antimutagenic, antioxidant, and anti-inflammatory activity. Stigmasterol isolated from *A. indica* shows chemo preventive activity against skin cancer (Ali et al., 2015). Sterols are extensively used in the treatment of AIDS (Ubaida-Mohien et al., 2017). Moreover, sterols are used in the prevention of coronary heart disease (Frye & Leonard, 1999). They are used as antifungal as well as anti-obesity agents (Bhatti & Khera, 2012). Sterols are key constituents of cell membranes and are essential for their stability, cell growth and proliferation. Some of them are used as an important source of raw materials for the production of steroids of the androstane, pregnane and estrane series (Sedlaczek, 1988; Garcia et al., 2015). Sterols advocate their dietary inclusion as an important strategy in prevention and treatment of cancer (Woyengo et al., 2009). They have also used for the treatment of some forms of breast and prostate cancer (Diaz-Chico et al., 2007; Siemes et al., 2010). Variety of steroids is widely used as anti-inflammatory, progestational, anabolic, immunosuppressive, diuretic and contraceptive agents (Ahmad et al., 1992; Mahato & Majumdar, 1993; Ko et al., 2000; Tuba et al., 2000; Bhatti & Khera, 2012). Sterols are widely used as replacement agents in the treatment of adrenal insufficiencies (Hohnston, 1987; Garcia et al.,

2015). Recently, Arthan et al. (2002) reported a steroidal glycoside which exhibits anti-viral activity on herpes virus type 1.

Cholesterol was the only sterol recorded in the egg mass of 17 aquatic molluscs collected along the Illawarra Coast, NSW, Australia (Benkendorff et al., 2005). Antimicrobial activity of ergocalciferol and cholesterol was studied by Galbraith et al. (1971). Kanazawa (2001) studied the nutritive value, composition and metabolism of sterols in different classes of marine molluscs. Kawashima et al. (2011) studied the differences in sterol composition of gonads of male and female lottiid limpets, *N. concinna* and *N. fuscoviridis* from Northeastern Japan. Sexual differences affect the sterol composition of male and female intertidal gastropods, *C. grata* and *C. toreuma* (Kawashima et al., 2008; Kawashima et al., 2013). According to Murphy et al. (2003), sterol composition in marine molluscs reflects the sterol profiling of phytoplankton, because molluscs consume a large variety of phytoplankton. According to Kawai et al. (2007) sterol content of two bivalves *C. soyoae* and *B. septemdierum* varies, due to feeding modes and metabolism of nutrients from their habitat. The anatomical distributions of sterols and the incorporation of dietary phytosterols into different organs of sea scallops *P. magellanicus* were studied by Napolitano et al. (1993). The sterol composition of the female gonads *P. maximus* reflected that of the diet; however, the cholesterol was maintained at stable levels independent of dietary supply (Soudant et al., 1996). Sterol composition of dark-grown *I. galbana* and its implication in the seed production of Pacific oyster, *C. gigas* were studied by Park et al. (2002). 13 different sterols were identified in muscle and viscera of the marine bivalve *M. zyonoensis* from coastal

waters of Hokkaido, Northern Japan (Kawashima et al., 2007). Sterol composition and their seasonal variations were investigated in digestive gland and gonads of adductor muscle, *P. maximus* from Malaga (South Spain) (Pazos et al., 2003).

The world-wide demand for steroidal products is increasing day by day. Steroid drug production industry demands more than 2000 tons of natural sterols annually and there is an increasing need for cheap and available sterol containing raw material (Sharma, 2006). Most of the natural sterols are derived from plants and marine invertebrate phyla, including the mollusca (Simmons et al., 2005; Benkendorff, 2010; Mayer et al., 2010; Blunt et al., 2014). Moreover, a number of marine molluscs are used as traditional medicines in China, India, South African and Middle Eastern countries (Herbert et al., 2003; Prabhakar & Roy, 2009; Benkendorff, 2010; Benkendorff et al., 2015) as well as in homeopathic remedies (Benkendorff et al., 2015). Steroid drugs today play a major role in human welfare. Screening studies of steroids using computational techniques flourish their clinical applications (Kapetanovic, 2008). Computational (*in silico*) methods have been developed and widely applied to pharmacology for testing new compounds (Paarakh & Ravichandra, 2016). These *in silico* methods include databases, quantitative structure-activity relationships, similarity searching, pharmacophores, homology models and other molecular modeling, machine learning, data mining and data analysis tools using computer (Ekins et al., 2007). Different soft wares were used in drug discovery and development process, which helps a rapid gain of popularity, implementation and appreciation of novel compounds. PASS (Prediction of Activity Spectra for Substances)

is a software product designed as a tool for evaluating the general biological potential of virtual molecules, prior to their biological testing (Lagunin et al., 2013). This tool helps to estimate the probable profile of biological activity of a drug like organic compound whose molecular mass ranges from 50 to 1250Da (Lagunin et al., 2013). A number of studies have reported on the chemical constituents of molluscs, but limited works were published on the steroid composition of molluscs. Present study deals with the isolation and characterization of sterols from the soft tissue of gastropods and bivalves collected from various locations, and predicting the *in silico* biological activity using the PAAS software technology.

6.2 Material and methods

6.2.1 Species collection

Marine gastropods, *T. curta, M. trapa, T. telescopium* & *B. spinosa*, and bivalves *P. viridis* & *V. cyprinoids* were collected and stored in deep freezer for analysis of steroids (details of taxonomical description of the organisms and sampling sites are described in Chapter 2).

6.2.2 Characterization of steroids

Neutral lipids were extracted from the species using 2:1 $CHCl_3$ and CH_3OH (Harvey, 1994). Steroids were separated from crude lipid through column chromatography using 15% ethyl acetate in n-hexane (Barnes & Blackstock, 1973) and analysed using GC-MS. Detailed procedures were explained in Chapter 2.

6.2.3 *In silico* biological screening of steroids

In silico biological screening of steroids from molluscs was studied using PASS (Prediction of Activity Spectra of Substances) and data of cytotoxicity recovered from ChEMBLdb (version 19) (https://www.ebi.ac.uk/chembldb/) (Paarakh & Ravichandra, 2016). Pa (probability "to be active") values greater than 0.5 for cytotoxicity studies and Pa values greater than 0.7 for biological activity studies were reported in this work. The details about the software tool were already mentioned at Chapter 2.

6.3 Results and discussion

6.3.1 Steroids profiling

From the 15% ethyl acetate fraction, seven sterols and one sterone were identified by comparison of their spectra with those in National Institute of Standard Technology (NIST) library (version 2.2). The total ion chromatogram of steroid factions is depicted in Figure 6.1. Pictorial representation of steroid compositions of all the six species of mollusc is given in Figure 6.2. A total of eight different steroids were found in the six mollusc species examined, of which four were major constituents (> 10% in at least 1 species) and all the eight compounds were already reported. Although the presence of sterols was almost similar for four gastropods and two bivalves, the relative abundance of individual sterols present in each tissue were different. Table 6.1 shows the percentage abundance of steroid content in each tissue. The sterols obtained were cholesterol, ergosta-7-en-3-ol, cholesta-5,22-dien-3-ol, cholesta-8-en-3-ol, gamma ergosterol and stigmasterol. Cholesta-4-en-3-one is the only sterol

present in all the six species. The structures and mass fragmentation values are illustrated in Table 6.2.

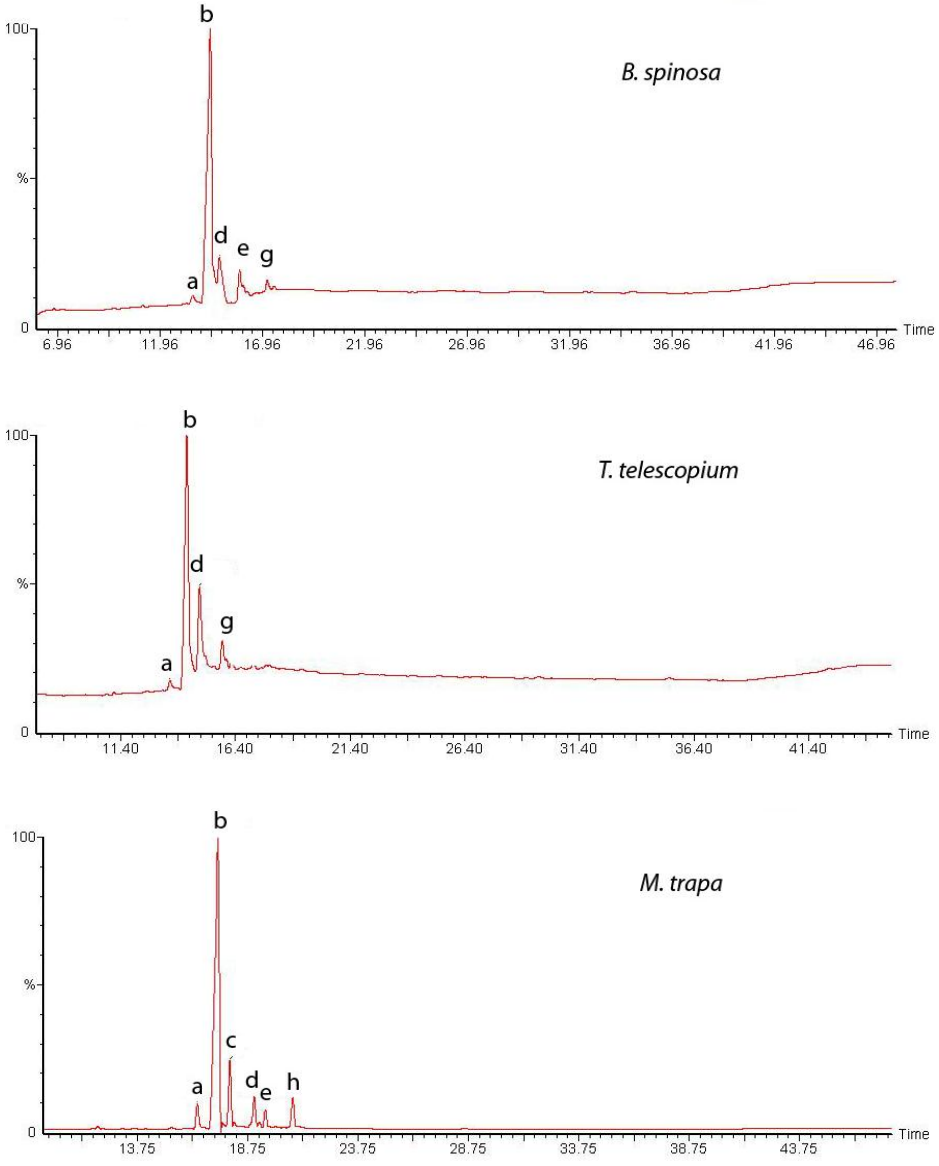
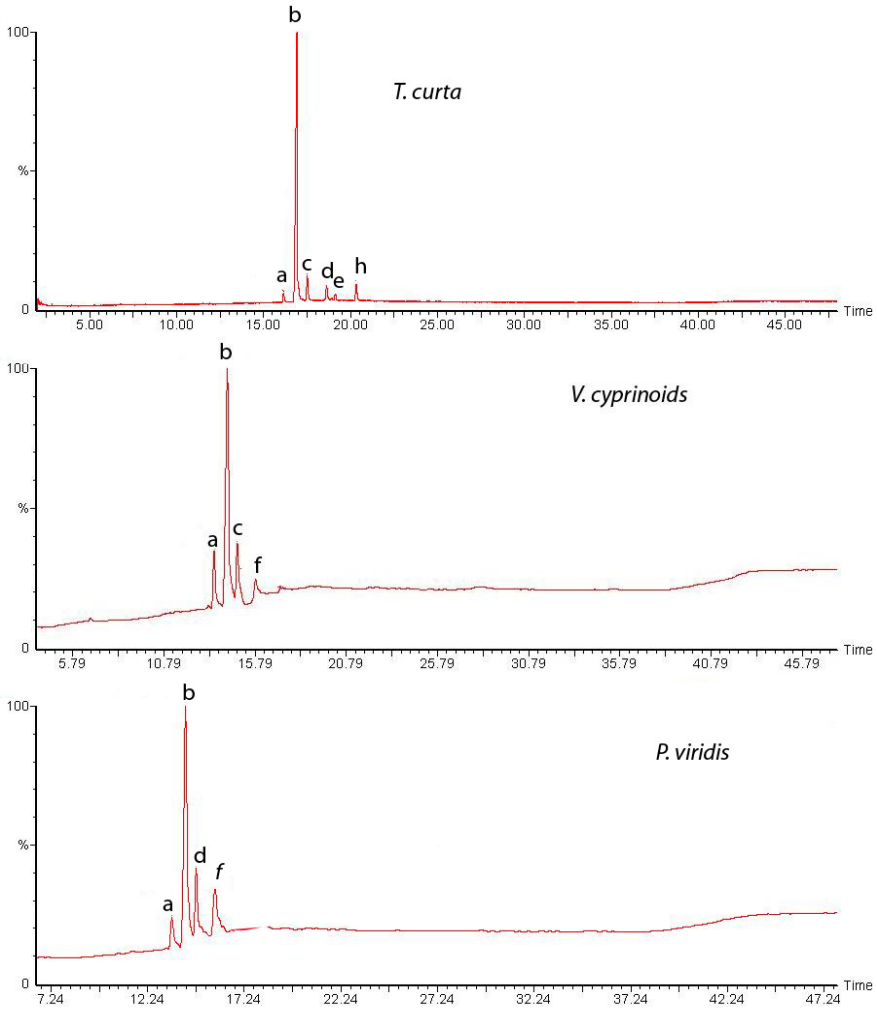


Figure 6.1 continued…



[a - cholesta-4-en-3-one; b – cholesterol; c – cholesta-5,22-dien-3-ol; d – cholesta-8-en-3-ol; e – ergosta-7-en-3-ol; f – gamma ergostrol; g – stigmasterol; h – gama sitosterol]

Figure 6.1: Total ion chromatogram of steroids from six species

Steroids of mollusc are generally complex mixtures that are composed of C27, C28 and C29 sterols. Cholesterol was the major sterol identified in all species and identified by comparing the mass spectrum, showed m/z with (relative intensity) values: 386(72), 368(36), 353(28), 301(65), 275(68), 255(30), 231(25), 213(35), 113(30), 55(86) and 43(100)

(Table 6.2). Percentage composition of cholesterol in *V. cyprinoids* was 69% and that of *B. spinosa* was 72%. Comparable cholesterol was observed in *P. viridis* (67%) and *T. curta* (67%). Cholesterol content of *M. trapa* (76%) and *T. telescopium* (79%) were analogous (Table 6.1). This study is in good agreement with the previous reports on sterols in mollusc (Bergmann 1949; Idler & Wiseman, 1972; Benkendorff et al., 2005; Orban et al., 2007). Cholesta-8-en-3-ol was the second most abundant sterol in all species and its ElMS m/z (relative intensity) values were 385(38), 367(16), 287(8), 245(12), 227(14), 161(18), 91(28), 81(46), 69(52), 55(64) and 43(100) (Table 6.2).

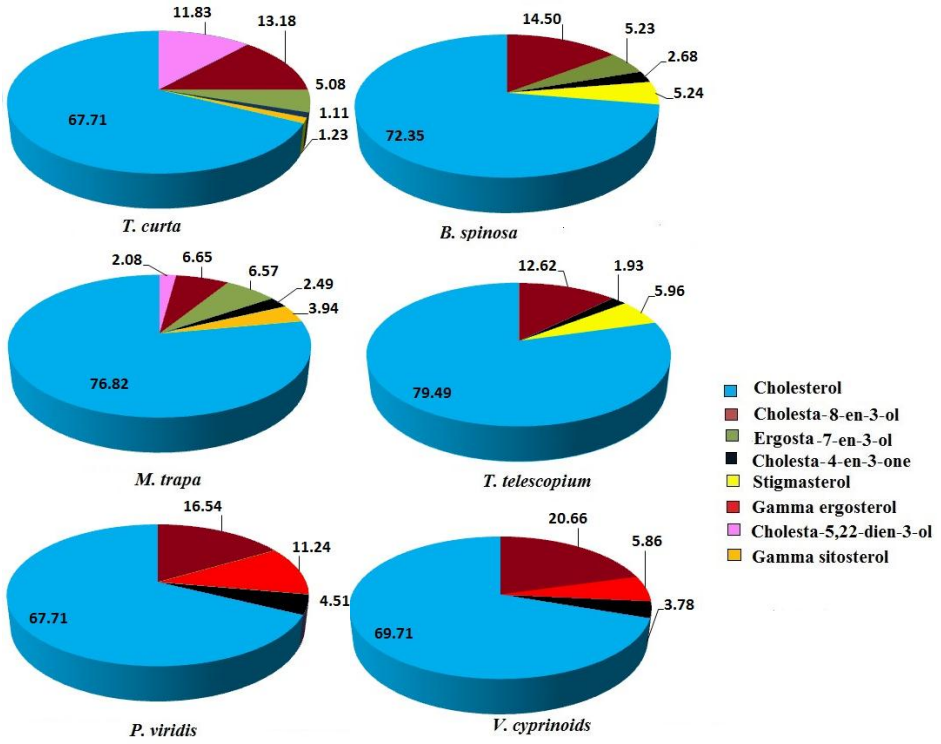
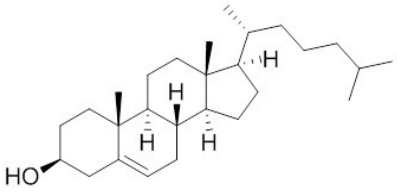
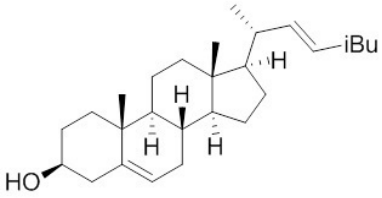
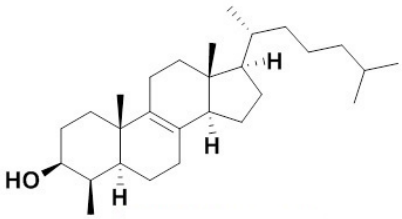
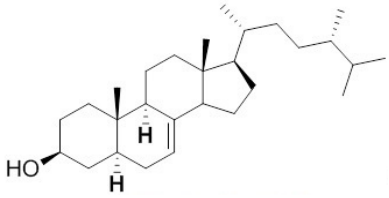


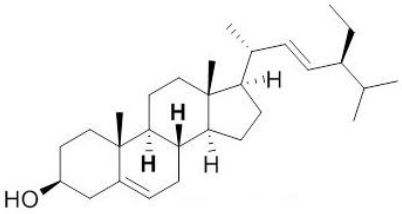
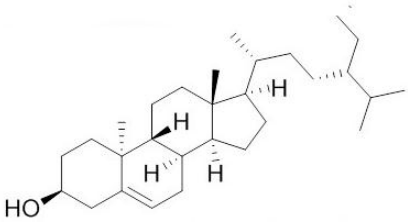
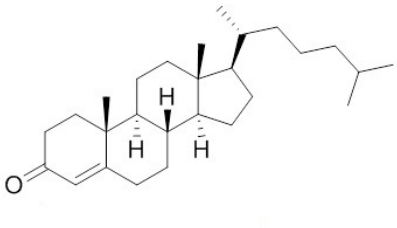
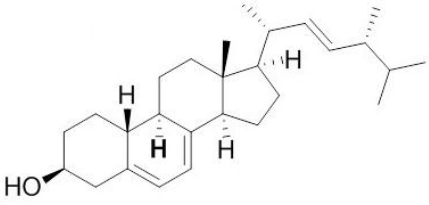
Figure 6.2: Pictorial representation of percentage composition of steroids identified in molluscs

Table 6.1: Relative abundance of steroids present in six species

Steroids	*M. trapa*	*T. curta*	*B. spinosa*	*T. telescopium*	*P. viridis*	*V. cyprinoids*
Cholesta-5,22-dien-3-ol	2.08 ± 0.16	11.83 ± 0.72				
Cholesta-4-en-3-one	2.49 ± 0.64	1.11 ± 0.14	2.68 ± 0.49	1.93 ± 0.64	4.51 ± 0.25	3.78 ± 0.29
Cholesta-8-en-3-ol	6.65 ± 1.15	13.18 ± 2.14	14.50 ± 0.86	12.62 ± 0.33	16.54 ± 1.16	20.66 ± 5.23
Cholesterol	76.82 ± 2.89	67.71 ± 6.16	72.35 ± 1.91	79.49 ± 3.46	67.71 ± 4.19	69.71 ± 7.21
Ergosta-7-en-3-ol	6.57 ± 1.92	5.08 ± 1.43	5.23 ± 0.78			
Gamma ergosterol					11.24 ± 2.16	5.86 ± 0.59
Stigmasterol			5.24 ± 0.67	5.96 ± 0.97		
Gamma sitosterol	3.94 ± 0.69	1.23 ± 0.59				

Table 6.2: Structure and major fragmentation of identified steroids

SL No.	Sterols	Major m/z
1	 Cholesterol	386, 368, 353, 301, 275, 255, 231, 213, 113, 55, 43
2	 Cholesta-5,22-dien-3-ol	384, 366, 351, 300, 285, 271, 255, 213, 55
3	 Cholesta-8-en-3-ol	385, 367, 287, 245, 227, 91, 81, 69, 55
4	 Ergosta-7-en-3-ol	398, 384, 351, 300, 271, 255, 229, 213, 159, 69

5	 Stigmasterol	412, 364, 271, 255, 213, 199, 159, 145, 133, 105, 91, 83, 69, 55. 41
6	 Gamma sitosterol	414, 396, 381, 329, 303, 273, 255, 231, 213, 145, 55, 43
7	 Cholesta-4-en-3-one	384, 369, 342, 299, 261, 229, 187, 124
8	 Gamma ergosterol	396, 386, 382, 315, 271, 213, 145, 107, 81, 55, 41

Percentage abundance of cholesta-8-en-3-ol in *P. viridis* was 16.54% and those of *V. cyprinoids* and *M. trapa* were 20.65% and 6.65% respectively (Table 6.1). Cholesta-8-en-3-ol content in *B. spinosa* (14.50%),

T. telescopium (12.62%) and *T. curta* (13.04%) were analogues. Gamma ergosterol was only present in *P. viridis* (5.86%) and *V. cyprinoids* (11.24%) and its ElMS m/z (relative intensity) values were: 396(85), 386 (30), 382(55), 315 (48), 271 (56), 213(45), 145(62), 107(68), 81(59), 55(68) and 41(100) (Table 6.2). Comparable ergosta-7-en-3-ol was present in *T. curta* (5.08%), *M. trapa* (6.57%) and *B. spinosa* (5.23%) and its ElMS m/z (rel int) values were 398(23), 384(18), 351(25), 300(20), 271(28), 255(32), 229(15), 213(21), 159(65) and 69(100). Cholesta-5,22-dien-3-ol content in *T. curta* (11.83%) was high compared to *M. trapa* (2.08%) and its ElMS m/z (rel int) values were 384(32), 366(12), 351(13), 300(38), 285(22), 271(32), 255(55), 213(30) and 55(100). Comparable stigma sterol was present in *B. spinosa* (5.24%) and *T. telescopium* (5.96%) and corresponding ElMS m/z (rel int) values were 412(20), 364(3), 255(16), 213(9), 199(8), 159(25), 145(29), 133(26), 105(32) 91(34), 83(64), 69(52), 55(100) and 41(39) (Table 6.2). Gamma sitosterol was present only in *T. curta* and *M. trapa*, and its relative abundance was 1.23% and 3.94% respectively (Table 6.1). ElMS m/z (rel int) of sitosterol was 414(42), 396(20), 381(15), 329(35), 303(31), 273(25), 255(28), 231(30), 213(38), 145(62), 55(65) and 43(100) (Table 6.2). Land snail, *C. nemoralis*, contains C29 sterols such as gamma sitosterol while stigmasterol has been detected only in a trace amount (Van der Horst & Voogt, 1972). Trace amount of sterone, cholesta-4-en-3-one was identified in all six species . Cholesta-4-en-3-one present in *M. trapa, T. curta, B. spinosa, T. telescopium, P. viridis* and *V. cyprinoids* were 2.49, 1.11, 2.68, 1.93, 4.51 and 3.78 respectively (Figure 6.2). ElMS m/z (rel int) values were 384(30), 369(5), 342(10), 299(8), 261(35), 229(42), 187(25) and 124(100) (Table 6.2).

General mass fragmentation pattern of sterol molecule is shown in Figure 6.3. Presence of unsaturation in the side chain of sterols can be deduced from the peak at m/z 257, which may be generated from the parent ion after the removal of hydrogen. Analogues peak observed at m/z 271 also indicates the loss of side chain along with transfer of hydrogen atom (Figure 6.4) (Wyllie & Djerassi, 1968). Sterols with saturated side chains having peak at m/z 217 were also inferred from the mass fragmentation pattern (Figure 6.5) (Sklarz, 2013). Mass fragmentation peaks at m/z at 275 and 301 represent different bond cleavages in the sterol skeleton (Figure 6.3). Peak at m/z 213 on cholesterol, cholesta-5,22-dien-3-ol, ergosta-7-en-3-ol, stigmasterol, gammasitosterol and gamma ergosterol indicates the presence of unsaturation in the ring system (Wyllie & Djerassi, 1968). The m/z at 42 and 342 in cholesta-4-en-3-one results from the ring fragmentation as shown in the figure 6.6. The m/z at 42 observed in sterols might be resulting from the ring fragmentation as shown in the figure 6.7.

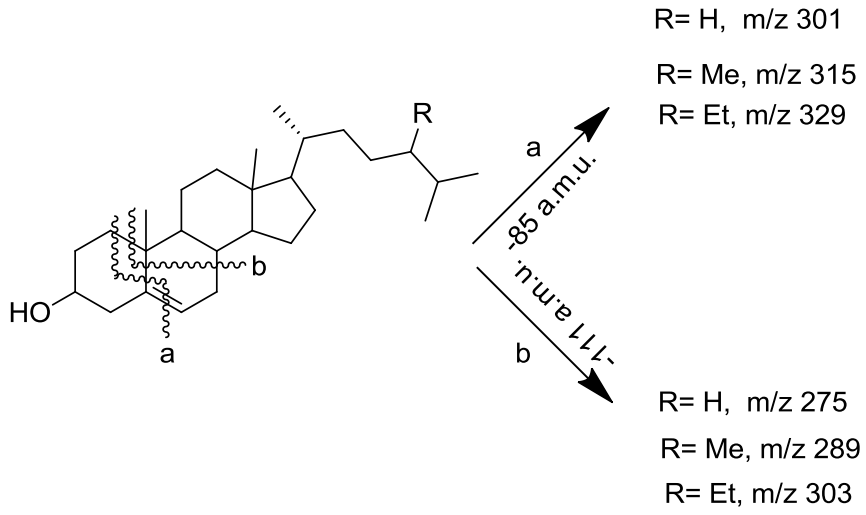


Figure 6.3: General mass fragmentation of sterol molecule ring cleavage.

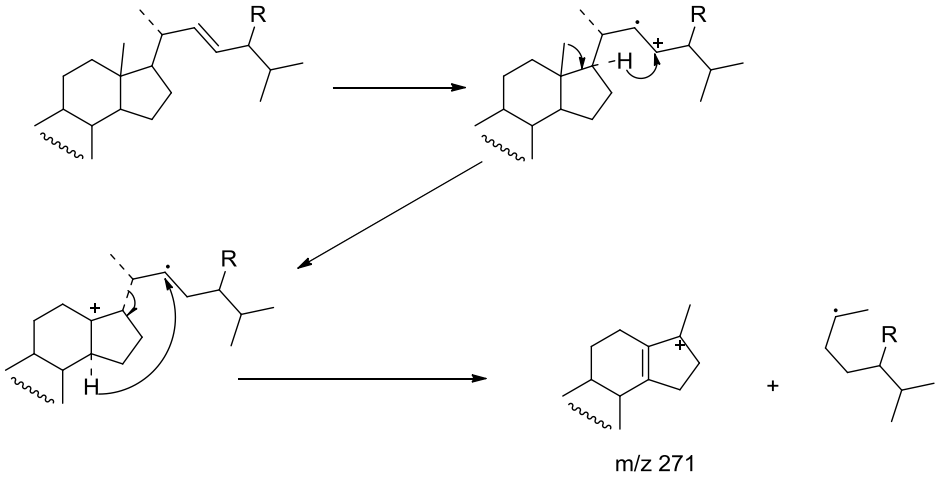


Figure 6.4: Mass fragmentation of unsaturated side chain of free sterol molecule producing m/z 271.

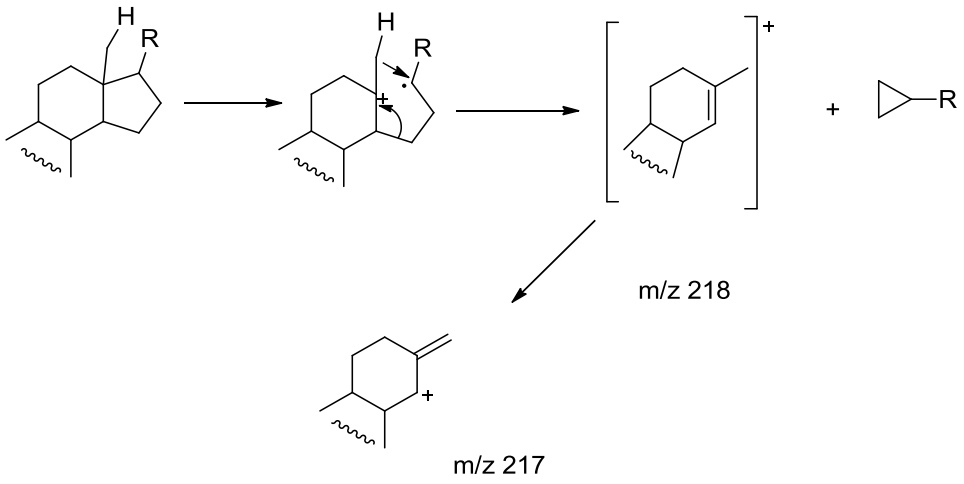


Figure 6.5: Mass fragmentation of saturated side chain of free sterol molecule producing m/z 217.

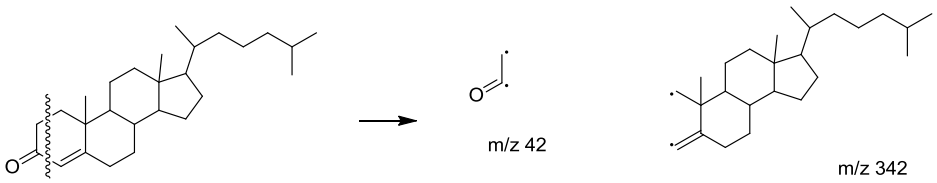
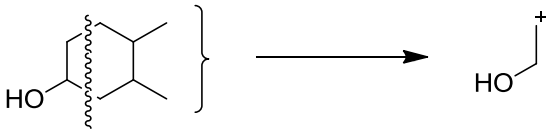


Figure 6.6: Mass fragmentation of sterone producing m/z 342.



m/z 43

Figure 6.7: Mass fragmentation of sterols producing m/z at 43

Sterol composition of gastropods collected from Illawarra Coast, Australia shows close resemblance with the present study (Benkendorff et al., 2005). According to Bergmann (1949), cholesterol is the only sterol occurring in cephalopods and gastropods. Moreover, numerous other sterols were detected in the gastropod spawn but in such small quantities that most remain unidentified. Cholesterol was the major sterol identified in the soft tissues of bivalves, *M. balthica, M. edulis, C. glaucum*, and *M. arenaria* collected from the Inner Puck Bay (Baltic Sea) (Jarzebski, 1985). Presence of C27 and C28 steroids in *M. edulis* has been reported by Reis-Henriques et al. (1990), of which cholesterol dominates. The gastropod *A. montereyensis*, order Nudibranchia, contains a complex mixture of sterols with cholesterol (60%) predominates (Idler et al., 1978). A preliminary investigation of the sterols in the squid *lllex* and the octopus *O. vuloaris* shows predominance of cholesterol in both species (Idler et al., 1976). Similar variety of steroids has been detected in bivalves, of which

cholesterol was the most abundant sterol with percentage content approximately 67% of total sterol. Comparable data have been obtained for the majority of other reported bivalve species (Goad, 1978). Steroid composition of bivalves such as *M. balthica*, *M. irus*, *M. incongrua* and *M. contabulata* also shows close resemblance with the present study (Jarzebski & Wenne, 1990).

Cholesta-5,22-dien-3-ol sterol is richly present in fresh water dinoflagellates (Robinson et al., 1987). Small amount of Cholesta-5,22-dien-3-ol was reported in *D. entale* obtained from the Bay of Fundy (Idler & Wiseman, 1972; Chung et al., 1998). Cholesta-5,22-dien-3-ol was the second most abundant sterol reported in muscle samples of *N. concinna* and *N. fuscoviridis* (Kawashima et al., 2011). Cholesta-4-en-3-one compound is a key intermediate in steroid chemistry, which is known as a cholesterol derivative occurring in both plant and animal tissues (Parish et al., 1991; Kendel et al., 2015). It may be derived from the biosynthesis or the autoxidation of cholesterol. Cholesta-4-en-3-one, known as an intestinal catabolite of cholesterol, has an anti-obesity effect on animals (Suzuki et al., 1998; Kendel et al., 2015). Reported data on sterol content in molluscs shows, stigmasterol and sitosterols were slightly present in both gastropods and cephalopods (Goad, 1981).

In the present study, comparable sterol content was observed in bivalve species such as *P. viridis* and *V. cyprinoids*. Sterol composition of crustaceans and molluscs are mixture of C28 and C29 sterols, of which cholesterol was found as major sterol (Kanazawa, 2001). A mixture of

sterols with cholastane (C27), ergostane (C28) and stigmastane (C29) nucleus was observed in the present study. Variation in sterol composition was detected in gastropod species. Sterol contents in the soft tissue of *T. curta* and *M. trapa* were analogues, while those of *B. spinosa* and *T. telescopium* were different. The difference in sterol composition of gastropod species may be linked either to the ability of synthesizing sterols or to their diet (Morais et al., 2003). Generally, several gastropod species have been shown to possess the ability for de novo synthesis of cholesterol and have a limited capacity for biosynthesis of remaining sterols (Idler et al., 1978). The presence of different phytosterols in molluscs, reflect the variety of phytoplankton food sources and may also be regarded as an indication of the complex metabolic transformations undergone by exogenous sterols after ingestion (Ozogul et al., 2015). Cholesta-5,22-dien-3-ol is significantly present in *T. curta* and *M. trapa*. From the studies of Idler et al. (1978), cholesta-5,22-dien-3-ol is generally abundant in many red algae.

In earlier days, the knowledge of origin of mollusc's sterols is scanty, but recently researchers give a reliable idea about the source of sterols in molluscs. Khan & Goad (1983) and Teshima et al. (1985) have mentioned that, generally sterols in molluscs were formed by three processes(1) de novo synthesis from lover molecule, (2) the accumulation of dietary sterols, and (3) the inter conversion of dietary sterols. Cholesta-5,22- dien-3-ol, cholesta-8-en-3-ol, ergosterol were significantly present in bivalves such as *D. Patagonia* and *D. varialilis* (Voogt, 1975). Bivalve obtained its nutrition from micro-organisms, bacteria, diatoms and planktons containing variety of sterols, of which cholesterol dominated. These data suggest the

possibility of accumulation of dietary sterols by bivalves (Soudant et al., 2000). Also, several reports have shown that some bivalves have the ability of de novo synthesis of alkylated sterols and others have the capability of dealkylating some high molecular sterol to cholesterol (Trider & Castell, 1980; Soudant et al., 2000). Some bivalve mussels synthesise sterols with incorporation of acetate (Voogt, 1975; Napolitano et al., 1993). Voogt (1975) reported that, incorporation of acetate into sterol fraction was not detected in bivalve such as *O. edulis*, *M. arenaria* and *C. islandica*. Generally bivalves do not have the ability to synthesise or bio convert sterols from lower molecules such as mevalonate and acetate (Patterson, 1991). This implies that a dietary supply of sterol is necessary for bivalve growth (Voogt, 1975; Holden & Patterson, 1991; Napolitano et al., 1993; Soudant et al., 2000). But gastropods have the ability of de novo synthesis of sterols with the incorporation of mevalonate and acetate (Voogt, 2014). From the previous studies, it is clear that gastropods have the ability of de-alkylating some phytosterols to cholesterol (Kanazawa, 2001). In the present study cholesta-5,22-dien-3-ol was present only in gastropods *T. curta*, and *M. trapa* and ergosta-7-en-3-ol was present in *T. curta*, *B. spinosa* and *M. trapa*. From the previous reports of Teshima et al. (1982), gastropods contained cholesta-5,7-dienol, cholesta-5,22-dienol, whereas the pelecypods consist of 24-methylcholesta-5,7,22-trienol and/or cholesta-5,7-dienol. This show to the dietary habits and abilities for modification of dietary sterols in gastropods.

6.3.2 *In silico* biological screening

Results of CLC-Pred (Cell Line Cytotoxicity Predictor) for *in silico* prediction of cytotoxic effect of sterols obtained from different molluscs against cancer cell lines based on their corresponding structural formula is expressed in Table 6.3. Five sterols, cholesta-5,22-diene-3-ol, cholesta-8-en-3-ol, ergosta-7-en-3-ol, stigma sterol and gamma ergosterol were selected for *in silico* studies according to the percentage availability in molluscs. The results emphasised the potential of these sterols against adenocarcinoma, gastric epithelial carcinoma, colon carcinoma, breast carcinoma and lung carcinoma cells. Probability to be active (Pa) value, 0.76 against stomach adenocarcinoma cells was obtained for stigmasterol. This was followed by 0.72 for cholesterol, 0.68 for cholesta-8-en-3-ol, 0.57 for cholesta-5, 22-dien-3-ol and 0.52 for ergosta-7-en-3-ol. Gamma ergo sterol is inactive against stomach adenocarcinoma cells. Another significant activity observed was against colon carcinoma cells and it was shown by all sterols except ergosta-7-en-3-ol and gamma ergo sterol. Gamma ergosterol exhibits activity against lung carcinoma cells and breast carcinoma cells. Cholesterol, ergosta-7-en-3-ol and stigma sterol showed significant activity against gastric epithelial carcinoma cells, affecting the stomach whereas, activity of other three sterols were insignificant. All sterols shows activity against breast carcinoma cells and the corresponding Pa values ranging from 0.58 to 0.72. Moreover, all sterols exhibited activity against lung carcinoma cells except ergosta-7-en-3-ol.

Table 6.3: Cancer cell line prediction of sterols using PASS software

Activity (Pa values) / Sterols	Stomach adenocarcinoma cells	Lung carcinoma cells	Gastric epithelial carcinoma cells	Colon carcinoma cells	Breast carcinoma cells
Cholesta-5,22-dien-3-ol	0.57	0.67	–	0.54	0.70
Cholesta-8-en-3-ol	0.68	0.62	–	0.62	0.64
Ergosta-7-en-3-ol	0.52	–	0.56	–	0.58
Stigmasterol	0.76	0.64	0.56	0.54	0.68
Gamma ergosterol	–	0.56	–	–	0.61

In silico predictions showed that the identified steroids exhibited remarkable biological activity. Steroids in this study mainly regarded as adenomatous polyposis, hypolipidemic agents, respiratory analeptic, antinfertility in females, antieczematic, antipruritic, dermatologic and prostate disorders treatment (Table 6.4). The Pa values ranging from 0.8 to 0.9 are expected to have high activity and values from 0.7 to 0.79 are considered unlikely to cover some positive biological activity. Pa values < 0.7 reflect certain biological activity. All the seven sterols mainly show anti pruritic, anti-eczematic, anti-infertility, respiratory analeptic, adenomatous polyposis treatment. The only one sterone shows maximum respiratory analeptic and anti-pruritic activity. Present study revealed that cholesta-5,22-dien-3-ol and gamma ergo sterol exhibited maximum activity for ant infertility in females, prostate disorders treatment, ovulation inhibitor and testosterone 17 beta- dehydrogenase. Stigma sterol, gamma sitosterol and gamma ergo sterol displayed activity against dermatological diseases.

Table 6.4: Biological activity prediction of sterols using PASS software

Sl.No.	Activity	Cholesta-8-en-3-ol	Cholesta-4-en-3-one	Stigma sterol	Cholesta 5,22-dien-3-ol	Gamma ergo sterol	Gamma sitosterol	Ergosta-7-en-3-ol
1	Adenomatous polyposis treatment	++	+	+	−	+	+	++
2	Hypolipemic	++	−	+++	++	+	+++	++
3	Respiratory analeptic	++	++	−	−	−	−	++
4	Antiinfertility, female	++	+	−	++	+	−	−
5	Antieczematic	++	+	++	++	++	+	++
6	Antipruritic	++	++	+	−	+	++	+
7	Dermatologic	+	+	++	++	++	+++	−
8	Bone diseases treatment	+	+	+	+	++	+	−
9	Antiosteoporotic	+	+	+	−	++	−	−
10	Analeptic	+	+	−	+	−	+	−
11	Immunosuppressant	+	+	+	+	−	+	−
12	Prostate disorders treatment	+	+	+	+++	−	−	−
13	Proliferative diseases treatment	−	+	+	−	+	−	−
14	Chemopreventive	−	−	++	+	++	+	++
15	Antipsoriatic	−	−	+	−	++	−	−
16	Testosterone 17 beta− dehydrogenase	−	−	−	+++	+++	+	++
17	Anti parkinsons agent	+	+	−	−	+	+	−
18	Ovulation inhibitor	+	−	−	+	+	−	−

'+++' – Pa ≥ 0.9; '++' – Pa ≥ 0.8; '+' – Pa ≥ 0.7; '−' < 0.6

There are sufficient confirmations from *in vitro* and *in vivo* studies to indicate that all steroids identified from six species of molluscs are therapeutically significant. Awad et al. (2003), Choi et al. (2003) and Varghese et al. (2017) reported that, cholesterol and β-sitosterol exhibit apoptosis agonist activity in cancer prevention. According to Lu et al. (2011), intracellular cholesterol accumulation induces apoptosis of pancreatic cells. Desai & Naik (2008) reported that sterols with cholastane and ergostane nucleus show anti-fungal activities. Cholesterol and its oxygenated derivatives (cholesterones) were used as acceleration of spermatogenesis in males, and increased ovarian atresia in females (Ryan et al., 2009). *In vivo* studies on stigma sterol, sitosterol and their oxygenated sterones prove apoptosis agonist activity in breast cancer prevention (Newill et al., 2007). *In vitro* studies on sitosterol and stigmasterol prove the efficacy of lowering plasma LDLC concentrations (Fernandez & Vega-Lopez, 2005) and suppress pro-inflammatory and matrix degradation mediators involved in osteoarthritis-induced cartilage degradation (Gabay et al., 2010; Thomas et al., 2016). Also, stigmasterol possesses activity on thyroid inhibitory, antiperoxidative and hypoglycemic effects (Panda et al., 2009). There is no proven report for supporting cholesta-5, 22-diene-3-ol, cholesta-8-en-3-ol and ergosta-7-en-3-ol, that these are apoptosis agonist but the PASS study conducted supports the findings of O' Callaghan et al. (2014).

6.4 Summary

This study has succeeded in isolating seven sterols namely cholesta-5,22-diene-3-ol, cholesterol, cholesta-8-en-3-ol, ergosta-7-en-3-ol, gamma ergosterol, stigmasterol and gamma sitosterol and one sterone

(cholesta-4-en-3-one) for the first time from gastropods viz., *T. curta, M. trapa, B. spinosa* and *T. telescopium*. Cholesterol, cholesta-8-en-3-ol, gamma ergosterol and cholesta-4-en-3-on were the four steroids isolated from the bivalves, *P. viridis* and *V. cyprinoids*. The steroids were characterized by using GC MS and identified from NIST library report. Cholesterol was the most dominant sterol and gamma sitosterol was the least abundant sterol observed in all the species. Cholesta-4-en-3-one was the single sterone, which is very low in quantity. Steroids present in gastropods and bivalves, were commonly observed in diatoms and dianoflagellates. Phytosterols are the major sterols in molluscs, since they are filter feeders. Sterols present in molluscs are either accumulated from dietary sterols or de novo biosynthesis with the incorporation of mevolonate and acetate.

From the PASS prediction, it was found that all the sterols exhibited activity against breast carcinoma cells. Cholesta-5,22-dien-3-ol, cholesta-8-en-3-ol, ergosta-7-en-3-ol and stigmasterol show maximum activity against stomach adenocarcinoma cells, lung carcinoma cells and colon carcinoma cells (Pa > 0.55). Only ergosta-7-en-3-ol and stigmasterol shows comparable activity against gastric epithelial carcinoma cells. *In silico* prediction shows that, all steroids exhibit activity against adenomatous polyposis, hypolipidemic agents, respiratory analeptic, ant infertility in females, antieczematic, antipruritic, dermatologic and prostate disorders treatment with Pa values greater than 0.7. Present study discovered that cholesta-4-en-3-one shows maximum activity for respiratory analeptic and anti-pruritic, while C29 sterols such as stigma sterol, gamma sitosterol and gamma ergo sterol displayed activity against

dermatological diseases. Also cholesta-5,22-dien-3-ol and gamma ergo sterol showed maximum activity for prostate disorders treatment, anti-infertility in females, testosterone 17 beta- dehydrogenase and ovulation inhibitor. From the *in silico* studies of sterols such as cholesta-5,22-dien-3-ol, cholesterol, cholesta-8-en-3-ol, ergosta-7-en-3-ol, stigma sterol and gamma ergosterol suggest these sterols as a drug candidate in cancer therapy.

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Chapter 7

CHARACTERIZATION OF FATTY ACIDS AND ITS BIOLOGICAL ACTIVITY STUDIES

Contents

7.1 Introduction

Marine molluscs are rich source of biologically active compounds (Ekin et al., 2008; Ekin & Bashan, 2010). In addition to the pharmacological value, they are excellent sources of nutritionally important lipid compounds (Ozogul et al., 2008; Latyshev et al., 2009; Usydus et al., 2011; Pereira et al., 2013). Lipids are the esters of glycerol and fatty acids. They are one of the key nutrients along with carbohydrates and proteins in human diet. Lipids stored in the cell membrane as oil droplets acts as energy reservoir. Fatty acids are the principal components of lipids in cell membrane and can vary between organisms (Willis et al., 1998). Also their properties show diversities in terms of chain length, degree of unsaturation, geometry and position of the double bonds (Gutnikov, 1995).

Fatty acid is an organic compound having a hydrocarbon chain normally, C12 to C32 and a terminal carboxyl group. Though fatty acids constitute the main part of lipids in living organisms, even numbers of carbon atoms predominated due to their enzymatic biosynthesis from acetyl (C2) units of glucose (Sargent et al., 1987). They are broadly classified into saturated fatty acids (SFAs) and unsaturated fatty acids, based on their structure (Figure 7.1). SFAs are commonly straight chains with even carbon number, represented by the general formula $CH_3(CH_2)_n$ COOH (Table 7.1). Odd chain fatty acids were also coming under saturated fatty acids group. Branched-chain fatty acids (BCFAs) are normally saturated and the branch is a methyl-group, methoxy or a hydroxy substitution. They have usually either an iso or anteiso structure. Iso-methyl branched fatty acids have the branch point on the penultimate carbon, while, anteiso-methyl-branched fatty acids have the branch point on the ante-penultimate carbon atom. An unsaturated fatty acid contains one or more double bonds in the fatty acid chain. If the unsaturation resides between single set of carbon atoms, then the fatty acid is monounsaturated (MUFAs) (Table 7.2) and if it contains more than one double bond, it is known as polyunsaturated (PUFAs), (Table 7.3). Non-methylene interrupted fatty acids (NMIs) are another variety of polyunsaturated fatty acids, only observed in marine organisms. They are formed from SFA through the unusual biosynthetic pathways and possess double bonds with more than one methylene group between ethylenic bonds (Tanaka et al., 1999).

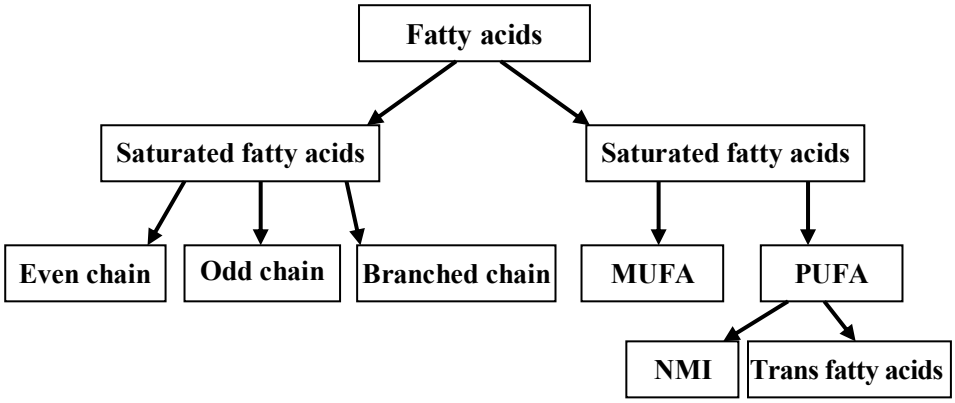


Figure 7.1: Classification of fatty acids in molluscs

Fatty acids are essential for life, due to their key role as a good source of energy, membrane constituents, as well as metabolic and signalling mediators (Pereira et al., 2013). Based on their role in animal body, researchers reported that fat content of diet should contain at least 25% of SFAs (Dannenberger et al., 2017). SFAs were obtained mainly from seven major plant crops: soybean, groundnut, cottonseed, rapeseed, palm, coconut and sunflower. In India, groundnut, sunflower, cotton seed and coconut are grown as oil crops (Chithra Som, 2012). Some unsaturated fatty acids cannot be synthesized from endogenous precursors by mammals. This reason directed to the description of essential and nonessential fatty acids (Ziboh, et al., 2002). The body can synthesize most of the fats it needs from the diet. However, linoleic (LA) and alpha-linolenic (ALA), cannot be synthesized in the body, known as essential fatty acids, and must be obtained from food. Most of the higher chain polyunsaturated fatty acids were formed by the oxidative desaturation and further elongation of LA and ALA.

Table 7.1: Common saturated fatty acids present in food

Common name	IUPAC name	Symbol
Butyric acid	Butanoic acid	C4:0
Caproic acid	Hexanoic acid	C6:0
Caprylic acid	Octanoic acid	C8:0
Capric acid	Decanoic acid	C10:0
Lauric acid	Dodecanoic acid	C12:0
Myristic acid	Tetradecanoic acid	C14:0
Palmitic acid	Hexadecanoic acid	C16:0
Stearic acid	Octadecanoic acid	C18:0
Arachidic acid	Icosanoic acid	C20:0
Behenic acid	Docosanoic acid	C22:0
Lignoceric acid	Tetracosanoic acid	C24:0

(Akoh & Min, 2008)

Table 7.2: Common monounsaturated fatty acids present in food

Common name	IUPAC name	Symbol
Caproleic acid	dec-9-enoic acid	C10:1
Lauroleic acid	(Z)-dodec-9-enoic acid	C12:1n3
Myristoleic acid	(Z)-tetradec-9-enoic acid	C14:1n5
Palmitoleic acid	(Z)-hexadec-9-enoic acid	C16:1n7
Oleic acid	(Z)-octadec-9-enoic acid	C18:1n9
Elaidic acid	(E)-octadec-9-enoic acid	*
Vaccenic acid	(E)-octadec-11-enoic acid	*
Gadoleic acid	(Z)-icos-9-enoic acid	C20:1n11
Erucic acid	(Z)-docos-13-enoic acid	C22:1n9
Brassidic acid	(E)-docos-13-enoic acid	*
Nervonic acid	(Z)-tetracos-15-enoic acid	C24:1n9

(*) is trans fatty acids; (Akoh & Min, 2008)

Table 7.3: Common polyunsaturated fatty acids present in food

Common name	IUPAC name	Symbol
Linoleic acid (LA)	(9Z,12Z)-octadeca-9,12-dienoic acid	C18:2n6
alpha-Linolenic acid (ALA)	(9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid	C18:3n3
gamma-Linolenic acid (GLA)	(6Z,9Z,12Z)-octadeca-6,9,12-trienoic acid	C18:3n6
Columbinic acid	(5E,9E,12E)-octadeca-5,9,12-trienoic acid	*
Stearidonic acid	(6Z,9Z,12Z,15Z)-octadeca-6,9,12,15-tetraenoic acid	C18:4n3
Mead acid	(5Z,8Z,11Z)-icosa-5,8,11-trienoic acid	C20:3n9
Dihomo-γ-linolenic acid (DGLA)	(8Z,11Z,14Z)-icosa-8,11,14-trienoic acid	C20:3n6
Arachidonic acid	(5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenoic acid	C20:4n6
Eicosapentaenoic acid (EPA)	(5Z,8Z,11Z,14Z,17Z)-icosa-5,8,11,14,17-pentaenoic acid	C20:5n3
Docosapentaenoic acid (DPA)	(7Z,10Z,13Z,16Z,19Z)-docosa-7,10,13,16,19-pentaenoic acid	C22:5n3
Docosahexaenoic acid (DHA)	(4Z,7Z,10Z,13Z,16Z,19Z)-docosa-4,7,10,13,16,19-hexaenoic acid	C22:6n3

(*) is trans fatty acids; (Akoh & Min, 2008)

Fatty acids, being an essential component in human diet are also important raw materials for the both cosmetic and pharmaceutical industry. Recent research and documents from the food and drug administration (FDA) and U.S. dietary guidelines advisory committee confirm the truth

about fatty acids - that all the three types of good fats (saturated fat, monounsaturated fat and polyunsaturated fat) should be consumed regularly, though research does indicate that it's healthiest to consume less saturated fat than the other two types (Jung et al., 2016; Zhao et al., 2016). Majority of naturally occurring unsaturated fatty acids exists in its *cis* form, while some food contains *trans* form also. It is formed by the process of hydrogenation (Ferlay et al., 2017). Diets with high amounts of *trans* fatty acids increase the risk of cardiovascular diseases and development of metabolic syndrome. So foods containing these fatty acids have been banned in major countries (King, 2017). PUFAs have importance in regulating membrane function, and are of major importance in the brain, retina, liver, kidney, adrenal glands and gonads (Das, 2006; Moya, 2017).

Fatty acids have pronounced hypocholesterolemic effect and act as carrier for vitamins such as A, D, E and K and promote their absorption (Margret, 2015). They lower LDL cholesterol, and reduce the risk of cardiovascular disease (Negele et al., 2015). MUFAs, especially palmitoleic acid (PA, 16:1n7) is considered to be a diatom marker and other SFAs are generally used as biomarkers (Alkanani et al., 2007). NMI fatty acids, most commonly present in marine molluscs and crustaceans are used for supporting the necessary fluidity of the cell membrane (Ackman & Hooper, 1973; Dernekbas et al., 2015). PUFAs have an important role in human health and nutrition, especially for cardiovascular diseases (Ferlay et al., 2017). Long chain n3 fatty acids specifically, eicosapentaenoic acid (EPA; 20:5n3) and docosahexaenoic acid (DHA; 22:6n3), play an important role in enhancing metabolic dysfunction, helping weight control in adults and

reducing the risk of inflammatory disease (Calder, 2012; Mingay et al., 2016). n3 Fatty acids are widely used against inflammatory diseases, migraine type headaches, joint rheumatism, certain types of cancers, high blood pressure, diabetes, certain types of allergies and eye retina development (Dernekbas et al., 2015; Ejike et al., 2017). Moreover, conjugated dienoic and trienoic acids exhibited activity against various cancerous cells (Shultz et al., 1992; Igarashi & Miyazawa, 2000; Ahmad et al., 2017). Reported data of conjugated linoleic acid and alpha linoleic acid show anti carcinogenic effects in animals (Cave, 1991; Hur et al., 2017). In vitro studies of n3 fatty acids, especially EPA and DHA show inhibition of the proliferation of human breast cancer cells (Durgam & Fernandes, 1997; Fasano et al., 2017), colon cancer cells (Shultz et al., 1992; Fan et al., 2017) and lung cancer cells (Schonberg & Krokan, 1995; Ren et al., 2017).

Consumption of molluscs and shellfish is considerably low, but the amount of fatty acid and the proportions of saturated, monounsaturated and polyunsaturated fatty acids in shellfish contribute to a healthy diet. Shellfish also contains significant amounts of "good" fats called n3 fatty acids and also provides high quality protein with all the dietary essential amino acids for maintenance and growth of the human body (King et al., 1990). Also, these are good sources of iron, zinc, copper, and vitamin B12 (Savvi et al., 2008). In marine bivalves, PUFAs and sterols are important biochemical constituents, used in several biochemical processes and give energy under critical nutritional conditions (Prato et al, 2010). Apart from their commercial value, molluscs are used as nutritional diet for humans and foodstuff for several marine crustaceans (Deshimaru et al., 1979;

Ekin & Bashan, 2010). Molluscan tissues contain major fraction of lipids, so the storage strategy and lipid composition of bivalves and molluscs have been studied (Voogt, 1983).

Fatty acids are important raw materials for the industry, because they are essential for human diet. Presently, bulk of these fatty acids is largely obtained from plant sources. Plants and animals are unable to meet the large demands of consumers and industries (Willis et al., 1998; Nigam, 1999). Aquatic organisms are looked upon as an alternative or additional source of fatty acids. For many years, researchers have keen interest in nutritional studies on seafoods such as fish, molluscs and crustaceans (Medale et al., 2003; Sirot et al., 2008). Nutritionists consider these products to be an important source of high-quality proteins, minerals, vitamin D and essential fatty acids. According to National Nutritional Health Programme (2001–2005), people consume fish at least twice a week, because it contains more n3 fatty acids. Lipids of marine origin are rich sources of n3 PUFAs and they provide 9.45 Kcal g^{-1} energy (Sellimi et al., 2017). Much research has been reported on the fatty acid profiling of gastropods and bivalves. Fatty acid composition varies with species (Sinanoglou et al., 2008). According to Sinanoglou et al. (2008), SFAs are predominant in two edible Mediterranean molluscs, *S. officinalis* and *T. sagittatus*, whereas PUFAs were predominated in *E. moschata*, edible molluscs collected from same area. The fatty acid profile of molluscs showed that the highest fatty acid of *C. obtusa* (sea snail) was MUFA, but *C. javanica* and *P. canaliculata* (freshwater snail) have the highest SFA (Purwaningsih et al., 2015). Structural analysis of the NMI fatty acid fraction isolated from lipids of the marine bivalve,

S. broughtoni gives mixture of n7 dienoic acids (Zhukova & Svetashev, 1986). Unsaturated fatty acids isolated from benthic spawn of aquatic molluscs shows activity against aquatic pathogenic bacteria relatively at low concentration (Benkendorff et al., 2005). The ability of fatty acids to interfere with bacterial growth and survival has been known for several decades (Kabara et al., 1977; Kabara, 1987; Ababouch et al., 1992; Bartsch et al., 2017).

Unsaturated FA were dominated in *P. lividus*, *H. forskali* and *Aplysia* sp., of which n6 fatty acids was higher than n3 fatty acids (Pereira et al., 2013). According to Barnathan (2009), the accurate determination of both NMI and PUFA has great importance to physiological and biochemical studies in marine molluscs. Biosynthesis of PUFA has been most extensively investigated in marine molluscs (Monroig et al., 2013). *M. galloprovincialis*, marine mollusc with the highest ecological and economic importance in the Black Sea ecosystem is an excellent source of n3 FA, especially EPA and DHA (Dernekbaş et al., 2015). Due to the filter feeding character of bivalves, fatty acid composition of bivalve molluscs varies with the type of symbiotic bacteria (Zhukova et al., 1992). EPA acids (20:5n3) were the predominant n3 FA and DHA (22:6n3), an important member fatty acid of marine organism, was unusually low in marine snail *T. coronatus* (Freije & Awadh, 2010). Benthic invertebrate egg masses are novel source of potential antimicrobial agents (Benkendorff et al., 2001). Antiviral activity of gastropods and bivalves were mostly reported against human viruses (Silvestri et al., 1995; Olicard et al., 2005a,b; Genova-Kalou et al., 2008; Defer et al., 2009a,b; Dang et al., 2011a,b). Recently antiviral activity

were reported in nine gastropods such as *H.laevigata*, *H. rubra*, *H. rufescens*, *L. littorea*, *B. corneum*, *T. gallina*, *R.venosa* and *B. undatum*, as well as in nine bivalve species viz., *M. mercenaria*, *M. arenaria*, *R. philippinarum*, *C. edule*, *M. galloprovincialis*, *C. grayanus*, *C. virginica*, *C. gigas* and *O. edulis* (Dang et al., 2015; Fasano et al., 2017). PUFAs, especially DHA and EPA show activity against numerous cancers including colorectal carcinoma (Baracos et al., 2004; Calviello et al., 2004; Serhan & Petasis; 2011), esophageal (Xue et al., 2007) and gastric cancers (Liu et al., 2015), hepatocellular carcinoma (Barber, 2002; Assisi et al., 2006), pancreatic cancer (Chalon et al., 2001), cholangiocarcinoma (Minami et al., 1997), breast (Xia et al., 1999; Hossain et al., 1999), ovarian (Hossain et al., 1999), prostate (Kyle et al., 1999; Akiyama et al., 2000) and bladder cancers (MacLean et al., 2006; Shivappa et al., 2017), neuroblastoma (Eslick & Talley, 2009) lung cancer (Serini et al., 2008; Yao et al., 2014), squamous cell carcinoma (SCC) (Arshad et al., 2014) and melanoma (Pompeia et al., 2000; Campos et al., 2002; D'Eliseo & Velotti, 2016).

The world-wide demand for fatty acid products is increasing day by day, both in food and pharmaceutical industry. Number of studies was reported on the lipid components of molluscs, but limited works were published on individual fatty acid composition of gastropods and bivalves. There are no work have been reported on *T. curta* and *M. trapa*. Present study deals with isolation and characterization of fatty acids from the soft tissue of four gastropods viz., *T. curta*, *M. trapa*, *B. spinosa* and *T. telescopium* and two bivalves such as *P. viridis* and *V. cyprinoids* collected from various locations. Moreover, both antimicrobial and

cytotoxic activity studies on PUFAs separated from *M. trapa* and *B. spinosa* were done.

7.2 Materials and methods

7.2.1 Species collection

Marine gastropods, *T. curta , M. trapa, T. telescopium, B. spinosa*, and bivalves *P. viridis, V. cyprinoids* were collected and stored in deep freezer for analysis of sterols (details of taxonomical description of the organisms and sampling sites were described in Chapter 2).

7.2.2 Characterization of fatty acids

Fatty acids were extracted from the species using 2:1 $CHCl_3$ and CH_3OH (Harvey, 1994). Extracted fatty acids were analysed as fatty acid methyl esters (FAME) in gas chromatography- mass spectrometry (GC-MS) (Perkin - Elmer Clarus 620 GC), with MS detector equipped with a non-polar HP ultra-double fused silica capillary column (30 m length, 0.32 μm internal diameter, 0.25 μm film thickness). Detailed procedures were explained in Chapter 2.

7.2.3 Biological activity of identified compounds

7.2.3.1 Anti-bacterial activity of fatty acid fractions

The crude PUFA fractions and a mixture of DPA and EPA separated from crude extracts were screened for antimicrobial activity via, Kirby-Bauer disc diffusion method using gram positive and gram negative bacteria (Bauer et al., 1966). Details about the analyses were already mentioned in Chapter 2.

7.2.3.2 *In vitro* antiproliferative effect of fatty acid extracts (MTT assay)

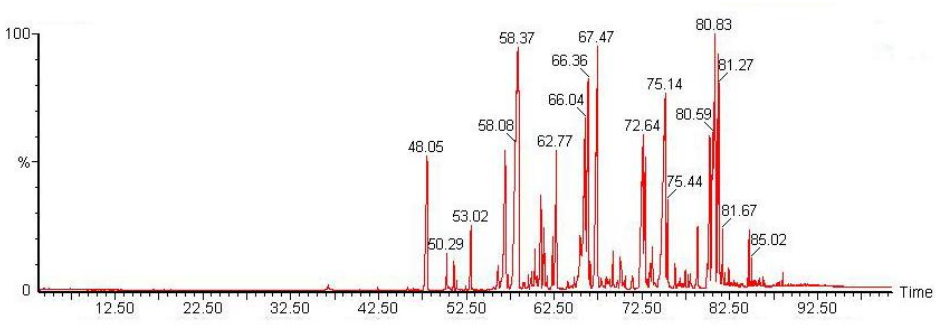
Human laryngeal carcinoma derived human epithelial type 2 cell lines were used for cytotoxic studies. Antiproliferative effect of fatty acid extracts were measured using MTT assay (Sylvester, 2011). MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5 dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The % difference in viability was determined by standard MTT assay after 24 hours of incubation. Detailed procedures were already mentioned in Chapter 2.

7.3 Results and discussion

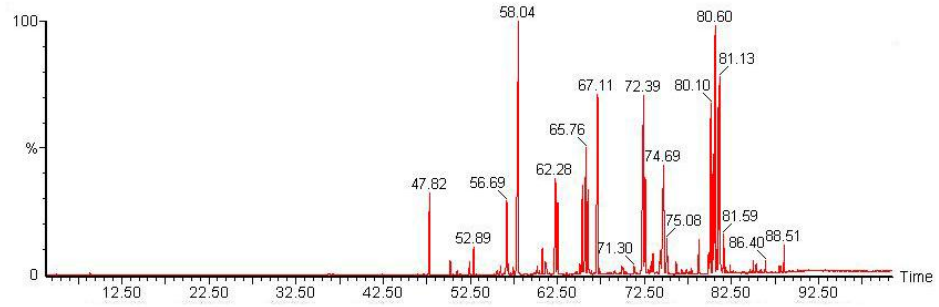
7.3.1 Characterization of fatty acids

Fatty acids separated from the total lipids, extracted from six species of mollusc consists of saturated fatty acids (SFAs), branched chain fatty acids (BCFAs), monounsaturated fatty acids (MUFAs) polyunsaturated fatty acids (PUFAs) and non-methylene interrupted fatty acids (NMI). Total ion chromatograms of fatty acids for each species are listed in Figure 7.2. Principal fatty acids observed in all species of molluscs were palmitic acid, stearic acid, oleic acid, docosadienoic acid, docosahexaenoic acid and docosapentaenoic acid (Table 7.4). This result showed a resemblance with the observations of Kochi (1975), Caers et al. (1999), Kawashima & Ohnishi (2003) and Zhukova (2014) on molluscs. Total SFAs of gastropods ranged from 15.41% to 38.54% and those of bivalves extended from 35.68% to 58.82%. In the present study, ΣSFA of gastropods ranged from 15.41% to 38.54% (Figure 7.3). These results are comparable with the total saturated fatty acids in *U. terminalis*

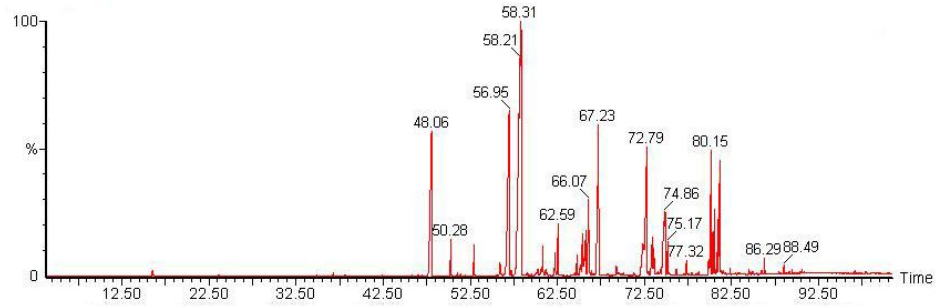
(32.13%) and in *P. littoralis* (30.21%) (Vernocchi et al., 2007). Moreover, total SFA content of *M. galloprovincialis* were approximately equal to 30% (Freites et al., 2002; Vernocchi et al., 2007).



B. spinosa



M. trapa



P. viridis

Figure 7.2 continued…

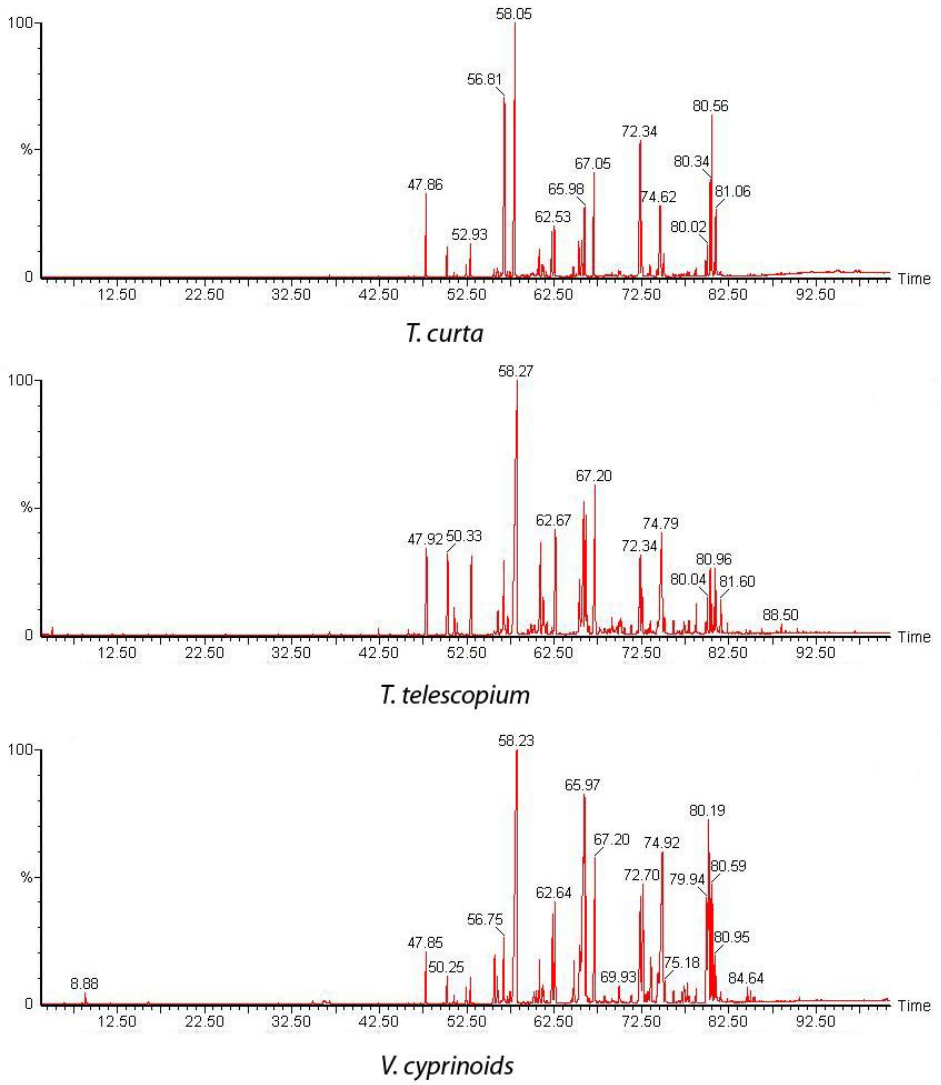
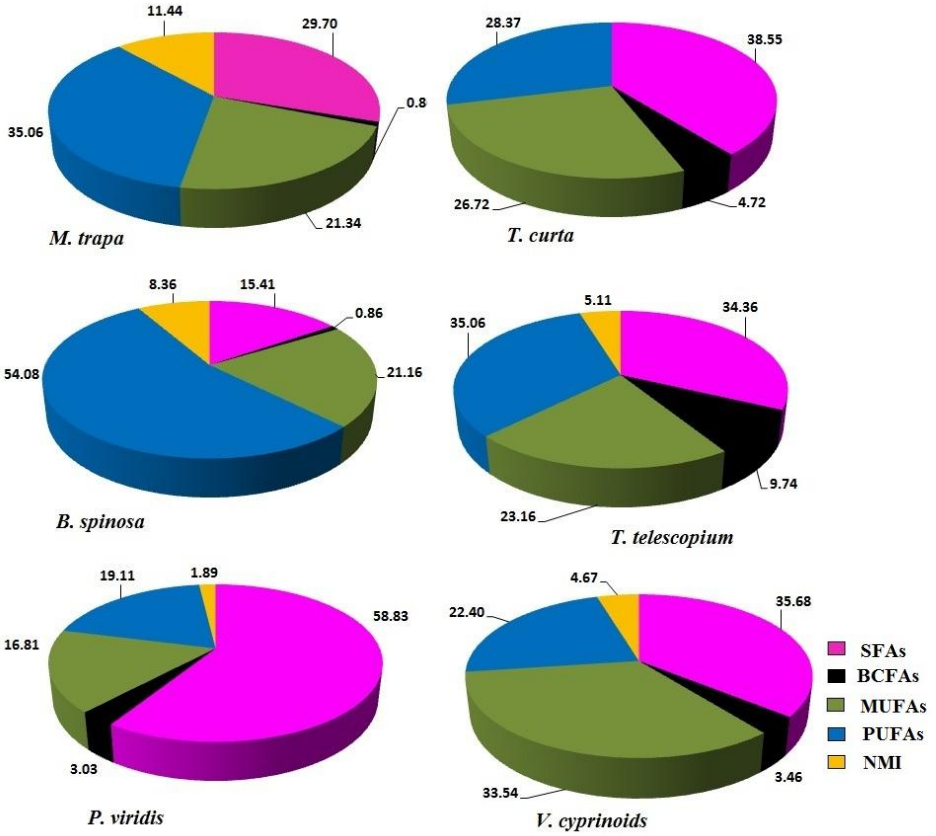


Figure 7.2: Total ion chromatogram of fatty acids

C16:0 and C18:0 were the major SFAs observed in all the six species. Considerable amount of C14:0 was observed in all species, of which *P. viridis* (10.61%) contains large quantity (Table 7.4). Equivalent, but low content of C14:0 was observed in *B. spinosa* (1.14%), *T. telescopium* (1.83%) and *V. cyprinoids* (1.46%). C14:0 contents

observed in *T. curta* and *M. trapa* was 4.98% and 2.89% respectively (Table 7.4). Maximum C16:0 was observed in *P. viridis* (35.46%) and minimum was in *B. spinosa* (6.11%). C16:0 content in *M. trapa* was 15.19% and also comparable C16:0 was observed in *T. curta* (22.65%), *T. telescopium* (20.17%) and *V. cyprinoids* (19.15%). Octadecanoic acid, commonly known as stearic acid (C18:0) was the second large SFA observed in all species except *P. viridis*.



[SFAs (saturated fatty acids), BCFAs (branched chain fatty acids), MUFAs (monounsaturated fatty acids), PUFAs (polyunsaturated fatty acids), NMI (non-methylene interrupted fatty acids)]

Figure 7.3: Percentage abundance of different type of fatty acids.

Table 7.4: Percentage abundance of fatty acids in molluscs

FA	RT	MT(%)	BS(%)	TC(%)	TT(%)	PV(%)	VC(%)
Saturated fatty acids							
C14:0	47.81	2.89	1.14	4.98	1.83	10.61	1.46
C15:0	52.94	0.88	0.70	1.76	1.82	1.05	0.68
C16:0	57.83	15.19	6.12	22.65	20.17	35.46	19.15
C17:0	62.49	0.89	2.346	2.42	2.83	2.06	3.41
C18:0	66.97	8.64	4.76	6.74	6.89	9.63	6.42
C19:0	67.10		0.23		0.34		
C20:0	76.13				0.49		0.35
C21:0	79.72	1.19	0.12				4.21
Branched chain fatty acids							
4,8,12-Me C13	50.25	0.47	0.14	1.60	1.83	1.23	0.69
iC15:0	50.95		0.14		0.48		
a-C15:0	51.03				0.19		0.21
iC17:0	60.75				7.14		
a-C17:0	60.83	0.39	0.36	2.73		1.02	1.19
9,10-Me C18	69.92			0.39			0.63
2-hydroxy 16:0	76.59		0.22			0.22	
2-hydroxyC18:2n6	77.38					0.55	0.74
Monounsaturated fatty acids							
C15:1	52.21	0.44					0.44
C16:1n3	56.45	2.77		14.76	0.94	12.18	2.27
C16:1n7	56.76		0.91				
C18:1n9	65.67	4.83	19.12	4.20	6.11		15.24
C18:1n6	65.81	3.08			6.13	3.70	2.84
C20:1n5	75.07	8.96	0.35	7.43	7.74	0.94	12.55
C20:1n9	75.12		0.39		0.92		
C22:1n9	81.64	1.27	0.39	0.34	0.92		
C24:1	85.83						0.19
Polyunsaturated fatty acids							
C20:2n7	74.27				1.09		
C20:5n3	81.64	3.71	5.97	12.38		10.51	4.54
C22:6n6	65.32	0.60		4.49		5.14	6.08
C22:6n3	72.19	4.29	44.09	7.66	8.31		2.21
C22:5n3	72.44	6.23	1.62	2.47	1.55		
C22:2n6	80.02	4.54	2.40	1.37	1.40	3.45	2.41
C18:3n6	80.39	4.16	5.72				1.28
C20:4n6	80.93	11.54					5.88
Nonmethlene interrupted fatty acids							
C18:2n6cis	65.32						
C18:4n4	65.81						
5,13-22:2	73.12				0.72		
C20:3n6	73.18					1.89	1.55
C22:4n6	75.44	8.36	1.62		2.67		
C22:5n6	75.86				0.98		

MT- *M. trapa*, BS- *B. spinosa*, TT- *T. telescopium*, TC- *T. curta*, VC- *V. cyprinoids*, PV- *P. viridis*

C16:0 was the most abundant SFA, C14:0 was the second largest SFA and C18:0 was the third most abundant SFA in *P. viridis*. Highest C18:0 was observed in *P. viridis* (9.63%) and minimum was observed in *B. spinosa* (4.76%). Stearic acid was the most abundant SFA in *G. tumidum*, bivalve collected from Gulf of Mannar (Babu et al., 2012). Comparable C18:0 was observed in *T. curta* (6.74%), *T. telescopium* (6.89%) and *V. cyprinoids* (6.42%) (Table 7.4). Palimitic acid and stearic acid was the most dominant fatty acids in both gastropods and bivalves, which is consistent with previous studies on the lipids of adult molluscs (Beninger & Stephan, 1985; Yamaguchi et al., 1992a,b; Rakshit et al., 1997; Carballeira et al., 2001; Benkendorff et al., 2005). Palmitic acid has been reported in numerous studies, as the major saturated fatty acids in mussels (King et al., 1990; Karakoltsidis et al. 1995; Freites et al., 2002; Orban et al., 2002; Otles & Sengor, 2005; Alkanani et al., 2007; Vernocchi et al., 2007). But in some bivalves, stearic acid was dominant (Fuentes et al., 2009).

In the present study, saturated fatty acids with an uneven chain length such as nonadecanoic acid (C19:0), heptadecanoic (C17:0) and pentadecanoic acids (C15:0) were also detected (Table 7.4). Similar results have been previously reported from both gastropods and bivalves (Beninger & Stephan, 1985; Rakshit et al., 1997; Benkendorff et al., 2005). Trace amount of C15:0 was observed in *M. trapa* (0.88%), *B. spinosa* (0.70%) and *V. cyprinoids* (0.68%) (Table 7.4). C15 content in *T. curta* (1.76%), *T.telescopium* (1.82%, *P. viridis* (1.05%) were comparable. High content of heptadecanoic acid (C17:0) was observed in *V. cyprinoids* (3.40%) and lowest concentration was observed in

M. trapa (0.89%). C17:0 content in *B. spinosa* (2.34%), *T. curta* (2.42%), *T. telescopium* (2.82%) and *P. viridis* (2.06%) were analogues (Table 7.4). Very low quantity of C19:0 was observed in *B. spinosa* (0.23%) and *T. telescopium* (0.34%). Trace amount of C20:0 was observed in *T. telescopium* (0.49%) and *V. cyprinoids* (0.34%). C21:0 was found only in *M. trapa* (1.19%), *B. spinosa* (0.12%) and *V. cyprinoids* (4.20%). Saturated fatty acids such as C14:0, C16:0 and C18:0 were most dominant and C15:0, C17:0 and C19:0 were very low in bivalve molluscs such as *L. annulata* and *P. tenuisculpta* (Fullarton et al., 1995). This result is comparable with the present study. According to Ekin & Bashan (2010), 21% of C16:0 and 5% of C18:0 were detected in *U. elongatulus*, freshwater mussel collected from Tigris River. Saturated and unsaturated fatty acids were equally present in the tissues of edible gastropod, *T. coronatus*, which is abundant in many areas in Bahrain (Freije & Awadh, 2010).

Among the six species, *T. telescopium* (9.74%) shows maximum abundance of total BCFAs and *T. curta* (4.72%) was on the second position. Comparable BCFAs content was observed between two bivalves such as *P. viridis* (3.03%) and *V. cyprinoids* (3.46%) (Table 7.4). BCFAs of *M. trapa* (0.89%) and *B. spinosa* (0.86%) were analogues. Branched SFAs found were 4,8,12- tri methyl tridecanoic acid (TMTD/4,8,12-Me C13:0), isopentadecanoic acid (iC15:0), anti isopentadecanoic acid (a-C15:0), isoheptadecanoic acid (*i*C17:0), anti isoheptadecanoic acid (*a*-C17:0), 9,10-di methyl octadecanoic acid (9,10-Me C18:0) and iso heptacosanoic acid (Table 7.4). Out of these detected branched chain fatty acids, TMTD was present in all six

species. The amount of 4,8,12 TMTD was observed as more abundant in *T. telescopium* (1.83%) and least in *B. spinosa* (0.14%) (Table 7.4). Previous studies have reported that TMTD was richly present in herbivorous mollusc, *C. lamyi* (Johns et al., 1979; Fuentes et al., 2009). In herbivorous species, TMTD is produced through the degradation of dietary chlorophyll (Joseph, 1982). Trace amount of anti iso heptadecanoic acid was present in all species except *T. telescopium*. Significant amount of iso heptadecanoic acid (7.14%) was detected in *T. telescopium*. Trace quantity of iso (0.48%) and anti iso (0.19%) pentadecanoic acid was observed in *T. telescopium*. Minimum amount of quantity of 9,10-Me C18:0 was present in both *V. cyprinoids* (0.63%) and *T. curta* (0.39%). Previous studies have reported 16-methyl-octadecanoic acid (ai-19:0) as a major FA in several calcareous sponge species mostly of the *calcinea* subclass (Schreiber et al., 2006), whereas low amounts were found in both *T. telescopium* and *B. spinosa*. Trace amount of iso and anti iso fatty acids were reported in marine bivalves and squids (Abdulkadir &Tsuchiya, 2008).

Trace amount of hydroxyl fatty acid was observed in some species. 2-Hydroxy fatty acid was identified by GC-MS as 2-hydroxy hexadecanoic acid (2-hydroxy C16:0) and 2-hydroxy 6-octadecadienoic acid (2-hydroxy C18:2n6) (Table 7.4). 2-Hydroxy C16:0 detected in *B. spinosa* and *P. viridis* were analogues (0.22%), and it was absent in all other four species. 2-Hydroxy C18:2n6 was present only in bivalve species such as *P. viridis* (0.55%) and *V. cyprinoids* (0.74%). According to Kawashima et al. (2003), 2-hydroxy fatty acids are generally derived from structural components of membranes called spingolipids. 2-

Hydroxy hexadecanoic and heptadecanoic acids were reported in some species of mussels (Fang et al., 1993; Murphy et al., 2002). 2-Hydroxy fatty acids were reported in marine bivalves such as *M. venulosus* and *M. zyonoensis* (Kawashima & Ohnishi, 2003).

Maximum abundance of MUFAs was observed in *V. cyprinoids* (33.54%) and minimum was detected in *P. viridis* (16.81%) (Figure 7.3). Similar MUFAs content were observed in *M. trapa* (21.34%) and *B. spinosa* (21.16%). MUFAs detected in *T. curta* (26.72%) and *T. telescopium* (23.16%) were analogues. 13-Hexadecenoic acid (C16:1n3), oleic acid (C18:1n9), 13- octadecenoic acid (C18:1n6) and 5-eicosenoic acid (C20:1n5) were the major MUFAs present in all species (Table 7.4). Total MUFA present in *B. spinosa*, *M. trapa*, *T. telescopium* and *T. curta* were analogues (21-26%). This result shows resemblance with the reported data of MUFAs from oceanic gastropod *X. pyrum* (Babu et al., 2010). In this study, C16:1 and C18:1 were the most predominant MUFA present in both gastropods and bivalves. Previous reports obtained from the fatty acid profile of gastropods and bivalves show good agreement with the present study, that they contain C16:1and C18:1 as major MUFAs (Ackman et al., 1971; Johns et al., 1979; Shanmugam et al., 2007). In the present investigation, bivalves showed approximately 12% of C16:1 and 15% of C18:1. These results are in close resemblance with the C16:1 and C18:1content of *D. cuneatus* (~11%) (Shanmugam et al., 2007). Equivalent amount of C16:1n3 was observed between *T. curta* (14.765) and *P. viridis* (12.18%). C16:1n3 contents in *M. trapa* (2.77%) and *V. cyprinoids* (2.27%) were analogues. Percentage compositions of C18:1n9 in *B. spinosa*, *T. telescopium* and

V. cyprinoids were 19.12%, 6.11% and 15.24% respectively. Comparable C18:1n9 was observed in *M. trapa* (4.83%) and *T. curta* (4.20%). Soft tissues of *M. galloprovincialis* were found to show high levels of C16:1n7 and C18:1n9 (Freites et al., 2002). Trace amounts of C16:1n7 and C20:1n5 were present in *B. spinosa* and *T. telescopium*. These results are comparable with the MUFAs present in *S. sachalinensis* (Tabakaeva & Tabakaev, 2017).

Present investigation showed a range of 4-19% 18:1n9 in gastropods. But in an earlier study MUFA content was reported as 23% in the frozen green lipped mussel in *P. canaliculus* (Murphy et al., 2003; Shanmugam et al., 2007). Oleic acid (C18:1) content of *C. obtusa*, *C. javanica*, *P. canaliculata*, *M. turbinate*, *G. cineraria* and *L. neritoides* were 11.9%, 3.22%, 6.44% 13.09%, 10.62%, and 13.16% respectively (Go et al., 2002; Purwaningsih et al., 2015). Generally, gastropods have been found to contain 18: 1 as major MUFA (Ackman et al., 1971; Johns et al., 1980). Oleic acid (18:1) contributed more than 10% in *C. tehuecha* (Pollero et al., 1979). According to Ekin et al. (2011), MUFAs of molluscs are usually containing dominant amount of C16:1n7 and C18:1n9 acids. Presences of these fatty acids in molluscs are associated with two mechanisms; (1) formation through the desaturation of C16:0 and C18:0 acids (endogenous) and (2) obtained from diet (diatoms) (exogenous).

Studies have shown that MUFAs help to reduce blood cholesterol levels and protect against heart disease, so these types of fatty acids coming under the category of good fat (Shanmugam et al., 2007). Like all fats, MUFAs contain 9 calories per gram and should be incorporated

in normal food, in order to regulate calories to acceptable daily intake levels (American Heart Association, 2015). Consuming higher levels of MUFAs than saturated fats have a protective effect against metabolic syndrome like cardiovascular diseases. (Estruch et al., 2013). Also daily intake of MUFAs containing food will lower the risk of atrial fibrillation (type of arrhythmia allied with decreased blood flow to the heart) in women (Chiuve et al., 2015). Compared to PUFAs, MUFAs have positive effects on children with high cholesterol and other cardiovascular disease risk factors (Negele et al., 2015). MUFAs are so important dietarily because they have anti-inflammatory and insulin resistance properties (Riserus, 2008; van Dijk et al., 2012). MUFAs have a positive effect on adipose dysfunction even in obese people and also it helps to reduce body weight (Aller et al., 2014; Finucane et al., 2015; Yang et al., 2016). Replacing saturated fats with monounsaturated fats in normal diet will decrease mental depression and osteoporosis (Sanchez-Villegas et al., 2011; Kien et al., 2013; Hryhorczuk et al., 2016; Wang et al., 2016). Large consumption of MUFA reduced the risk of certain cancers like liver cancer, breast cancer, etc (Duarte-Salles et al., 2015; Jung et al., 2016; Zhao et al., 2016).

All gastropods species show large quantity of PUFAs except *T. curta*. Maximum PUFA contents were observed in *B. spinosa* (54.08%) and minimum was detected in *P. viridis* (19.11%) (Figure 7.3). Comparable PUFAs were observed between *M. trapa* (35.06%) and *T. telescopium* (35.06%). PUFAs of *V. cyprinoids* (22.40%) and *T. curta* (28.37%) were analogues. Comparable amount of docosadienoic acid (C22:2n6) was present in between *B. spinosa* (2.40%) and *V. cyprinoids*

(2.41%). Percentage abundances of C22:2n6 in *T. curta* (1.37%) and *T. telescopium* (1.40%) were analogues. Comparatively high content of C22:2n6 was observed in both *M. trapa* (4.54%) and *P. viridis* (3.45%). γ-Linolenic acid (C18:3n6) was detected only in *M. trapa* (4.16%) and *V. cyprinoids* (1.28%). Significant amount of arachidonic acid (C20:4n6) was observed in *M. trapa* (11.54%) and *V. cyprinoids* (5.88%). Substantial amount of eicosapentaenoic acid (EPA) was observed in all species except *T. telescopium*. EPA content of *M. trapa*, *B. spinosa*, *T. curta* , *P. viridis* and *V. cyprinoids* were 3.71%, 5.97%, 12.38%, 10.51% and 4.54% respectively. Docosahexaenoic acid was (DHA) highest in *B. spinosa* (44.09%) compared to other species. Comparatively minute amount of DHA was detected in *V. cyprinoids* (2.21%) and *M. trapa* (4.29%). Comparable amount of DHA was observed in *T. curta* (7.66%) and *T. telescopium* (8.31%). Significant amounts of docosatetraenoic acid (8.36%) and docosapentaenoic acid (6.23%) were observed in *M. trapa*. Equivalent amounts of DPA were observed in between *B. spinosa* (1.62%) and *T. telescopium* (1.55%).

In the present study, n3 fatty acids contribution is higher in all species, so it can be suggested the inclusion of such animal in normal diet of patients suffering from neurological diseases. C20:4n6 (characteristic to freshwater mollusc) and C20:5n3 (characteristic to marine mollusc) are important mediators in basic physiological functions, ion regulation, renal function and reproductive process in molluscs (Ekin et al., 2012). Reported data give the knowledge that C20:4n6 is mostly associated with reproductive process and not with growth. The reported data of PUFAs in the mantle of *T. telescopium* was found to be high (Rakshit

et al., 1997; Ekin & Bashan., 2010). According to Ekin & Bashan (2010), marine molluscs possess very low level of C20:4n6 acid, but in fresh water bivalves, its amount is relatively high. Consumption of EPA and DHA stimulates blood circulation, increase the breakdown of fibrin, a compound involved in clot and scar formation and, in addition, may reduce the blood pressure. So these fatty acids can have medicinal applications for certain circulatory problem such as varicose veins. *T. telescopium* contains 5% EPA and 8% DHA of the total PUFAs. DHA is normally one of the most abundant fatty acids in marine animals, including marine molluscs. Comparable results were obtained in the present study. Previous reports also show that n3 fatty acids reduce blood triglyceride levels; regular intake of this may reduce the risk of primary and secondary heart attack (Ekin & Bashan, 2010).

Large quantity of C20:5n3 (EPA) was detected in sea gastropod *B. undatum* (Arakelova et al., 2009). EPA and DHA are important dietary requirements in fish nutrition (Sargent et al., 1995; Sargent et al., 2002; Bell & Sargent, 2003). PUFAs such as linolenic (18:3n3) and EPA acids (20:5n3) were the predominant n3 fatty acids in marine snail *T. coronatus* collected from Arabian Gulf. Also low content of DHA (22:6n3), an important member fatty acid of marine organism is due to seawater characteristic in the Arabian Gulf (i.e. warm water with high salinity) and the food availability (Freije & Awadh, 2010). Marine molluscs such as *A. punctata*, *P. lividus* and *H. forskali* displayed higher amounts of unsaturated fatty acids when compared with saturated derivatives (Pereira et al., 2013). Also, similar results were observed in some sea cucumbers (Aydin et al., 2011). In marine molluscs, DHA and EPA are

formed from linolenic FA as a result of elongation of its hydrocarbon chain (Arakelova et al., 2009). PUFAs in microalgae is formed by de novo synthesis of saturated fatty acids, mainly 18:0 and 16:0 through an aerobic pathway. This process involving sequential addition of double bonds to SFA via ω6 desaturases to produce 18:2n6. 18:2n6 undergoes further desaturation using ω3 desaturase to produca 18:3n3 and further convert to EPA and DHA using n3 elongase. (Guschina & Harwood, 2006). Recently this desaturase and elongase genes have been identified in sponges, cnidarians, annelids, echinoderms, hemichordates, tunicates and crustaceans, particularly in molluscs (Monroig et al., 2013).

Molluscs, like other marine invertebrates (Zhukova, 1986; Kornprobst & Barnathan, 2010), possess a particular group of PUFA called non-methylene-interrupted (NMI) fatty acids that can be biosynthesized endogenously (Barnathan, 2009). NMI fatty acids in bivalves were formed as a result of the elongation and Δ5 desaturation of 20:2 Δ5,11 and 20:2 Δ5,13 acids respectively into the 22:2 Δ7,13 and 22:2 Δ7,15 acids with the active incorporation of 1- ^{14}C acetate (Zhukova, 1986). Fatty acyl desaturases having Δ5-like specificity appear to be widely distributed among molluscs. Δ5-like desaturase were isolated from common octopus, *O. vulgaris*, abalone *H. discus hannai*, clam *M. mactroides*, etc. (De Moreno et al., 1976; Guillou et al., 2010; Monroig et al., 2013). Recently these biosynthetic processes were experimentally proved in the marine bivalve, *S. broughtoni* (Zhukova, 1991). Further experiments with the mussel *M. edulis* and the mollusc *C. brevisiphonatas* showed that 22:2 Δ7,13 and 22:2 Δ7,15 acids arose from C2 elongation of the 20:2 Δ5,11 and 20:2 Δ5,13 acids (Barnathan, 2009).

In the present study, small amount of non-methylene interrupted (NMI) fatty acids were detected in almost all species. C18:2n6, C18:4n4, C20:3n6, 5,13-C22:2, C22:5n6 and C22:4n6 were the NMI detected in the present study (Table 7.4). Maximum amount of NMI was observed in *M. trapa* (11.44%) and minimum was in *P. viridis* (1.89%). Comparable amount of NMI was observed in between *V. cyprinoids* (4.67%) and *T. telescopium* (5.11%). NMI was absent in soft tissues of *T. curta* and 8.36% was observed in *B. spinosa* (Figure 7.3). Maximum amount of C18:2n6 was observed in *M. trapa* (3.08%) and *V. cyprinoids* (3.12%) and minimum was observed in *B. spinosa* (1.02%) and *T. telescopium* (0.74%). Also it was absent in both *T. curta* and *P. viridis*. This result showed good agreement with the results of gastropods obtained from Mediterranean Sea and Red Sea (Go et al., 2002). C18:4n4 was only present in *B. spinosa* (5.72%). C20:3n6 was present only in bivalves such as *P. viridis* (1.89%) and *V. cyprinoids* (1.55%) (Table 7.4). Very small amounts of 5,13-C22:2 (0.72%) and C22:5n6 (0.98%) were present in *T. telescopium*, while absent in others. C20:3n6 were observed in marine mussels from genera *Mytilus* and *Perna,* but 5,13-C22:2 was detected only in *M. galloprovincialis* (Trigari et al., 2001; Freites et al., 2002; Murphy et al., 2002; Treschow et al., 2007; Ventrella et al., 2008). Highest amount of C22:4n6 was present in *M. trapa* (8.36%) and lowest in *B. spinosa* (1.62%), *T. telescopium* (1.55%) and *T. curta* (2.47%). In the present study, C22:4n6 was only present in gastropods. NMI fatty acids are usually present in many marine invertebrates such as mussels, oysters and gastropods (Kawashima et al., 2008), but the functions are not fully understood. Less amounts of NMI

fatty acids were observed in marine molluscs such as *S. sachalinensis* and *S. broughtoni* and it varied from 0.7% to 20.7% (Zhukova & Svetashev, 1986). The nudibranchs, *Chromodoris* sp. and *P. coelestis*, from tropical waters of the Northwestern Pacific, exhibited a wide diversity of NMI (Zakhartsev et al., 1998). Further, less amount of dienoic and trienoic NMI were observed in *A. opercularis* and *P. maximus* (Kraffe et al., 2004). Recently, novel C24 polyenoic NMI FA were observed in female gonad (Kawashima et al., 2008).

Table 7.5: n3/n6 ratio of different species

Species	n6 PUFA	n3 PUFA	n3/n6 PUFA
M. trapa	16.68	14.23	0.85
B. spinosa	2.4	51.68	21.53
T. curta	5.86	22.51	3.84
T. telescopium	2.38	9.86	4.14
P. viridis	8.59	10.51	1.22
V. cyprinoids	14.37	6.75	0.47

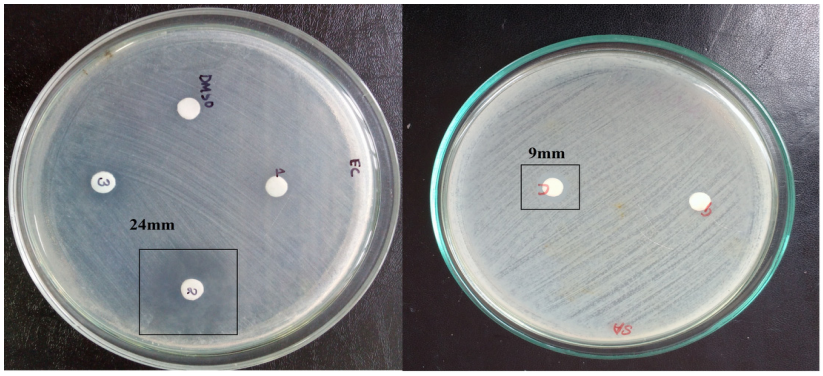
n3 and n6 are two types of PUFAs, and are derived from linolenic acid through the same path way. They are both required for the body to function but have opposite effects when it comes to the inflammatory response and cardiovascular health. Too much n6 and n3 are among the causes for many diseases in modern society (Williams et al., 2011). The proportions of n3 PUFA were particularly high in muscle tissue of *megangulus* variety (Kawashima et al., 2008). PUFAs were the predominant fatty acids in marine species and n3 fatty acids are vital for normal metabolism. In the present study, n3 fatty acids were higher for *B. spinosa*, *T. curta* and *T. telescopium* than n6 fatty acids. n6 fatty acids

were predominated in *V. cyprinoids* (Table 7.5). Comparatively equal amount of n3 and n6 fatty acids was observed in *M. trapa* and *P. viridis*. According to Arakelova al. (2009), predominance of n3acids is revealed in marine molluscs, n3/n6 < 1 in freshwater gastropods and n3/n6 > 1 in the marine ones. El-Badry et al. (2007) and Vuppalanchi et al. (2007) suggested that the ratio of n6 to n3 should be approximately 3:1. In recent years, biopsy results show that tendency for some diseases, including breast cancer and asthma were increased, because the modern diet is rich in n6 foods (Masterton et al., 2010). Reports shown that reduction in cardiovascular risk is linked to the total amount of n3 fatty acids rather than the n6:n3 ratio (Griffin, 2008). Experimental studies suggest omega-3 (n3) PUFAs suppress and n6 PUFAs promote prostate tumour carcinogenesis (Williams et al., 2011). Liu et al. (2015) suggest that n-3 and n-6 polyunsaturated fatty acids (PUFAs) have opposite effects on type2 diabetes mellitus (T2DM). n6 PUFAs promotes the development of insulin resistance (IR) while, n3 PUFA has opposite effect (Siriwardhana et al., 2012). The suggested dietary ratio of n6/n3 fatty acids for health benefits is 1-2 (Simopoulos, 2009). Dietary balance of n3 to n6 PUFA helps to reduce prostate cancer risk (Kobayashi et al., 2006; Kelavkar et al., 2009; Williams et al., 2011) and unbalanced ratio tend to increase atherosclerosis, obesity, and diabetes (Adler et al., 1994; Schraer et al., 1999). n3 PUFAs were used as weight reducing agents and also produced conflicting effect due to the unbalanced mixing of n3/n6 PUFAs in diet (Belury et al., 2003; Fontani et al., 2005; Hill et al., 2012).

7.3.2 Biological activity of identified compounds

7.2.3.1 Anti-bacterial activity of fatty acid fractions

Antimicrobial activity assay of natural compounds leads to the discovery of new effective drugs (Ababouch et al., 1992; Choi et al., 2001). Compounds extracted from marine invertebrates, including gastropods, bivalves, squids, etc. possess antimicrobial activities (Mitta et al., 2000a; Karthikeyan et al., 2014). There are numerous previous studies carried out on antimicrobial activity screening of compounds isolated from marine sources, especially from Indian coasts (Tilvi et al., 2004; Asthana et al., 2006; Chandrasekaran et al., 2008; Alagan et al., 2017). Fatty acids isolated from marine sponge *T. curta* sp. show antimicrobial activity against various bacteria and fungi (Berge & Barnathan, 2005). Δ5,9 dienoic fatty acids are widely used as antimalarial drugs (Carballeira, 2008). So many studies have reported antimicrobial activity of extracts from marine molluscs (Benkendorff et al., 2000a,b; 2001; 2004; 2005; Haug et al., 2004; Chandrasekaran et al., 2008, Liu et al., 2015). Compounds isolated from *M. edulis*, *M. galloprovincialis* and *D. auricularia* show wide range of antimicrobial activity (Charlet et al., 1996; Hubert et al., 1996; Mitta et al., 1999; 2000a,b; Iijima et al., 2003; Karthikeyan et al., 2014). Potent antibacterial activity in haemocytes and haemolymph has been detected in various molluscs (Anderson & Beaven, 2001; Benkendorff et al., 2005).

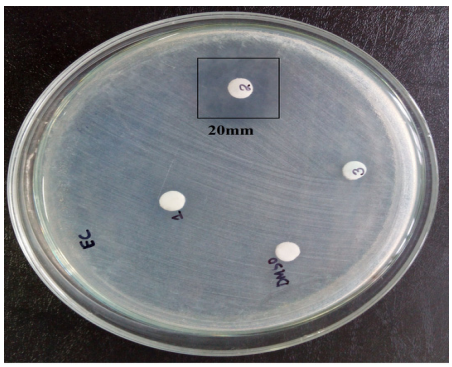


E. coli *S. aureus*

Figure 7.4: Antimicrobial activity of PUFA of *B. spinosa*

Table 7.6: Anti-microbial activity of PUFA from *B. spinosa* and *M. trapa*

PUFA samples	Inhibition level expressed in mm	
	S. aureus (gram + ive bacteria)	*E. Coli* (gram − ive bacteria)
B. spinosa	9 ± 0.1	24 ± 0.3
M. trapa	No inhibition	20 ± 0.4



E. coli

Figure 7.5: Antimicrobial activity of PUFA of *M. trapa*

Antimicrobial activity of PUFAs separated from crude fatty acids mixture of *B. spinosa* and *M. trapa*, which measures diameter of growth of inhibition is summarised in the Table 7.6. According to the results, PUFAs of *B. spinosa* were active against bacterial pathogens associated

with human diseases viz. *Escherichia coli* (24 ± 0.3 mm) and *Staphylococcus aureus* (9 ± 0.1 mm) and inactive against *Aeromonas hydrophila* (Figure 7.4). PUFAs of *M. trapa* was also active against *E. coli* (20 ± 0.4 mm) and inactive against *S. aureus* and *A. hydrophila* (Figure 7.5). Ohta et al. (1995) reported that unsaturated fatty acids containing DHA have antibiotic activity against MRSA. This reported data were a supporting evidence for the biological activity of PUFAs isolated from *B. spinosa*, because DHA constitutes 44% of total fatty acid. From the previous study, it is clear that DHA- rich extracts were highly active against gram positive and negative bacteria (Findlay & Patil, 1984; Shin et al., 2007a,b). DHA- rich PUFAs isolated from marine fish, *S. fimbriata* shows higher activity against *S. aureus* and *E. coli* which also supports the current study (Chitra Som & Radhakrishnan, 2011).

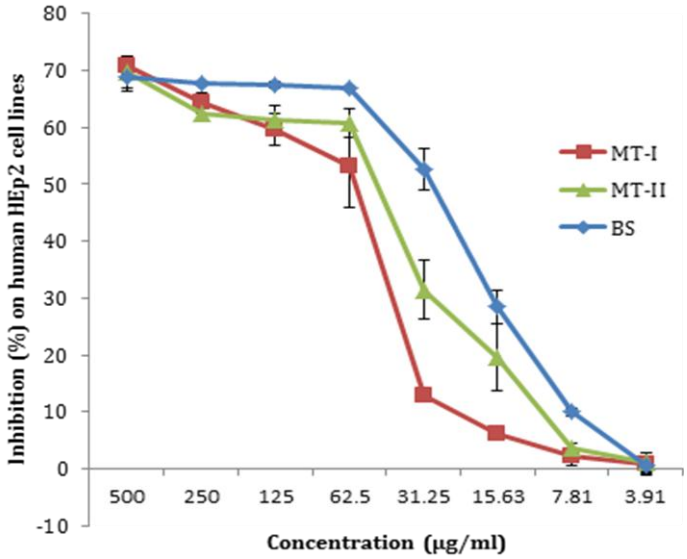
PUFAs of both *M. trapa* and *B. spinosa* were inactive against bacterial pathogens associated with fish diseases viz., *Vibrio parahaemolyticus*, *Vibrio harveyi*, and *Vibrio cholera*. DHA and EPA contents of *B. spinosa* were higher than those of *M. trapa*. PUFA isolated from *B. spinosa* exhibited activity against both gram positive (*S. aureus*) and negative (*E. coli*) bacteria, of which latter showed greater inhibition. This is because, inhibitory effects on bacterial strains increased with level of unsaturation (Chitrasom & Radhakrishnan, 2011). Fatty acids isolated from diatoms, *P. tricornutum* are powerful antibacterial agent against multidrug resistant *S. aureus* (MRSA) (Desbois et al., 2008). Fatty acids extracted from wedge clam *D. cuneatus* exhibited appreciable bacteriostatic and bactericidal activities against tubercle bacilli (Shanmugham et al., 2007).

7.2.3.2 *In vitro* anti-proliferative effect of fatty acid extracts (MTT assay)

Cancer is a serious worldwide issue, especially in developed countries. Chemotherapy is one of the potential treatments for cancer through the application of cancer-destroying drugs. This treatment technique uses one or more anti-cancer drugs as chemotherapeutic agents (Senthilraja & Kathireshan, 2015). Almost 60% of anticancer drugs are of natural origin (Grever, 2001; Newman & Cragg, 2016). Introduction of new compounds as anticancer drug is a gruelling task, which require chemical as well as biological efforts. This is because cancers cells are just like normal cells. Nowadays more researchers have come to the awareness that compounds isolated from marine sources are widely used for cancer treatment. Comparing with terrestrial origin, approximately 16000 compounds were isolated from marine sources (Bhakuni & Rawat, 2005), though only very few compounds have subjected to comprehensive biological evaluation as chemotherapeutic agents. At present about 85 marine derived natural products are in clinical trials as a drug candidate for anticancer efficacy (Newman & Cragg, 2016).

Sawada et al. (2012) reported that large consumption of omega-3 polyunsaturated fatty acids (n3 PUFA), mainly eicosapentaenoic acid (EPA 20:5) and docosahexaenoic acid (DHA, 22:6) protect against the development of hepatocellular carcinoma (HCC). Also n3 PUFA containing high concentration of DHA down regulates Her-2/neu oncogene expression in human breast cancer cells (Menendez et al., 2016). Horii et al. (2007) reported that conjugated octadecatetraenoic acid from garden balsam seed oil shows strong cytotoxic effect on human leukemic cells.

Conjugated linoleic acid inhibited strong cytotoxic activity against lung, breast, and colorectal cancer but have no effect on the glioblastoma cell line (Shultz et al., 1992; Schonberg & Erokan, 1995). Suzuki et al. (2001) reported that conjugated diene system is the reason for this cytotoxic effect and the cytotoxicity increases with number of unsaturation. Jakobsen et al. (2008) reported that DHA has strong cytotoxic activity against SW620 (colon cancer cell line). Giros et al. (2009) reported that both DHA and EPA exerts an important proapoptotic effect in different colorectal cancer cell lines such as Caco-2, HT-29, HCT116, LoVo, SW480 by the down regulation of two key regulatory elements viz., FLIP and XIAP through extrinsic and intrinsic pathways and not affect the viability of normal human colon mucosal epithelium (NCM460) cells.



[MT-I: EPA, DPA and DHA mixture of *M. trapa;* MT-II: PUFA of *M. trapa;* BS: PUFA of *B. spinosa*]

Figure 7.6: Cytotoxicity of PUFAs of *B. spinosa*, PUFAs of *M. trapa* and DPA, EPA, DHA mixture of *M. trapa* against human laryngeal carcinoma cell line (HEp-2)

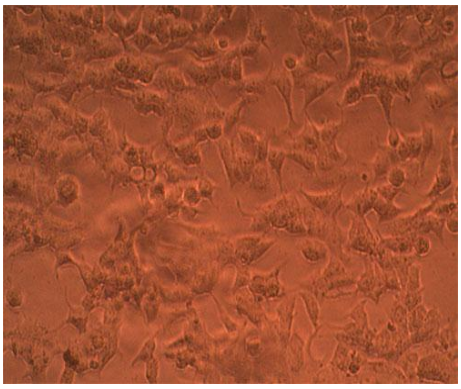


Figure 7.7: Phase contract image of HEp-2 cell line control

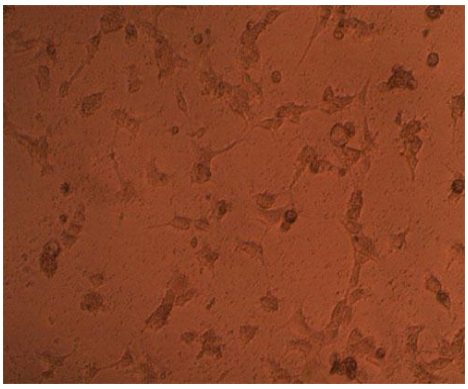


Figure 7.8: Morphological alteration of HEp-2 cell line exposed to PUFA of *M. trapa*

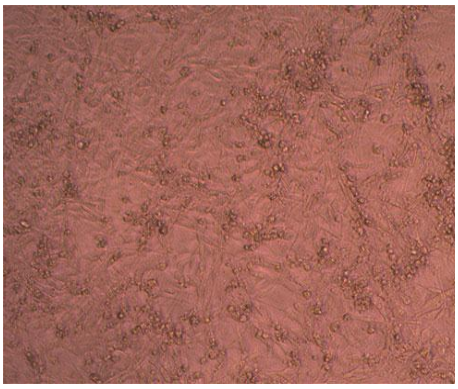


Figure 7.9: Morphological alteration of HEp-2 cell line exposed to PUFA of *B. spinosa*

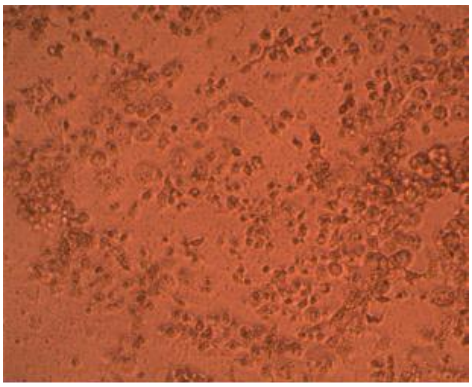


Figure 7.10: Morphological alteration of HEp-2 cell line exposure of mixture containing EPA, DPA and DHA of *M. trapa*

Table 7.7: IC_{50} values for samples against human laryngeal carcinoma cell line (HEp-2)

Samples	IC_{50} values
PUFA of *B. spinosa*	27.5 µg mL^{-1}
PUFA of *M. trapa*	54.15 µg mL^{-1}
EPA, DPA and DHA mixture of *M. trapa*	59.25 µg mL^{-1}

Cytotoxicity testing is the primary assay, which focuses mainly on cell death or some measure of growth impairment (Ferro & Doyle, 2001). Many different assays have been developed to determine in vitro studies. Popular assays that are widely used are the total cellular protein assay (sulforhodamine B), the neutral-red uptake assay, the LDH leakage assay and the tetrazolium dye assays (Wang et al., 2010). The best known tetrazolium assays is probably the MTT assay for mammalian cells in which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazolium salt is reduced by the mitochondria of viable cells to insoluble, purple formazan crystals (Loveland et al., 1992; Wang et al., 2010).

To evaluate the cytotoxic activity of PUFAs separated from crude fatty acid mixture of *B. spinosa* and *M. trapa* against human laryngeal carcinoma cell line (HEp-2) was incubated with different doses (500 to 3.9 μg mL^{-1}) of extract. After 24 h of incubation, cell viability was determined by the MTT assay. Moreover, cytotoxic activity of mixture containing EPA, DPA and DHA separated from crude PUFAs of *M. trapa* was measured by same procedure. The results of cytotoxicity assay are presented in (Figure 7.6). All samples were able to inhibit the proliferation of the cancer cell line (HEp-2). Phase contract image of HEp-2 cell line control morphology is shown in Figure 7.7. Morphological alteration of HEp-2 cell line exposed to PUFAs of *B. spinosa* and *M. trapa* are shown in Figure 7.8 and 7.9 respectively. The morphological changes of HEp-2 cell line with the exposure of mixture containing EPA, DPA and DHA is illustrated in Figure 7.10. According to the American National Cancer Institute guidelines (NCI), IC_{50} value of crude extracts

must be within the limit of 30 mg/ml after an exposure time of 24 h (Senthilraja & Kathiresan, 2015). In this study, IC_{50} value obtained for sample 1 (59.25 μg mL^{-1}), sample 2 (54.15 μg mL^{-1}) and sample 3 (27.5 μg mL^{-1}) (Table 7.7) were less than limit approved by NCI. This is a preliminary data in antitumor studies, which demonstrated that all the are samples showing anticancer effect.

From this study, crude PUFAs of *B. spinosa* showed maximum activity against human laryngeal carcinoma. This is because, in *B. spinosa*, DHA constitutes 44% of total fatty acids. According to de Boer et al. (2009), cytotoxicity of fatty acids increased with unsaturation. The toxicity of unsaturated fatty acids was found to be up to 8-fold higher than that of saturated ones (Mancini et al., 2011). Comparing the crude PUFAs of *M. trapa* and mixture of EPA, DPA and DHA separated from crude PUFAs of *M. trapa*, former is more active against Hep-2 than latter one. Combined interactions between DHA and taxanes were show higher IC_{50} value than the IC_{50} value of DHA itself against breast carcinoma cell lines (Menendez et al., 2016). This study is a supporting evidence for the present study. PUFAs is a mixture of linolenic acid (LA), arachidonic acid (AA), docosatetraenoic acid, eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), obsbond acid, docosahexaenoic acid (DHA). All these unsaturated fatty acids have its own cytotoxic activities and its synergistic effects is higher than those of individual ones (Zhang et al., 2015). Results of cytotoxicity of PUFAs and EPA/DHA mixture against colon cancer (COX-2) show that crude PUFAs exhibit higher activity against COX-2 than EPA and DHA mixture (Zhang et al., 2015). Even though, EPA and DHA have similar

biochemical effects, studies, particularly those related to cytotoxic investigations are carried out using PUFAs (Price & Trisdale, 1998). EPA and DHA rich PUFAs isolated from sardine fishes such as *S. fimbriata* and *S. longiceps* exhibited cytotoxic activity against MCF-7 (breast cancer) and DU-145 (prostate cancer) (Chitra Som et al., 2017).

6.4 Summary

Fatty acid compositions of different mollusc (four gastropods and two bivalves) collected from Kerala coast has been investigated. In this study, both saturated and polyunsaturated fatty acids were higher than monounsaturated fatty acids and branched chain fatty acids. Tetradecanoic acid (C14:0), pentadecanoic acid (C15:0), hexadecanoic acid (C16:0), heptadecanoic acid (C17:0) and octadecanoic (C18:0) acid were the major saturated fatty acids observed in all species, of which C16:0 and C18:0 were predominant. Trace amount of C14:0 was observed in all species, of which *P. viridis* shows highest concentration. Saturated fatty acids were common in animal food products as well as in vegetables. 4,8,12-trimethyltridecanoic acid (TMTD) (4,8,12-Me C13:0), isopentadecanoic acid (iC15:0), anti isopentadecanoic acid (a-C15:0), isoheptadecanoic acid (iC17:0), anti isoheptadecanoic acid (a-C17:0) and 9,10-di methyl octadecanoic acid (9,10-Me C18:0) were the major branched chain fatty acids identified in the present study. TMTD was present in both gastropods and bivalves, but others are not common. Iso and anti-iso pentadecanoic acid and isoheptadecanoic acid were detected in *T. telescopium*. These varieties of fatty acids were formed from bacteria via symbiotic process.

Maximum amount of monounsaturated fatty acids was observed in *V. cyprinoids* and minimum was detected in *P. viridis*. C16:1n3, C18:1n9, C18:1n6, C20:1n5, C20:1n9 and C22:1n9 were major MUFAs detected in the present study, of which C16:1 and C18:1 were the most predominant MUFAs present in both gastropods and bivalves. Fewer amounts of 2-hydroxy fatty acids was noticed in *P. viridis* and *V. cyprinoids*. All gastropods species shows large quantity of polyunsaturated fatty acids except *T. curta*. Among the gastropod species, *B. spinosa* shows maximum PUFAs content. Eicosapentaenoic acid (EPA), docosadienoic acid, docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) were the major PUFAs observed in the present study. The concentration of γ-linolenic acid (ALA) (C18:3n6) was very small in *M. trapa* and *V. cyprinoids* and it was absent in all other species. In molluscs, linoleic acid and ALA were desaturated by ω3 desaturase to give 18:3n-3, further convert to eicosapentaenoic acid and docosahexaenoic acid using n3 elongase. Small amount of non-methylene interrupted (NMI) fatty acids such as C18:2n6, C18:4n4, C20:3n6, 5,13-C22:2, C22:5n6 and C22:4n6 were detected in almost all species. In the present study, n3 fatty acids were higher for *B. spinosa*, *T. curta* and *T. telescopium* than n6 fatty acids, but the reverse was observed in *V. cyprinoids*. Virtually equal amount of n3 and n6 PUFAs were observed in *M. trapa* and *P. viridis*. Generally, marine molluscs are rich source of n3 PUFAs while, fresh water molluscs contain large quantity of n6 PUFA.

PUFAs of *B. spinosa* and *M. trapa* show activity against bacterial pathogens associated with human diseases viz., *Escherichia coli* but inactive against *Aeromonas hydrophila*. PUFAs of *B. spinosa* show

activity against *Staphylococcus aureus* but PUFAs of *M. trapa* are inactive against *S. aureus*. This is because, activity of PUFAs against gram positive bacteria increased with level of unsaturation. In *B. spinosa*, 44% of total fatty acids was DHA. PUFAs of both *M. trapa* and *B. spinosa* were inactive against bacterial pathogens associated with fish diseases viz., *Vibrio parahaemolyticus*, *Vibrio harveyi*, and *Vibrio cholera*. This indicates that *M. trapa* and *B. spinosa* act as a significant source for new antimicrobial compound as a future drug candidate, and more specific studies are required for the conformation of compound.

From this study, crude PUFAs of *B. spinosa*, *M. trapa* and DPA, EPA, DHA mixture of *M. trapa* showed maximum activity against human laryngeal carcinoma (HEp-2 cell line). IC_{50} value for DPA, EPA, DHA mixture separated from PUFAs of *M. trap*a was 59.25 μg mL^{-1}. IC_{50} value of PUFAs isolated from *M. trapa* and *B. spinosa* were 54.15 μg $mL^{-1}$and 27.5 μg mL^{-1}respectively. IC_{50} values of all samples were less than limit approved by NCI. PUFA of *B. spinosa* were more active than *M. trapa* against HEp-2 cell line. This is because cytotoxicity of fatty acids increased with unsaturation. IC_{50} value of EPA, DPA, DHA mixture shows lower value as compared to the IC_{50} value of PUFA. This is because, almost all unsaturated fatty acids have its own cytotoxic activities and its synergistic effects are higher than those of individual ones. This is a preliminary data in antitumor studies, which demonstrated that all the samples showing anticancer effect.

All the species selected for the study are cheaply available in marine world. Fatty acids isolated from all the gastropods and bivalves

show maximum abundance of saturated and polyunsaturated fatty acids. PUFAs separated from the fatty acids show both antibacterial as well as cytotoxic effect. This is a preliminary study and more specific studies required for the conformation of mechanism of apoptosis and will leads to the development of new drugs. So this study helps to find out an easily available source for both nutritional and pharmacological food products.

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Chapter 8

SUMMARY

Molluscs are the most diverse, biochemically rich and biologically active marine living resources widely distributed in the coastal, mangrove and shoreline area. They are being used as food, ornamental purpose, fertilizer and pollution monitoring purpose for the past decades. Biochemical constituents such as proteins, carbohydrates, lipids, calorific value, amino acids, fatty acids and steroids indicate their commercial and nutritional importance. Furthermore, the *in silico* activity studies, antimicrobial activity studies and cytotoxic studies pointed that, they are pharmaceutically important resources. The present study investigates the biochemical composition and biological activities of some mollusc species from the Kerala coast. The study focused on the quantification of biochemical constituents in gastropods viz., *Telescopium telescopium, Bursa spinosa, Murex trapa* & *Tibia curta,* and bivalves viz., *Perna viridis* & *Villorita cyprinoids*. Moreover, this study also concentrated on the primary screening of biological activity of the secondary metabolites in molluscs. The information acquired from the present investigation supports the utilization of molluscs as a nutritive food material. The study also evaluates the cytotoxicity of polyunsaturated fatty acids separated from the crude lipid fraction of *M. trapa* and *B. spinosa*

against human laryngeal carcinoma diseases. The highlights of present study are summarized here under.

Six mollusc species (four gastropods and two bivalves) were collected from Kerala coast. Gastropods such as *M. trapa*, *T. curta* and *B. spinosa* were collected from the fishing zone, at which Ashtamudi lake opens to the Arabian sea. Two bivalve species viz., *P. viridis* and *V. cyprinoids* were collected from Ashtamudi estuary. *T. telescopium*, a gastropod collected from the mangrove estuary situated in Pappinissery, Kannur. Molluscs species examined in this study were found to contain substantial amounts of proteins and carbohydrates. Protein contents of gastropods vary from 30.54% to 53.36% and those of bivalves fluctuate from 39.16% to 42.41%. With respect to species division; gastropods shows large concentration of protein than carbohydrates. Carbohydrate levels in gastropods and bivalves vary from 8.36% to 15.58% and 18.39% to 21.26% respectively. Compared to gastropods, bivalve species show the highest amounts of carbohydrates. Lipid contents of the gastropods range from 2.38 to 11.56% and those of bivalves vary from 7.56 to 8.94%. Meanwhile, ash content of molluscs varies from 0.89 to 3.23%. In gastropods it range from 0.89% to 2.12% and in bivalves, it varies from 2.57% to 3.23%. Water contents exhibit a range of 65.15 to 82.18% in gastropods and 70.18 to 78.15% in bivalves. The average energy contents (calorific value) of gastropods and bivalves exhibited similar trends. Calorific value in bivalves varied from 3.82 Kcal g^{-1} to 4.01 Kcal g^{-1} and those of gastropods ranging from 3.17 Kcal g^{-1} to 3.76 Kcal g^{-1}.

Essential elements such as Ca, Mg, Zn, Fe, Cu and Mn were most abundant and toxic elements such as Pb and Cd were low in these organisms. Present study revealed that, metals present in all the species were below the permissible limit approved by World Health Organizations, Malaysian Food Regulation, Ministry of Public Health, Thailand, National Health and Medical Research Council, Food Safety and Standards Authority of India, and General Standard for Contaminants and Toxins in Food and Feed. Compared to gastropods, bivalves possess higher protein, lipid, calorific value and water holding capacity. Proximate composition analysis of the investigated mollusc species shows that all the species were fit for human consumption due to low-fat, high-carbohydrate and protein content. Energy contribution was found to be highest in all species, so these organisms can act as food and energy alternatives.

Study on the amino acid compositions of mollusc species shows the presence of both essential and non-essential amino acids. During the analysis of amino acids in mollusc species, 15 amino acids were identified. Among these 15 amino acids, lysine, methionine, and leucine are the major essential amino acids, and glutamic acid and proline are the major non-essential amino acids. Gastropods such as *B. spinosa*, *T. curta*, and *T. telescopium* were observed to be rich in amino acids, especially essential amino acids. Taste active value of glutamic acid and methionine were high in *M. trapa,* while leucine and lysine showed intermediate taste active values. Sweet tasted methionine is high in *B. spinosa, T. curta* and *V. cyprinoids*. Moreover, sweet tasted lysine and arginine were significantly present in *T. curta* and *B. spinosa*. In *T. telescopium,* bitter taste of

histidine was balanced by the sweet taste of lysine and methionine. Sweet amino acids such as methionine and lysine were predominated in the soft tissue of *P. viridis*.

Alkane fraction separated from the crude lipid content of *M. trapa*, *T. curta, B. spinosa* ranged from C_{11} to C_{27}, while those of *T. telescopium* and *P. viridis* vary from C_{11} to C_{28}. Alkanes range from C_{11} to C_{27} with the absence of C_{16} was observed in *V. cyprinoids*. Molluscs acquire alkanes from the surrounding environment through diet, since they are filter feeders. *T. curta* and *M. trapa* showed similarities in the values of CPI, TAR and LMW/HMW, because of the resemblance in sampling location. Predominance of even chain n-alkanes was observed in *T. telescopium, P. viridis, V. cyprinoids* and *B. spinosa*. n-Alkanes in molluscs were either accumulated from the surrounding environment or formed as a by-product of chemical reactions occurred inside the body.

Mixture of ethyl acetate and hexane fractions (3:20 v/v) of gastropods contain seven sterols namely; cholesta-5, 22-diene-3-ol, cholesterol, cholesta-8-en-3-ol, ergosta-7-en-3-ol, gamma ergosterol, stigmasterol and gamma sitosterol and one sterone (cholesta-4-en-3-one). Cholesterol, cholesta-8-en-3-ol, gamma ergosterol and cholesta-4-en-3-on were the four steroids isolated from bivalves. Cholesterol was the most dominant sterol and gamma sitosterol was the least abundant sterol detected in all the species. Furthermore cholesta-4-en-3-one was the only sterone present in all species. *In silico* activity studies of all the steroids exhibit activity against breast carcinoma cells. Out of the eight steroids, cholesta-5,22-dien-3-ol, cholesta-8-en-3-ol, ergosta-7-en-3-ol and

stigmasterol displayed maximum activity against stomach adenocarcinoma cells, lung carcinoma cells, and colon carcinoma cells and ergosta-7-en-3-ol and stigmasterol show comparable activity against gastric epithelial carcinoma cells. Moreover, all steroids exhibit activity against adenomatous polyposis, hypolipidemic agents, respiratory analeptic, antinfertility in females, antieczematic, antipruritic, dermatologic and prostate disorders treatment with probability to be active (Pa) values greater than 0.7.

Study on the fatty acid fractions of gastropods and bivalves revealed the presence of saturated fatty acids (SFAs), branched chain fatty acids (BCFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs) and non-methylene interrupted fatty acids (NMI). Both SFAs and PUFAs were higher than those of MUFAs and BCFAs. C14:0, C15:0, C16:0, C17:0 and C18:0 were the major SFAs observed in all species, of which C16:0 and C18:0 were predominated. Very small amount of C14:0 was observed in all species, of which *P. viridis* shows highest concentration. TMTD/4,8,12-Me C13:0, *i*-C15:0, *a*-C15:0, *i*-C17:0, *a*-C17:0 and 9,10-Me C18:0 were the major BCFAs identified in all the species. *i*-C15:0, *a*-C15:0, and *i*-C17:0 were detected in *T. telescopium* and those are accumulated from sedimentary microbes via symbiotic process.

C16:1n3, C18:1n9, C18:1n6, C20:1n5, C20:1n9 and C22:1n9 were the major MUFAs detected in all species, of which C16:1 and C18:1 were predominated. Minute amount of 2-hydroxy fatty acids were noticed in bivalves. All gastropods species show large quantity of polyunsaturated fatty acids except *T. curta*. Out of the gastropod species, *B. spinosa* shows

maximum PUFAs content. Eicosapentaenoic acid (EPA), docosadienoic acid, docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) were the major PUFAs observed in both gastropods and bivalves. C18:2n6, C18:4n4, C20:3n6, 5,13-C22:2, C22:5n6 and C22:4n6 were the NMI detected in gastropods and bivalves. In both gastropods and bivalves, n3 fatty acids were predominated over n6 fatty acids.

PUFAs of *B. spinosa* and *M. trapa* shows activity against bacterial pathogens associated with human diseases viz., *Escherichia coli* but inactive against *Aeromonas hydrophila* as well as bacterial pathogens associated with fish diseases viz., *Vibrio parahaemolyticus*, *Vibrio harveyi*, and *Vibrio cholera*. PUFAs of *B. spinosa* show activity against *Staphylococcus aureus* but PUFAs of *M. trapa* was inactive against *S. aureus*. PUFAs of *B. spinosa* and *M. trapa* showed maximum activity against human laryngeal carcinoma (HEp-2 cell line), the corresponding IC_{50} values of them recorded as 27.5 µg mL^{-1} and 54.15 µg mL^{-1}, furthermore DPA, EPA, DHA mixture of *M. trapa* showed IC_{50} value of 59.25 µg mL^{-1}.

In short, the thesis highlights the scope of gastropods (*B. spinosa, T. curta, T. telescopium* and *M. trapa*) and bivalves (*P. viridis* and *V. cyprinoids*) as potential sources of proteins, carbohydrates, lipids, calorific value, alkanes, amino acids, steroids and fatty acids, which are needed for human and animal nutrition. Also, polyunsaturated fatty acid fraction shows antimicrobial and cytotoxic activities. Facts obtained in this investigation also support these molluscs as pharmaceutically important nutritional source of food. *In silico* studies of sterols such as

cholesta-5,22-dien-3-ol, cholesta-8-en-3-ol, ergosta-7-en-3-ol, stigmasterol and gamma ergosterol suggest these sterols as a drug candidate in the cancer therapy.

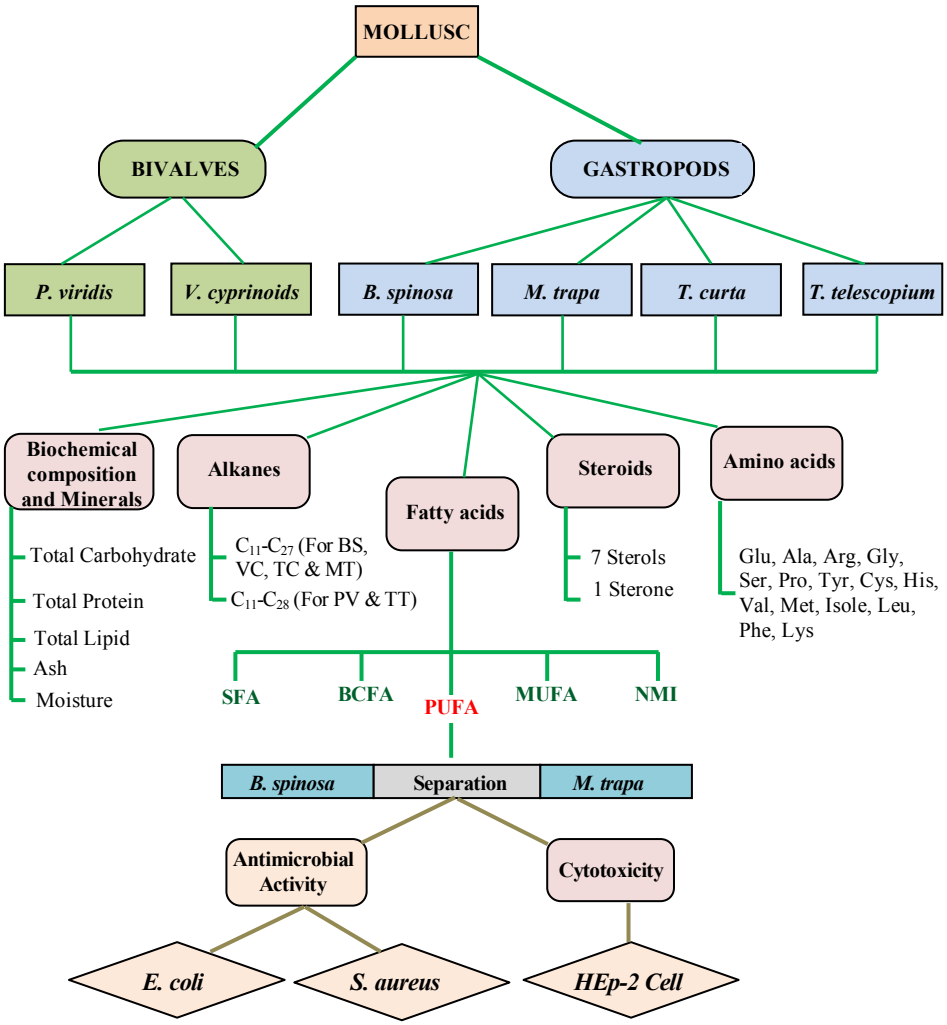

Figure 8.1: Schematic representation of the summary of the study

In future, with respect to the scenario of the over usage of fishery resources and malnutrition, the study strongly supports the possibilities of utilization of these molluscs in day to day diets. In India, bivalves are

used as nutritional diet, but gastropods are uncommon, due to the lack of knowledge on their nutritional quality. Based on the values of average energy content and the presence of large quantity EAA, both gastropods and bivalves can be recommended as a delicious food for humans. Also, isolation and characterization of individual fatty acids and sterols from the molluscs and its cytotoxic and apoptotic properties may expand research in natural product chemistry, and find application in pharmaceutical industry.

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List of Publications

Ragi, A. S.*, Leena, P. P., & Nair, S. M. (2015). Study of lipids and amino acid composition of marine gastropod, *Tibia curta* collected from the Southwest coast of India. *World Journal of Pharmacy and Pharmaceutical Sciences*, 5, 1058−1076.

Ragi, A. S., Leena, P. P., & Nair, S. M.* (2015). Fatty acid composition of the marine gastropods *Telescopium telescopium* and *Bursa spinosa* collected from the Southwest coast of India. *Natural Product Communications* (under review).

Ragi, A. S.*, Leena, P. P., Cheriyan, E., & Nair, S. M. (2017). Heavy metal concentrations in some gastropods and bivalves collected from the fishing zone of South India. *Marine Pollution Bulletin*, 118, 452−458.

Ragi, A. S.*, Leena, P. P., Prashop peter, K. J., & Nair, S. M. (2017). *In silico* biological activities of steroids from the marine gastropods *Telescopium telescopium, Avicenna Journal of Medical Biotechnology*, (Manuscript id: A-10-1412-1) (in press and will be published in vol. 10(3).

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