BIOLOGIA (PAKISTAN)
June, 2020, 66 (I), 47-59

PKISSN 0006 – 3096 (Print)
ISSN 2313 – 206X (On-Line)

The molecular and biochemical characterization of two Marine Gastropods of the Genus *Nerita*

FOZIA HUMAYUN[1], IQRA MUNIR[1], MUHAMMAD SAMEE HAIDER[3], ZARRIEN AYUB[2] & SYED ABID ALI[1,*]

[1]*H.E.J. Research Institute of Chemistry, International Center of Chemical and Biological Sciences (ICCBS), University of Karachi, Karachi-75270, Pakistan*
[2]*Center of Excellence for Marine Biology, University of Karachi, Karachi-75270, Pakistan*
[3] *Food & Marine Resources Research Centre, PCSIR Labs Complex, Karachi-75280, Pakistan*

ARTICLE INFORMAION

Received: 09-08-2019
Received in revised form: 20-04-2020
Accepted: 07-07-2020

***Corresponding Author:**

Syed Abid Ali:
dr.syedabidali@gmail.com

Original Research Article

ABSTRACT

In the present work, two marine gastropods (*Nerita albicilla* and *N. undata*) were subjected to detailed investigation highlighting the biomass distribution, phenotypic and genotypic identification, biochemical composition including total lipid, carbohydrate, protein, fatty acid and amino acid profiles. The sampling was done at Paradise Point and Buleji located at the Sindh Coast of Pakistan. Microstructure and chemistry of the shell was examined via Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectrometry (EDX). The biochemical composition of *Nerita sp.* showed high concentration of proteins followed by lipids and carbohydrates. Hemocyanins, the medicinally important protein was abundantly found in the hemolymph of both species and was confirmed by SEC FPLC and SDS-PAGE analysis. Fatty acid profile showed eight different types of fatty acids, while amino acid analysis showed rich composition of non-essential and essential amino acids. Our results present successful method of identification at genetic level by amplification of mitochondrial gene 16SrDNA. The data from current study also exhibit nutritionally rich status of *Nerita sp.* which can be utilized as significant and valuable marine resource of high energy and can also be an alternative source of balanced diet (food/feed) to overcome the problem of malnutrition in underdeveloped countries.

Keywords: Pakistan, Gastropods, Phylogeny, Fatty acid, *Nerita*

INTRODUCTION

Mollusca, the second largest phylum of animals, contributes over 23% of all marine species and are recognized as dominant taxa of marine ecosystems. Among the mollusks, 80% of the species are represented by gastropods which are the most dominant and species rich class of Mollusca with several economically significant marine organisms reported in the field of fisheries and aquaculture. Ecologically, they are also considered as a valuable model in environmental bio monitoring of genotoxic pollutants (Tewari *et al*., 2002; Sarkar *et al*., 2013; Amin *et al*., 2014).

They have been extensively studied for possessing therapeutically significant proteins and peptides which exhibits antiviral, antimicrobial, antifungal activities (De Smet *et al*., 2011; Cheung *et al*., 2014; Dolashka *et al*., 2016). In addition to this, some of them particularly hemocyanin (respiratory protein) can be used as adjuvant in cancer therapy to boost the immune response (Antonova *et al*., 2014; Gesheva *et al*., 2015; Mora *et al*., 2019; Dolashki *et al*., 2019a; Coates and Paiva *et al*., 2020). Besides exhibiting immunotherapeutic benefits, hemocyanin is potentially antibacterial, antiviral and antineoplastic (Dolashki *et al*., 2019b). Pakistan's coastline is separated into the Baluchistan coast (640 km) starting from westward to the Indian border and Sindh Coast (240km) extending from Karachi eastward to Indian border. Both Sindh and Baluchistan coasts are enriched with various invertebrate fauna including diverse species of Gastropods, which are found to inhabit intertidal zones of sandy, rocky and muddy shores. Semidiurnal tidal range along the coast of Pakistan ranges from 1.8 m to 3.2 m with2.3 m tidal range of Karachi port (Khan *et al*., 2002).

Major coastal areas of Pakistan include,

Author's Contribution: S.A.A. & Z.A., Designed study concept; **F.H., Z.A., M.S.H., S.A.A. & I.M**., Did acquisition of data; **F.H., Z.A., M.S.H., S.A.A. & I.M**., Analyzed and did interpretation of data; **F.H. & S.A.A**., Drafted manuscript: **S.A.A & Z.A**., Revised criticall

Manora channel, Mubarak Village, Manora rocky ledge, Somiani, Old and New Korangi fish harbor, Pacha and Cape Monze (Ahmed & Hameed, 1999; Nasreen *et al*., 2000; Hameed and Ahmed, 2000; Afsar *et al*., 2012; Rahman & Barkati, 2012).These coastal areas of Pakistan support valuable living resources and contribute largely to the annual national economy. Around 617,470 hectares of the Indus delta is covered by Mangroves. As per a recent report, the area of Sindh coast lying under these forests is the sixth largest in the world and it has been estimated that around 200 Kg Mollusks/ ha of mangroves can be produced per annum, if managed properly (Khan, 2010; Ullah *et al*., 2015). The mangrove harbors the commercially important shrimps, gastropods as well as a variety of fishes. Mangroves also serve as a valuable resource of charcoal, timber and fodder for domestic animals (Qureshi *et al*., 2002; Barkati & Rahman, 2005).

As per the previous studies reported from different costal sites of Pakistan so far, gastropods dominate the rocky shore fauna. Several studies reported the species diversity, abundance and biomass of gastropods found on the various rocky shores of Pakistan that includes Buleji, Manora, Cape Monze and Pacha (Ahmed and Hameed, 1999; Hameed & Ahmed, 2000; Nasreen *et al*., 2000; Rahman & Barkati, 2012). However, the taxonomic profiling of mollusks at genomic level remain scarce. The present study is in continuation to our earlier contribution which presented data on morphological identification, biochemical profiles and occurrence of hemocyanin in the predominant species of genera *Siphonaria* and *Thais* for the very first time from Pakistan (Ali *et al*., 2011; Ali *et al*., 2018; Humayun *et al*., 2019).

Genus *Nerita*, the well represented genus in fossil record (since most of species of this genus are extinct now) captured our attention for further species level molecular phylogeny. However, more than 60 known species of this genus are still extant. *Nerita* are usually found restricted to the tropical rocky shore grazing on rocky substrates and few of them inhabit mangroves (Frey and Vermeij, 2008; Postaire *et al*., 2014; Chee *et al*., 2015; Uribe *et al*., 2016). This genus is comprised of more than 450 species, which are though primitively marine, but now the group has adapted to colonize fresh water, ground water and terrestrial habitat (Arquez *et al*., 2014). *Nerita albicilla* has been previously reported for its abundance and biomass from different coastal sites of Pakistan including high tidal zone of Buleji, Manora, Nathiagali, Cape Monze and Sindh mangroves (Ahmed & Hameed, 1999; Rahman and Barkati, 2012; Nazim *et al*., 2015). It is one of the gastropods that contributes the highest biomass at rocky ledge of Buleji (Rahman & Barkati, 2004). *N. albicilla* and *N. undata* have also been reported from the rocky shore of Pacha (Hameed & Ahmed, 2000).

In the present study, specimens of *N. albicilla* and *N. undata* were obtained from Paradise Point and Buleji. Paradise Point is situated between Nathiagali & Buleji. The shoreline is marked with a slope showing a sharp gradient towards the sea, thus the intertidal zone at this site is narrow. The bottom of the shore at Paradise Point is rather flat in comparison to Buleji, which shows elevations and depressions. The other sampling site Buleji, lies in the south west of Karachi facing the Arabian Sea and is defined with rocky ledge along with few sandy pockets. Buleji was chosen as the sampling site since it is an ideal place to study the sea life and has been extensively reported in different studies for its diverse environment and biomass (Afsar *et al*., 2013). Amino acids, the building blocks of protein serve as nutritionally important compound in human diet. They are involved in number of vital cellular processes like synthesis of vitamins, maintenance of growth and reproduction etc. Several studies report that marine gastropods are a good source of non-essential amino acids (NEAA) and specifically the essential amino acids (EAA) that humans cannot synthesize (Srilatha *et al*., 2013; Pereira *et al*., 2013). Marine invertebrates especially mollusks have the capability to synthesize long chain polyunsaturated fatty acids (lcPUFAs) by converting the dietary poly unsaturated fatty acids (PUFAs) into lcPUFAs. Omega-3 fatty acids hold an important place in human and animal diet for its several beneficial effects. Owing to this fact, marine gastropods are being consumed as a potential source containing omega-3 lcPUFAs to meet the growing demand, since they not only have relatively higher proportion of omega-3 but they are also able to desaturate and elongate precursor fatty acids to generate lcPUFAs endogenously (Afsar *et al*., 2014; Bano *et al*., 2014; Zhukova *et al*., 2014; Surm *et al*., 2015). Despite the fact, that gastropods are being consumed as a valuable dietary resource and still holds a special place in the menu of different countries, the information related to its nutritional qualities is still limited to some species only. Several studies shave been conducted on gastropods, but they are poorly characterized in case of their chemical composition and nutritional status (Luo *et al*., 2017).

MATERIALS AND METHODS

Sampling and Identification of specimens

The specimens of *Nerita sp.* (Fig. 1) were collected from Buleji by transect line and quadrate method from the arbitrarily divided three zones that is low, mid and high tidal zones. In comparison to Buleji, the other sampling site i.e. Paradise point has very narrowed intertidal zones therefore, 16 quadrates were randomly fixed in the sampling area and followed by collection of gastropods. The samples after reaching to the laboratory were washed, identified and stored as per the protocol described earlier (Ali *et al*., 2018).

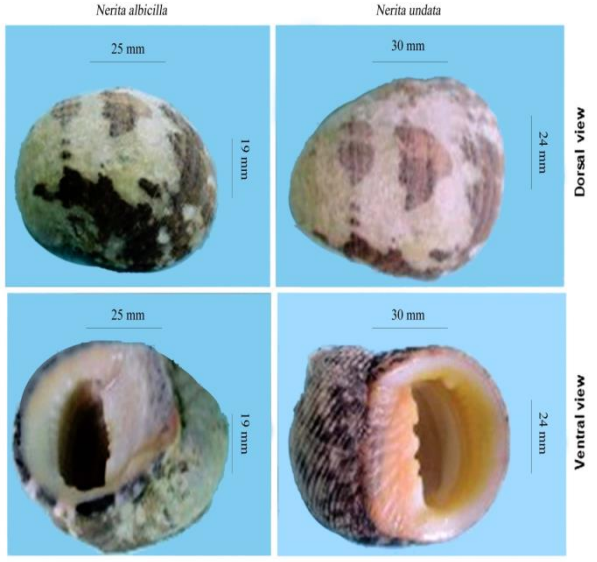

Fig. 1: Dorsal and ventral view of the shells of two major *Nerita sp*. used for shell-based morphological identification.

Scanning electron microscopy (SEM)

To identify the shell microstructure and elemental composition, the cross sections of the shells were coated with a thin layer (approximately 300 Å) of gold coater by JFC-1500 Jeol Japan. The samples were then placed under the microscope SEM (JSM-6380A, Jeol, Japan) operating at 20kV. EDS detector (EX-54175jMU, Jeol Japan) was used for Element characterization. EDS Analysis Station (analytical program) was used for data analysis.

Isolation of Genomic DNA and PCR amplification

Extraction of genomic DNA was done with DNA purification kit (Promega, USA) as per the protocol described earlier (Humayun *et al*., 2019). The samples were estimated for their quantity and integrity of genomic DNA isolated from the tissue by Nano-Drop (ND-2000, Thermo Scientific, USA). The gene,16S rDNA was amplified using the primer 16SH/16S-R (Postaire *et al*., 2014). PCR based amplification was performed using kit (KAPA Biosystems, USA) and PCR (Master cycler ProS, Eppendorf Germany) (Ali *et al*., 2018). Information for the PCR reaction mix, sequences of primers, thermal condition and amplicon size of the amplified gene is provided in Table I. To determine the integrity of amplified products, 2% agarose gel was run for 45 min at 100 volts analyzed via agarose gel electrophoresis with 1kb and Ultralow DNA ladder (Fermentas, USA). After electrophoresis, the bands stained with ethidium bromide were viewed under UV light via UV transilluminator (UVP, UK) and the images were saved.

Table I. List of the PCR primers and conditions used for the amplification of genes for molecular identification of *Nerita* species.

Gene region	Specie	Gen Bank Acc. No	Amplicon Size (bp)	Primers Name	Sequences 5' → 3'	PCR program	References
16S rDNA	*Nerita albicilla*	KU987440	528	16S-R	CGCCTG TTTATC AAAAACAT	94 °C 5 min, (94 °C 40 sec, 52 °C 1 min, 72 °C 5 min) x30, 72 °C 10 min	Dayrat *et al*., 2011
	Nerita undata	KU987441	527	16S-R	CCGGTC TGAACTC AGATCACGT		

Phylogenetic analysis

The amplicons were purified using ExoSap from Affymetrix/USB products and cycle sequencing was carried out at the Centralized Science Laboratory (CSL), University of Karachi on a Genetic Analyzer (Model-3130, Applied BioSystems, USA) following the manufacturer protocol. Sequences were deposited in Gen Bank via Bank IT submission tool. Obtained sequence data was used to perform comparative analysis of *Nerita sp.* and a phylogenetic reconstruction including the 16S rDNA sequences of other *Nerita sp.* available in GenBank. Molecular Evolutionary Genetics Analysis (MEGA) software was used to obtain multiple sequence alignments and phylogenetic relationship (Tamura *et al*., 2013). Phylogenetic analyses of datasets were analyzed via neighbor joining method including the sequence of *N. albicilla* and *N. undata.* and 32 sequences from GenBank comprising of 16S rDNA from other reported *Nerita sp.* to study the relationship and differences among the two *Nerita sp*ecies subjected in the present study and other species reported so far.

Protein finger printing

Molecular sizes of crude proteins from the hemolymph of the two *Nerita sp.* were determined via SDS-PAGE utilizing PROTEAN®3 Cell (Bio-Rad Lab, UK) following the protocol recently described by us (Ali *et al*., 2018).After running for 1 hr at 140 volts, the gels were stained with the dye 0.2% Colloidal Coomassie Brilliant Blue G-250 (Ali *et al*., 2013; 2015).

Size Exclusion Chromatography-Fast Protein Liquid Chromatography (SEC-FPLC) was also performed to verify the results obtained from SDS-PAGE (Ali *et al*., 2011). The supernatant obtained from crude protein samples after centrifugation for 15 min at 14,000 rpm were subjected to FPLC (AKTA-Basic, GE Healthcare, UK) using the column TSK-3000SW (300 x 7.5 mm; 10µ; Tosoh Bioscience, USA). 100 mM Phosphate buffer (pH 7.4) was used to equilibrate, calibrate and run the column. Peaks for proteins and hemocyanins (Hcy) were differentially observed at 280 nm for 340 nm, respectively. UNICORN-5(GE Healthcare, UK) software was used to compare and analyze the chromatograms.

Estimation of total protein

Folin – Ciocalteu Phenol method was applied for the estimation of total content of protein following the protocol described in (Lowry *et al*., 1951).

Estimation of total carbohydrate

The total carbohydrate portion was quantified via phenol – sulphuric acid method (Dubois *et al*., 1956).

Estimation of total lipid

The lipids (percentage) in the tissue were extracted and estimated via chloroform-methanol extraction procedure (Barnes & Blackstock, 1973). Details of the aforementioned three protocols can be obtained from our earlier contribution (Humayun *et al*., 2019).

Amino acid analysis

The tissue samples (2g) of experimental gastropods (*Nerita sp.*) were oven dried at 60 °C for 24 hrs. The dried samples were then finely grounded for estimating the amino acid analysis via amino acid analyzer (10A, Shimadzu Japan) associated with Shim-Pack Amino-Na column (4.6 mm, I.D x 100 mm) following the protocol recently described by Humayun *et al*., (2019).

Extraction and GC analysis of fatty acids

Dry and meshed samples were first processed for fatty acid extraction and subjected to Gas Chromatography was described by Ali *et al*., (2018). The GC Analysis for the determination of the composition of different fatty acids in the sample was performed via Gas Chromatograph (Shimadzu-2014, Japan) associated with Supelco Sp238 column (30 m x 0.25 µm x 0.2 µm). The obtained chromatogram was further analyzed by using software GC Solution.

RESULTS

Biomass of gastropods

Our results reflect an increased biomass (number of animals/m^2 and tissue weight g/m^2) of gastropod at Buleji in comparison to the biomass found at Paradise Point for the same species (Table II and III).

Table II. Variation in the number of animals and tissue dry weight of *Nerita sp*. during the study period of October 2014 to September 2015 at Buleji and Paradise point.

Species	Animal number/m^2		Dry Tissue (g/m^2)	
	Buleji	Paradise Point	Buleji	Paradise Point
Nerita albicilla	122.7	61.7	67.2	18.6
Nerita undata	26.3	-	25.5	-

Table III. Seasonal variation in the total weight (g/m^2) of *Nerita sp*. during the period from October 2014 to September 2015 at Buleji.

Species	Oct' 14	Nov' 14	Dec' 14	Jan' 15	Feb' 15	Mar' 15	Apr' 15	May' 15	Jun' 15	Jul' 15	Aug' 15	Sep' 15	Total
High Tidal Zone													
Nerita albicilla	5.73	0.47	4.29	2.21	3.83	4.29	2.89	2.8	6.03	4.42	5.73	5.82	48.51
Nerita undata	0.67	2.47	-	3.67	1.15	-	-	2.56	-	2.68	-	-	13.2
Mid Tidal Zone													
Nerita albicilla		2.8	0.99	1.69	0.15	2.91	-	1.62	2.8	-	2.8	1.69	18.32
Nerita undata	0.87	1.73	-	0.09	2.91	2.91	-	-	-	-	1.73	2.9	12.27
Low Tidal Zone													
Nerita albicilla	-	-	-	0.42	-	-	-	-	-	-	-	-	0.42

Scanning electron microscopic analysis

SEM analysis of both shells' microstructure revealed identical combination of various elements. SEM-EDX results showed the presence of O, C, Cl, Ca, Na and Ti in the shell microstructures of *N. albicilla* whereas the shell of *N. undata* was also found with the same elements with exception of Ti (Fig. 2; Table IV).

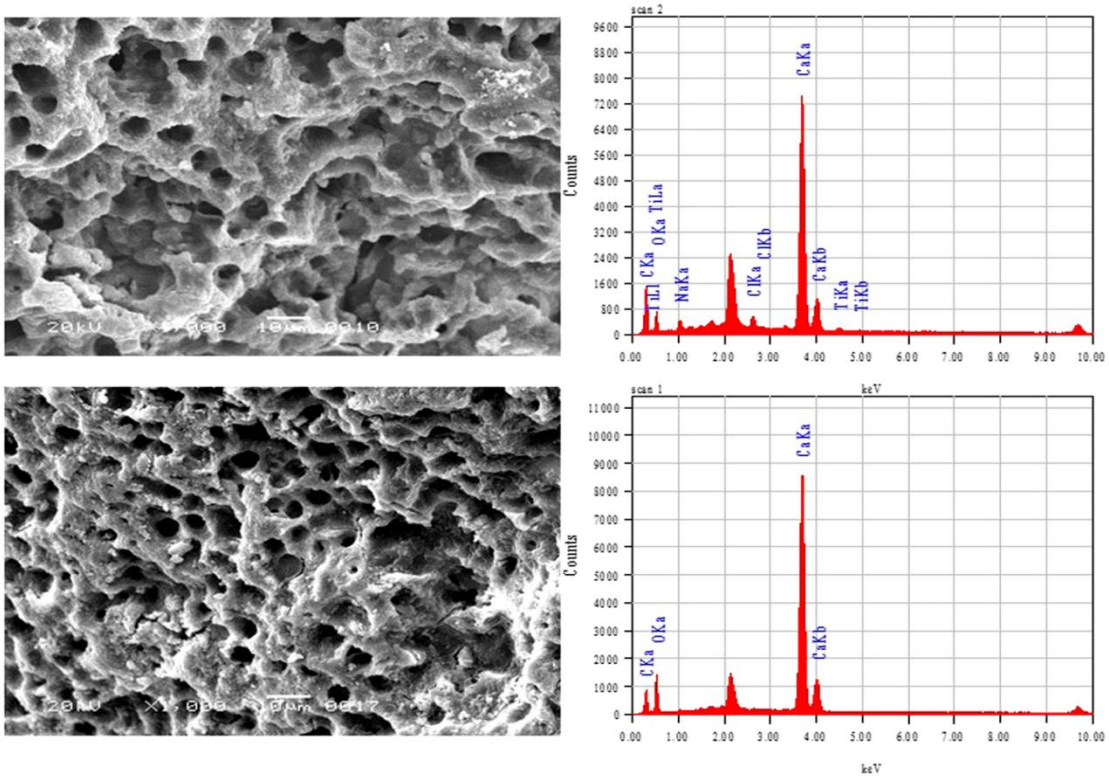

Fig. 2: SEM and EDX spectra obtained from the shell of two *Nerita sp*. showing calcium as the main inorganic component. See "Materials and Methods" for detail.

16S rDNA Amplification and gene Sequencing

PCR based amplification of the mitochondrial gene (i.e. 16S rDNA) was performed successfully (Fig. 3). The morphological differences were further complemented by phylogenetic analysis of 16S rDNA and DNA sequencing (Fig. 4).

The sequences are available from GenBank, under Accession number KU987440 (*N. albicilla*) and KU987441 (*N. undata*). Sequence fragments represented 528bp 16S rDNA in *N. albicilla* and 530bp 16S rDNA in *N. undata* (Table I).

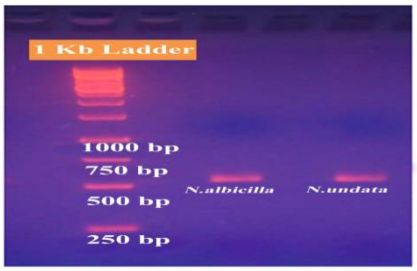

Fig. 3: PCR based amplification of 16S rDNA from two major species of genus *Nerita*.

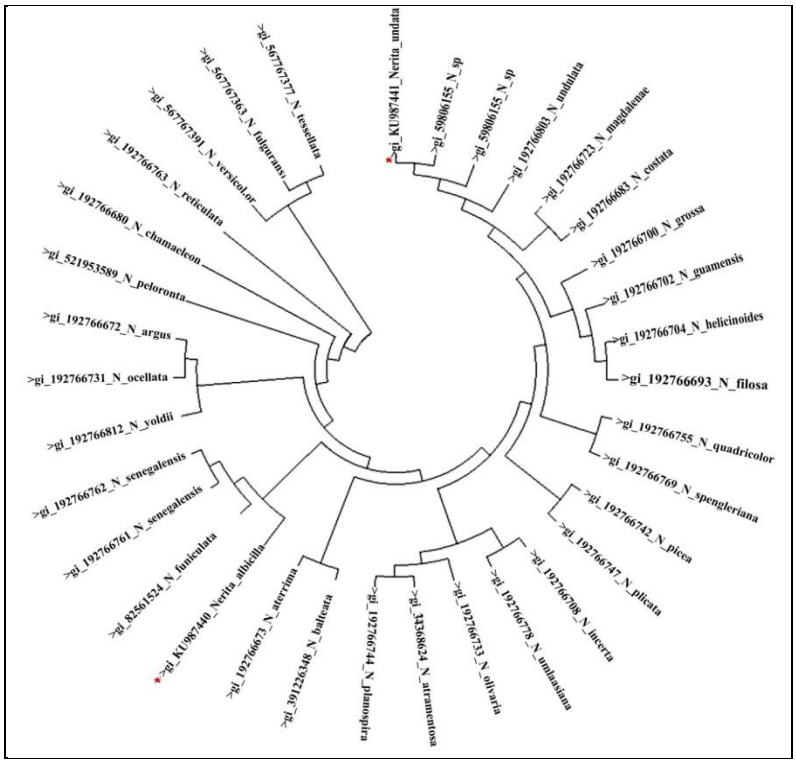

Fig. 4: Phylogeny of out groups based on analysis of 16S rDNA * Indicates the two *Nerita sp*. subjected for molecular identification in the present study

Biochemical characterization

Protein finger printing - A chemotaxonomic approach

Results from electrophoresis (SDS-PAGE) and chromatography (SEC FPLC analysis), showed hemocyanin (the giant respiratory protein complexes) as the major hemolymph protein present in the total hemolymph collected from the two specimens (Fig. 5).

The bands for the high molecular mass Hcy subunits were observed ~380 - 400 kDa. Some bands for proteins which are less abundant were also observed in the two species of the genus *Nerita* with molecular weight falling in the range 45-95 and 20-32 kDa (Fig. 5). Detection of Hcy via chromatography at specific wavelength (i.e. 340 nm) separated the Hcy from other distinct proteins (Fig. 6).

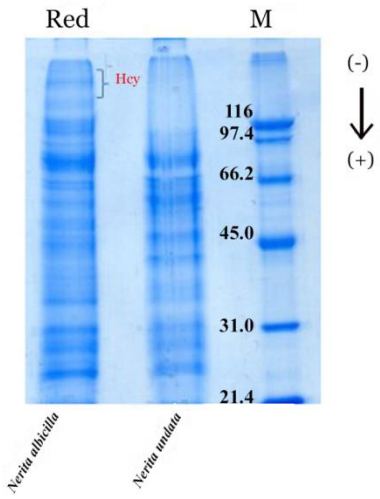

Fig. 5: Biochemical characterization of two *Nerita sp.* using 10% polyacrylamide gel electrophoresis under dissociating/denaturing (Red, reduced) conditions. Lane M is the known molecular weight marker proteins. * Indicates Hcy for predominant blue cupper-containing oxygen carrier protein (i.e. hemocyanins).

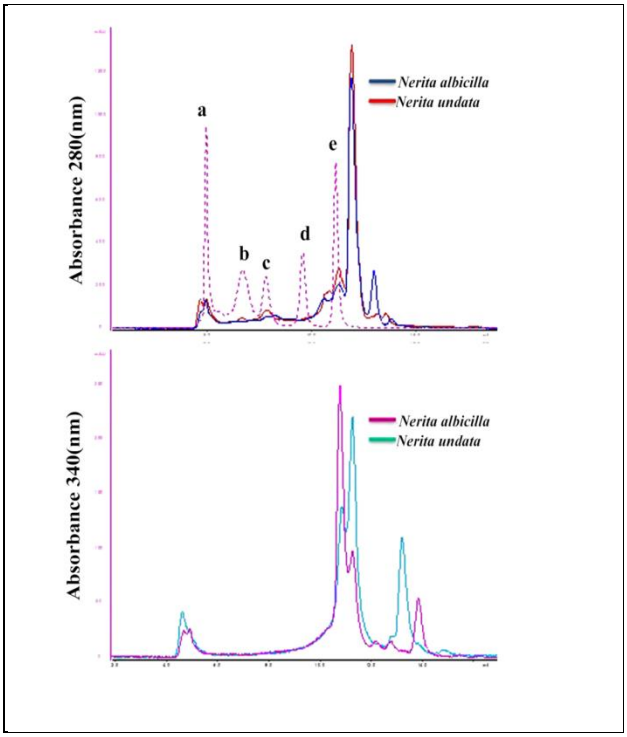

Fig. 6: Size exclusion (SEC FPLC) analysis of the two *Nerita sp.* Top chromatograms detected at 280nm for proteins and bottom 340nm specific for predominant blue cupper-containing oxygen carrier protein (i.e. hemocyanins). Known molecular weight marker proteins (profile with broken line) are labeled as a-e (i.e. 670, 158, 41, 17, and 1.3 kDa, respectively)

Quantification of protein, carbohydrate and lipids content

The total proportion of protein in *N. albicilla* and *N. undata* was found to be 34.33±0.43 and 35.67±3.67, respectively. No significant difference was observed in the total protein content among the two species (F = 0.46; df = 1; P = 0.53). The total carbohydrate portion in *N. albicilla* and *N. undata* were estimated to be 7.43±0.47 and 10.63±0.93 mg/g, respectively with no significant difference among the two species (F=27.98; df= 1; P = 0.006). Similarly, the total lipid contents were determined as 18.6±2.68 and 18.1±0.75 mg/g in *N. albicilla* and *N. undata*, respectively. The lipid content also showed no significant difference (F=0.09; df= 1; P=0.77). The protein proportion was found to constitute the major portion in the tissue followed by lipids and carbohydrates (Fig. 7).

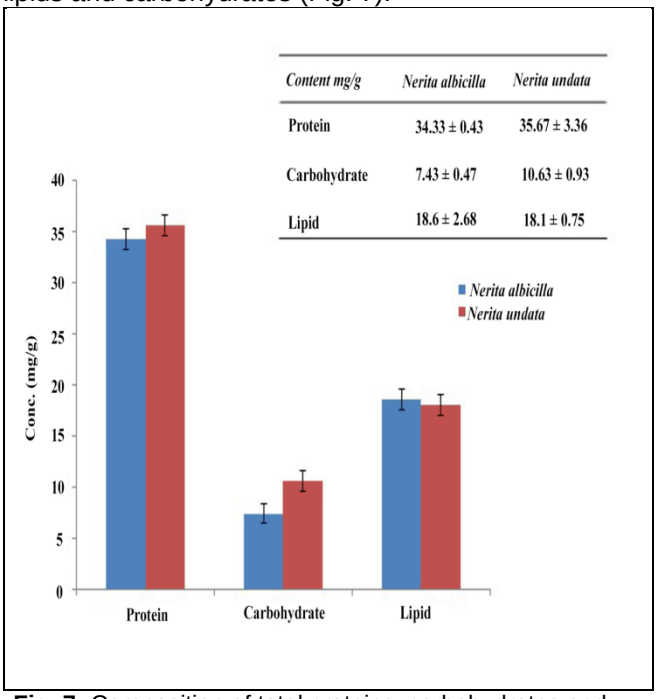

Fig. 7: Composition of total proteins, carbohydrates and lipids in the tissues of *N. albicilla* and *N. undata*. Data is expressed as the mean ± standard deviation of the triplicate of triplicate experiments.

Amino acid analysis

The results from amino acid profiling of the two *Nerita sp.* showed 18 amino acids (EAA and NEAA) (Table V). EAA including (Lysine, Valine, Threonine, Methionine, Leucine, Isoleucine, Histidine, Phenylalanine and Tryptophan) and NEAA (Serine, Proline, Glutamic acid, Aspartic acid, Cysteine, Glycine, Alanine, Tyrosine and

Arginine) were found in the two *Nerita sp.* The total amino acid composition of muscle comprising both EAA and NEAA was estimated to be 598.4 and 579.8mg/g in *N. albicilla* and *N. undata*, respectively.

Table IV. Elemental composition of the shell material determined using energy-dispersive X-ray spectroscopy (SEM-EDS).

Element	*Nerita albicilla*	*Nerita undata* (% mass)
C	13.69	7.99
O	42.57	42.30
Ca	41.63	48.73
Na	1.78	1.53
Cl	1.87	1.36
Ti	0.85	-

Table V. Amino acid composition analysis of the two *Nerita* species.

Amino Acids	*Nerita albicilla* (mg/g sample)	*Nerita undata* (mg/g sample)
Non-Essential Amino Acids		
Aspartic acid	61.4	39.4
Serine	6.3	6.2
Glutamic acid	80.4	88.7
Proline	35.3	26.7
Glycine	75.3	78.7
Alanine	79.2	69.8
Cysteine	7.4	5.7
Tyrosine	In trace	23.6
Arginine	125.5	7.1
Essential Amino Acids		
Threonine	3.6	3.9
Valine	22.2	13.5
Methionine	14.3	15
Isoleucine	In trace	17.2
Leucine	33.2	55.4
Phenylalanine	38.2	24.3
Histidine	In trace	2.3
Tryptophan	22	76.6
Lysine	0.4	25.7
Total	598.4	579.8

Fatty acid analysis

Fatty acid profile of the two *Nerita sp.* showed the presence of eight (8) fatty acids (Table VI). Eicosanoic acid (20:0), Stearic acid (18:0), Palmitic acid (16:0), and Myristic acid (14:0) were found among the saturated fatty acids while, Palmitoleic acid and Oleic acid were found from the monounsaturated class of fatty acids. Eicosapentaenoic and Linolenic acid were reported from the PUFAs. Among the total fatty acids found in *N. albicilla* and *N. undata*, SFA constituted around 54.74% and 36.26%, respectively. While, MUFAS make up 3.79% and 9.3% of the total fatty acids in *N. albicilla* and *N. undata*, respectively. PUFAS in *N. albicilla* and *N. undata* were estimated as 5.60% and 6.77%, respectively. The total no of fatty acid in relative amount (% composition) is estimated as 64.13% and 52.33% in *N. albicilla* and *N. undata*, respectively.

Table VI. Fatty acid profiles of two *Nerita sp.* PUFAs- polyenoic unsaturated fatty acids. SFAs- saturated fatty acids; MUFAs; monoenoic unsaturated fatty acids.

Fatty Acids	*Nerita albicilla*	*Nerita undata* (% Total FA)
Saturated Fatty Acids		
Myristic acid (C14:0)	4.87	4.69
Palmitic acid (C16:0)	36.63	21.22
Stearic acid (C18:0)	12.59	10.00
Eicosanoic acid (C20:0)	0.65	0.35
∑SFA	54.74	36.26
Monounsaturated Fatty Acids		
Palmitoleic acid (C16:1)	1.95	2.61
Oleic acid (C18:1)	1.84	6.69
∑MUFA	3.79	9.3
Polyenoic unsaturated Fatty Acids		
Linolenic acid (C18:3)	5.60	6.11
Eicosapentaenoic acid (C20:5)	-	0.66
∑PUFA	5.60	6.77
Total	64.13	52.33

DISCUSSION

The Biomass distribution was found higher at the site of Buleji as compared to the other sampling site the Paradise point. The possible reason for variation in the biomass at the two sampling sites could be due to the availability of food. Another factor behind the bare fauna at paradise point might be due to the less dense algal population since the variation in the gastropod's population can be correlated to higher algal

productivity (Ahmed and Hameed, 1999). Karachi Nuclear Power Plant, (operational since 1972) located near paradise point is another possible reason behind its scant fauna. The targeted species were found more in number at the high tidal zone in comparison to the *Thais* species recently reported by us (Ali *et al*., 2018) which was more favored at the mid tidal zone.

Molluskan shell is a combination of different biocomposites, mainly comprising of a brittle material (calcium carbonate). This provides high mechanical strength to the shell along with high fracture toughness (Rajabi *et al*., 2014; Upadhyay *et al*., 2016). It has been reported in a study that the thickness of the shell is correlated with the temperature of water. In cold water, thinner shells are frequently found since, the large amount of calcium carbonate is required to saturate cold water in comparison to warm water. Also, with decreasing water temperature, the dissolution rates increase (Bourdeau *et al*., 2015). The results obtained via SEM-EDX were found consistent in the two studied shells. A prominent calcium peak was observed showing Ca as the dominant element of the shell and other smaller peaks that relates to minor elements (i.e. O, Na, Mg, Cl, Ti) were also observed.

Identification of gastropods at genomic level via the amplification of housekeeping gene (16S rDNA) revealed differences among the two studied gastropods at species level and supported the purpose of establishing the phylogenetic analysis and evaluation of the common ancestors of the *Nerita sp.* studied in the presented study. A high level of homoplasy is reported in the morphological characters of shells and the shells often share common characters which makes the identification of shells at morphological level more controversial, but this problem can be resolved by establishing phylogenetic relationships among different taxons of mollusks via amplification of housekeeping genes, like 16S, 18S, 28S and COI (White *et al*., 2011; Silva *et al*., 2017). Previous studies on the morphological identification of shells reported factors like extreme temperature, desiccation stress, habitat, plasticity in shell morphology that are responsible for the changes in the shell pattern and color. Thus, identification and phylogenetic relationships of shells based on the DNA barcoding provides fruitful results in such studies (Teske *et al*., 2007; Teske *et al*., 2011; Kumbhar & Rivonker, 2012).

The biochemical composition of the studied gastropods in the present study showed high protein content followed by lipid and carbohydrates. Comparing the percentage composition of macronutrients in *Nerita sp.*, with other reported studies *Babylonia spirata* (family Babyloniidae), was found to possess carbohydrate (16.65%), protein (53.86%) and lipid (9.3%) in its body tissue (Periyasamy *et al*., 2011). Another gastropod, *Burssa spinosa*, was estimated for percentage composition of macronutrients in different parts of tissue (i.e. foot, mantle and gonad), with gonad comprising maximum content of protein (27.91%), carbohydrate (7.7%) and lipid (4.9%) (Babu *et al*., 2010). The protein content from edible part of *Rapana venosa* meat was reported to be 191.5 mg/g (Luo *et al*., 2017). *Siphonaria sp*. in one of our earlier reported study, showed 107.1 mg/g protein, 36.1 mg/g carbohydrate, and 92.5 mg/g lipid in the body tissue (Ali *et al*., 2011). In another recent study reported by us *Thais sp*. was found with 36.02 mg/g protein, 17.26 mg/g carbohydrate, and 17.11 mg/g lipid in the body tissue (Ali *et al*., 2018).

Amino acids hold an important portion of human diet. Besides being building blocks of proteins, they also play vital role in several cellular processes. Marine gastropods have been found as good alternative source of diet rich in amino acids and PUFAS (Pereira *et al*., 2013). *N. albicilla* and *N. undata* were detected with eighteen amino acids. Among the NEAA, glutamic acid was found maximum with 80.4mg/g and 88.7mg/g in *N. albicilla* and *N. undata*, respectively. Majority of the amino acids were detected in both the samples, except the tyrosine and histidine which were detected in trace amount in *N. albicilla.* The sum of amino acid composition from muscle (including EAA and NEAA) in *N. albicilla* (598.4mg/g) and *N. undata* (579.8 mg/g) is quite higher in comparison to the total amino acid composition of other reported gastropods, as 9.911 mg/g in *Babylonia spirata* (Periyasamy *et al*., 2011) whereas *Bursa spinosa* was reported with both EAA (50.01%) and NEAA (46.79%) (Babu *et al*., 2010). A recent study reported seventeen different amino acids from marine snail *Rapana venosa*, among those eight were EAA. The essential amino acids index (EAAI) from three different parts is reported to be 70.99, 85.70 and 38.53 in meat, visceral mass and operculum respectively (Luo *et al*., 2017). The total amino acid composition in *Siphonaria sp.* was reported in the range of 529.4 - 673.8mg/g (Humayun *et al*., 2019) and from *Thais sp.* it is reported as 653.2-709.2 mg/g of the total amino acid of the body tissue (Ali *et al*., 2018) which are quite comparable to the amino acid composition shown in the present study.

Several marine organisms have been investigated and reported to be an alternative

source of polyunsaturated fatty acids (PUFA) for their reported anti-inflammatory and anticancer effects. Among the PUFA, ω3 compounds, like eicosapentaenoic acid, 20:5*n*-3 and docosahexaenoic acid, 22:6*n*-3 are being regarded as bioactive compound in the prevention of several cardiovascular diseases (Babu *et al*., 2010; Pereira *et al*., 2013; Zhukova *et al*., 2014). Moreover, they are also a perfect alternative diet source providing several fatty acids significant to the human health (Afsar *et al*., 2014; Bano *et al*., 2014; Saito & Aono, 2014; Saito & Ioka, 2019). Considering this fact, marine gastropods may be a potential source of lipid bioactive compounds; and thus, studied gastropods were screened for their fatty acid profile. The total fatty acid composition in *N. albicilla* and *N. undata* was found to be 64.13% and 52.33%, respectively. Saturated fatty acids (SFA) were found predominant comparative to MUFA and PUFA. Eicosanoic acid, Stearic acid, Palmitic acid and Myristic acid were reported from the class of SFA. Oleic acid and Palmitoleic acid were observed among the MUFA. Linolenic acid and Eicosapentaenoic acid were reported from the group of PUFA. However, Eicosapentaenoic acid was not detected in the *N. undata.*

To compare our results with fatty acid profile of other reported gastropod species, it is noted that around 27.40% to 28.99% of SFA and 45.97% to 47.84% of PUFA was reported in *Siphonaria sp*. (Bano *et al*., 2014) which are quite comparable to the fatty acid profile of *N. albicilla* and *N. undata to Thais sp.* which are reported with 36-67.8% of the total fatty acid composition recently reported by us (Ali *et al*., 2018). In another such study, *T. dolium,* showed 3.13% SFA, 1.05% MUFA and 3.55% PUFA, whereas *P. glaucum* was found to possess 2.22% SFA, 0.96% MUFA and 3.16% PUFA (Babu *et al*., 2011). In a recently reported study, *Rapana venosa* (marine snail) was found to contain 26 different kinds of fatty acids including SFA, MUFA and PUFA from different parts of body including meat, visceral mass and operculum (Luo *et al*., 2017).

There is an immediate need for detail bioinformatics, phylogenetic studies and more sequence data, especially from other set of genes. Approaching mitochondrial protein-coding genes analysis could be another possibility (Castro *et al*., 2010). Besides the recent efforts for taxon sampling and identification of new species based on shell morphology, several taxons are still relatively poorly studied at the genomic level. One of the reasons behind the lagged genomics of gastropod mollusks could be due to undeveloped genomic resources for many species (Amin *et al*., 2014).

CONCLUSIONS

The present study documents the identification of the genus *Nerita* by shell-based morphology along with SEM-EDS well supported with genetic evidence (genetic bar-coding) through molecular analysis. Similarly, the results obtain from protein finger printing and chemotaxonomy by SDS-PAGE under denaturing/dissociated conditions is quite complementary with the results obtained by SEC-FPLC. Thus, the genotypic and phenotypic techniques provided excellent protein/DNA fingerprints for the identification based on chemotaxonomy of the *Nerita* species. Moreover, the results from the biochemical composition of the studied gastropods in the present work reflects their nutritional status and shows that the reported *Nerita sp.* can be a potential valuable resource of high-quality proteins, lipids and carbohydrates.

ACKNOWLEDGEMENTS

This project was financially supported by Pakistan Science Foundation (PSF/Res/S-KU/Bio (422) to SA Ali and Z Ayub) and Higher Education Commission (HEC No. 20-1339/R&D/09; to SA Ali) Islamabad, Pakistan.

REFERENCES

Afsar, N., Siddiqui, G., Ayub, Z. & Fatima, H., 2014. Fatty acid profile of Neogastropod species *Babylonia spirata* from Manora channel, the intensive shipping area of Pakistan. *International Journal of Biology and Biotechnology*.,11: 23-28.

Afsar, N., Siddiqui, G. & Ayub, Z., 2013. Study of reproductive cycle of two Archaeo gastropods, *Turbo coronatus* and *Monodonta canalifera*. *Pakistan Journal of Zoology*.,45: 459-467.

Afsar, N., Siddique, G. & Ayub, Z., 2012. Update of records of selected Prosobranch gastropod species found along the coasts of Sindh and Baluchistan, Pakistan. *Pakistan Journal of Zoology*.,44: 267-275.

Ahmed, M. & Hameed, S.,1999. A comparative study on the biomass of animals and seaweeds of the rocky shore of Buleji near Karachi, Pakistan. *Pakistan Journal of Biological Sciences*.,2: 365-369.

Ali, S. A., Humayun, F., Munir, I., Ahmad, S., Ayub, Z., Fatima, H., Zehra, L. & Haider, M. S., 2018. Morphological, Molecular, Biochemical and Nutritional Characterization of Three Major *Thais* Species from the

Sindh Coast of Pakistan. *The Open Food Science Journal*., 10(1):33-45.

Ali, S.A., Jackson, T.N., Casewell, N.R., Low, D.H., Rossi, S., Baumann, K., Fathinia, B., Visser, J., Nouwens, A., Hendrikx, I., Jones, A., Undheim, E. & Fry, B.G., 2015. Extreme venom variation in Middle Eastern vipers: a proteomics comparison of *Eristicophis macmahonii*, *Pseudocerastes fieldi* and *Pseudocerastes persicus*. *Journal of Proteomics*.,116: 106-13.

Ali, S.A., Yang, D., Jackson, T.N.W., Undheim, E.A.B., Koludarov, I., Wood, K., Jones, A., Hodgson, W.C., McCarthy, S., Ruder, T. & Fry, B.G., 2013. Venom proteomic characterization and relative antivenom neutralization of two medically important Pakistani Elapid snakes (*Bungarus sindanus* and *Naja naja*). *Journal of Proteomics*.,89: 15-23.

Ali, S.A., Ayub, Z., Bano, A. & Fatima, H.,2011. Biochemical studies on *Siphonaria* (Gastropoda: Pulmonata) from the Karachi coast of North Arabian Sea. *Pakistan Journal of Zoology*.,43: 1085-1093.

Amin, S., Prentis, P.J., Gilding, E.K. & Pavasovic, A., 2014. Assembly and annotation of a non-model gastropod (*Nerita melanotragus*) transcriptome: a comparison of de novo assemblers. *BioMed Central Research Notes*.,1:7e488.

Antonova, O., Dolashka, P., Toncheva, D., Rammensee, H.G., Floetenmeyer, M. & Stevanovic, S., 2014. In vitro antiproliferative effect of *Helix aspersa* hemocyanin on multiple malignant cell lines. *Zeitschrift für Naturforschung*. C., 69: 325-34.

Arquez, M., Colgan, D. & Castro, L.R.,2014. Sequence and comparison of mitochondrial genomes in the genus Nerita (Gastropoda: Neritimorpha: Neritidae) and phylogenetic considerations among gastropods. *Marine Genomics*.,15:45-54.

Babu, A., Venkatesan, V. & Rajagopal, S., 2011. Fatty acid and amino acid compositions of the gastropods, *Tonna dolium* (linnaeus, 1758) and *Phalium glaucum* (linnaeus, 1758) from the gulf of mannar, southeast coast of India. *Annals Food Science and Technology*.,12: 159-163.

Babu, A., Kesavan, K., Annadurai, D. & Rajagopal, S., 2010. *Bursa spinosa* - a Mesogastropod fit for human consumption. *Advance Journal of Food Science and Technology*.,2: 79-83.

Bano, A., Ayub, Z. & Siddiqui, G., 2014. Fatty acid composition of three species of *Siphonaria* (Gastropoda: Pulmonata) in Pakistan. *Pakistan Journal of Zoology*.,46: 813-818.

Barkati, S. & Rahman, S., 2005. Species composition and faunal diversity at three sites of Sindh Mangroves. *Pakistan Journal of Zoology*.,37: 17-31.

Barnes, H. & Blackstock, J., 1973. Estimation of lipids in marine animals and tissues: detailed investigation of the sulphophosphovanillin method for total lipids. *Journal of Experimental Marine Biology and Ecology*.,12: 103-118.

Bourdeau, P.E., Butlin, R.K., Brönmark, C., Edgell, T.C., Hoverman, J.T. & Hollander, J.,2015. What can aquatic gastropods tell us about phenotypic plasticity? A review and meta-analysis. *Heredity (Edinburgh)*., 115:312-21.

Castro, L.R. & Colgan, D.J., 2010. The phylogenetic position of Neritimorpha based on the mitochondrial genome of *Nerita melanotragus* (Mollusca: Gastropoda). *Molecular Phylogenetics and Evolution*., 57:918-23.

Chee, S.Y., 2015. Limitations of cytochrome oxidase I for the barcoding of Neritidae (Mollusca: Gastropoda) as revealed by Bayesian analysis. *Genetics and Molecular Research*., 14:5677-84.

Cheung, R.C., Wong, J.H., Pan, W.L., Chan, Y.S., Yin, C.M., Dan, X.L., Wang, H.X., Fang, E.F., Lam, S.K., Ngai, P.H., Xia, L.X., Liu, F., Ye, X.Y., Zhang, G.Q., Liu, Q.H., Sha, O., Lin, P., Ki, C., Bekhit, A.A., Bekhit, Ael-D., Wan, D.C., Ye, X.J., Xia, J. & Ng, T.B., 2014. Antifungal and antiviral products of marine organisms. *Applied Microbiology and Biotechnology*., 98: 3475-3494.

Coates, C. J. & Costa-Paiva, E. M., 2020. Multifunctional roles of hemocyanins. In: *Vertebrate and Invertebrate Respiratory Proteins, Lipoproteins and other Body Fluid Proteins* (pp. 233-250). Springer, Cham, Switzerland.

De Smet, L., Dimitrov, I., Debyser, G., Dolashka-Angelova, P., Dolashki, A., Van Beeumen, J. & Devreese, B., 2011. The cDNA sequence of three hemocyanin subunits from the garden snail *Helix lucorum*. *Gene*., 487: 118-28.

Dolashka, P., Dolashki, A., Van Beeumen, J., Floetenmeyer, M., Velkova, L., Stevanovic, S. & Voelter, W., 2016. Antimicrobial activity of Molluscan hemocyanins from *Helix* and

Rapana snails. *Current Pharmaceutical Biotechnology*., 17: 263-270.

Dolashki, A., Dolashka, P., Stenzl, A., Stevanovic, S., Aicher, W. K., Velkova, L., Velikova, R. & Voelter, W., 2019a. Antitumour activity of *Helix* hemocyanin against bladder carcinoma permanent cell lines. *Biotechnology, Biotechnological Equipment*., 33(1): 20-32.

Dolashki, A., Velkova, L., Voelter, W. & Dolashka, P., 2019b. Structural and conformational stability of hemocyanin from the garden snail *Cornu aspersum*. *Zeitschrift für Naturforschung C.*, 74(5-6): 113-123.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. & Smith, F., 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*.,28: 305-356.

Frey, M.A. & Vermeij, G.J., 2008. Molecular phylogenies and historical biogeography of a circumtropical group of gastropods (Genus: *Nerita*): implications for regional diversity patterns in the marine tropics. *Molecular Phylogenetics and Evolution*.,48: 1067-86.

Gesheva, V., Chausheva, S., Stefanova, N., Mihaylova, N., Doumanova, L., Idakieva, K. & Tchorbanov, A., 2015. *Helix pomatia* hemocyanin - A novel bio-adjuvant for viral and bacterial antigens. *International Immunopharmacology*., 26: 162-168.

Hameed, S. & Ahmed, M., 2000. Distribution and abundance of intertidal macro-invertebrates on the rocky bench of Pacha, near Karachi. *International Journal of Ecology and Environmental Sciences*.,26: 149-200.

Horwitz, W. & Latimer, G.W.,2012. Amino Acids in feed, AOAC official method 994.12, Official Methods of Analysis of AOAC international, nineteenth ed. AOAC international press, Maryland, USA.

Humayun, F., Ayub, Z., Haider, M.S. & Ali, S.A., 2019. Morphological identification, molecular bar-coding and nutritional value of *Siphonaria* species from Sindh Coast, Pakistan. *Biologia (Pakistan)*. 65(1): 1-10.

Khan, N., 2010. Marine resources in Pakistan: A tentative inventory. *National Institute of Oceanography, Karachi*,pp834-841.

Khan, T.M.A., Razaq, D.A., Chaudhry, Q.U.Z., Quadir, D.A., Kabir, A. & Sarker, M.A., 2002. Sea level variations and geo-morphological changes in the coastal belt of Pakistan. *Marine Geodesy*.,25: 159-174.

Kumbhar, J.V. & Rivonker, C.U., 2012. A new record of *Morula anaxares* with a description of the radula of three other species from Goa, central West Coast of India (Gastropoda: Muricidae). *Turkish Journal of Fisheries and Aquatic Sciences.,*12: 189-197.

Luo, F., Xing, R., Wang, X., Peng, Q. & Li, P., 2017. Proximate composition, amino acid and fatty acid profiles of marine snail *Rapana venosa* meat, visceral mass and operculum. *Journal of the Science of Food and Agriculture.*, 97(15): 5361-5368.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L.& Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*.,193: 265-275.

Mora, J.J, Campo, M.D, Villar, J., Paolini, F., Curzio, G., Venuti, A., Jara, L., Ferreira, J., Murgas, P., Lladser, A., Manubens, A. & Becker, M.I., 2019. Immunotherapeutic potential of Mollusk hemocyanins in combination with human vaccine adjuvants in murine models of oral cancer. *Journal of Immunology Research*., 2019: e1-19

Nasreen, H., Ahmed, M. & Hameed, S., 2000. Seasonal variation in biomass of marine macro-invertebrates occurring on the exposed rocky ledge of Manora Island, Karachi, Pakistan. *Pakistan Journal of Zoology*.,32: 343-350.

Nazim, K., Ahmed, M., Khan, M.U., Shaukat, S.S., Khokhar, A. & Durrani, A.T.H., 2015. Population distribution of Mollusks in Mangrove Forests, Pakistan. *Fuuast Journal of Biology*., 5: 37-41.

Pereira, D.M., Valentão, P., Teixeira, N. & Andrade, P.B., 2013. Amino acids, fatty acids and sterols profile of some marine organisms from Portuguese waters. *Food Chemistry.,*141: 2412-7.

Periyasamy, N., Srinivasan, M., Devanathan, K. & Balakrishnan, S., 2011. Nutritional value of gastropod *Babylonia spirata* (Linnaeus, 1758) from Thazhanguda, Southeast coast of India. *Asian Pacific Journal of Tropical Biomedicine*,1: S249–S252.

Postaire, B., Bruggemann, J.H., Magalon, H. & Faure, B., 2014. Evolutionary dynamics in the southwest Indian ocean marine biodiversity hotspot: a perspective from the rocky shore gastropod genus *Nerita*. *PLoS One*., 9: e95040.

Qureshi, R.M., Mashiatullah, A., Fazil, M., Ahmad, E., Tasneem, M.A., Khan, H.A. & Sajjad, M.I., 2002. Contributions of Pakistan in the IAEA/RCA/UNDP regional project on

management of marine coastal environment and its pollution. *Science. Vision.,*7, 210-223.

Rahman, S. & Barkati, S., 2012. Spatial and temporal variations in the species composition and abundance of benthic molluscs along 4 rocky shores of Karachi. *Turkish Journal of Zoology*.,36: 1-16.

Rahman, S. & Barkati, S., 2004. Development of abundance – biomass curves indicating pollution and disturbance in molluscan communities on four beaches near Karachi, Pakistan. *Pakistan Journal of Zoology*., 36: 111-123.

Rajabi, H., Darvizeh, A., Shafiei, A., Eshghi, S. & Khaheshi, A., 2014. Experimental and numerical investigations of Otalalactea's shell-I. Quasi-static analysis. *Journal of the Mechanical Behavior of Biomedical Materials.,*32:8-16.

Saito, H. & Ioka, H., 2019. Lipids and fatty acids of Sea Hares *Aplysia kurodai* and *Aplysia juliana*: High levels of Icosapentaenoic and n-3 Docosapentaenoic acids. *Journal of Oleo Science*., 68(12): 1199-1213.

Saito, H. & Aono, H., 2014. Characteristics of lipid and fatty acid of marine gastropod *Turbo cornutus*: high levels of arachidonic and n-3 docosapentaenoic acid. *Food Chemistry*.,145:135-44.

Sarkar, A., Bhagat, J., Ingole, B.S., Rao, D.P. & Markad, V.L., 2013. Genotoxicity of cadmium chloride in the marine gastropod *Nerita chamaeleon* using comet assay and alkaline unwinding assay. *Environmental Toxicology*.,30:177-87.

Silva, C.O., Simões, T., Novais, S.C., Pimparel, I., Granada, L., Soares, A.M., Barata, C. & Lemos, M.F., 2017. Fatty acid profile of the sea snail *Gibbula umbilicalis* as a biomarker for coastal metal pollution. *The Science of the Total Environment*., 586: 542-550.

Srilatha, G., Chamundeeswari, K. Ramamoorthy, K., Sankar, G. & Varadharajan, D., 2013. Proximate, amino acid, fatty acid and mineral analysis of Clam, *Meretrix casta* (Chemnitz) from Cuddalore and Parangipettai Coast, South East Coast of India. *Journal of Marine Biology & Oceanography*., 2: 1000111

Surm, J.M., Prentis, P.J. & Pavasovic, A., 2015. Comparative analysis and distribution of Omega-3 lcPUFA biosynthesis genes in Marine Molluscs. *PLoS One*., 10:e0136301

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S., 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*.,30:2725-9

Teske, P.R., Papadopoulos, I., Mmonwa, K.L., Matumba, T.G., McQuaid, C.D., Barker, N.P. & Beheregaray, L.B., 2011. Climate-driven genetic divergence of limpets with different life histories across a southeast African marine biogeographic disjunction: different processes, same outcome. *Molecular Ecology*., 20: 5025-5041.

Teske, P.R., Barker, N.P. & McQuaid, C.D.,2007. Lack of genetic differentiation among four sympatric southeast African intertidal limpets (Siphonariidae): phenotypic plasticity in a single species?. *Journal of Molluscan Studies.,*73: 223-228.

Tewari, A., Raghunatan, C., Joshi, H.V. & Khambhaty, Y., 2002. Imposex in rocks whelks *Thais* and *Ocenebra* species (Mollusca, Neogastropoda, Muricidae) from Gujrat coasts. *Indian Journal of Marine Sciences*.,31: 321-328.

Ullah, Z., Zehra, I., & Gondal, M. A., 2015. Species diversity, distribution and seasonal abundance in Mangrove associated Molluscs along the Karachi Coast, Pakistan. *Journal of Bioresource Management*., 2(3):9-22.

Upadhyay, A., Thiyagarajan, V. & Tong, Y., 2016. Proteomic characterization of oyster shell organic matrix proteins (OMP). *Bioinformation.*, 12: 266-278.

Uribe, J.E., Colgan, D., Castro, L.R., Kano, Y. & Zardoya, R., 2016. Phylogenetic relationships among super-families of *Neritimorpha* (Mollusca: Gastropoda). *Molecular Phylogenetics and Evolution*., 104:21-31.

White, T.R., Conrad, M.M., Tseng, R., Balayan, S., Golding, R., de Frias Martins, A.M. & Dayrat, B.A., 2011. Ten new complete mitochondrial genomes of Pulmonates (Mollusca: Gastropoda) and their impact on phylogenetic relationships. *BioMed Central Evolutionary Biology*.,10: 11e295.

Zhukova, N.V., 2014. Lipids and fatty acids of nudibranch Mollusks: potential sources of bioactive compounds. *Marine Drugs.,*12: 4578-4592.