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MOLECULAR CHARACTERIZATION OF PROTEIN AND ANTIOXIDANT CAPACITY OF *TURBO BRUNNEUS* R. *CYPRAEA ANNULUS* L. AND *BABYLONIA SPIRATA* L.

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ABSTRACT: The aim of this study was to analyze the molecular weight of protein and *in-vitro* antioxidant activity of marine gastropods *Turbo brunneus*, *Cypraea annulus,* and *Babylonia spirata*. The molecular weight of the protein was determined using SDS-PAGE, and its antioxidant potential was carried out by DPPH radical scavenging activity, reducing power activity and H_2O_2 radical scavenging activity. The molecular weight of protein varies from 54 kDa in *T. brunneus*, 38 kDa to 60 kDa in *C. annulus*, and 44 kDa to 116 kDa in *B. spirata*. The DPPH scavenging effect was high in *Cypraea annulus* (78.30%) followed by Babylonia spirata (65.20%) and Turbo brunneus (64.89%). *C. annulus* showed the highest reducing power of 95.36% at 500 µg/ml concentrations and lowest reducing the power of 52.07% at 100 µg/ml concentrations in the *T. brunneus*. The maximum radical scavenging activity was reported in *T. brunneus* (71% at 150 µg/ml concentrations), and minimum activity was reported in *C. annulus* (8.34% at 50 µg/ml concentration). The results show that tissue extracts of three marine gastropods found to possess good antioxidant activity and confirm their use as natural antioxidants in the future.

INTRODUCTION: Marine organisms are considered to be a magnificent source of bioactive molecules. Over the few decades, several new therapeutic agents derived from marine origin have entered preclinical and clinical trials [1]. Molluscs are viewed as one of the important organisms to derive bioactive compounds. They also contain rich nutrients that are valuable to people of all ages [2]. Marine and freshwater products have become attractive as a nutraceutical and functional foods and as a material for the development of drugs and specific health foods [3].

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Proteins are biologically active compounds abundantly present in living organisms consisting of two or more amino acids linked by a peptide bond. Thousands of peptides have been identified from animals, plants, and microorganisms. Recent research has been paid attention on peptides from marine animals since they have been found as secondary metabolites from sponges, ascidians, tunicates, and molluscs.

The structure characteristics of these include various unusual amino acid residues which may be responsible for their bioactivity. In the present scenario, there has been an increment in the number of studies focused on marine bioactive peptides [4]. Biologically active peptides are observed to have diverse activities, including opioid agonistic, mineral binding, immune-modulatory, antimicrobial, antioxidant, anti-thrombotic, hypo cholesterols and antihypertensive

actions [5]. Molluscs are species that have a wide range of uses in pharmacology. Peptides present in protein hydrolysates have biological activities depending on their molecular weights and amino acids-sequences. *Conus magus* [6] contains Ziconotide, a 25 amino acid peptide with three disulphide bond. Marine gelatin derived peptides are expected to exert high antioxidant effect [7]. Therefore, marine-derived bioactive peptides with anti-oxidative properties may have great potential for use as nutraceuticals and pharmaceuticals and could be a substitute for synthetic antioxidants [8].

In our body, the oxidation process leads to cell damage, cancer, and degenerative diseases; antioxidant molecules present in different molluscs prevent cell damage from oxidation reaction[4]. Oxidation reaction can produce free radicals, which start chain reactions that damage cells [9]. Free radicals are charged molecules, *i.e.,* they have an unpaired electron, which causes them to seek out and capture electrons from other substances in order to neutralize themselves. The harmful action of the free radicals can, however, is blocked by antioxidant substances, which scavenge the free radicals and detoxify the organism [10]. Current research in free radicals has confirmed that antioxidants rich foods play an essential role in the prevention of cardiovascular diseases, cancer, neurodegenerative diseases, as well as inflammation and problems caused by cell and cutaneous aging [11].

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Antioxidant molecules present in different molluscs prevent cell damage from oxidation reaction [4]. The present study was designed to evaluate the antioxidant activity of the whole body tissue of gastropod *T. brunneus, C. annulus* and *B. spirata*.

MATERIALS AND METHODS: In the present study the gastropods *Turbo brunneus*, *Cypraea annulus,* and *Babylonia spirata* were collected from the Gulf of Mannar coastal region of Thoothukudi. The freshly collected samples were brought to the laboratory, cleaned, and washed with fresh seawater to remove all impurities. The shells were broken; tissues were removed and then dried in hot air oven at 56 °C for 48 h and used for further studies.

Molecular Characterization of Protein (SDS PAGE): SDS-PAGE is the most widely used method for analyzing protein mixture qualitatively. SDS - PAGE was performed following the method described by Laemmli UK (1970) [12].

Antioxidant Activity:

DPPH Radical Scavenging Activity: The antioxidant activity of the chloroform extracts of *T. brunneus, C. annulus,* and *B. spirata* (20 to 100 µg/ml) was determined using the DPPH radical scavenging activity. The scavenging ability of DPPH radical was assessed following the method of Harborne JB and Baxter H (1995) [13].

Reducing Power Activity: The reducing power was evaluated by the method of Athukorala Y *et al.,* (2006) [14]. The chloroform extract of different concentrations (100 to 500 µg/ml) of *T. brunneus, C. annulus* and *B. spirata* were investigated.

H_2O_2 Radical Scavenging Activity: The ability of the chloroform extracts of gastropods to scavenge hydrogen peroxide was assayed according to the method of Ruch RJ *et al.,* (1989) [15].

RESULTS:

Molecular Characterization of Protein (SDS-PAGE): The gastropod samples *Turbo brunneus*, *Cypraea annulus,* and *Babylonia spirata* were subjected to SDS polyacrylamide gel electrophoresis to estimate the molecular weight of proteins.

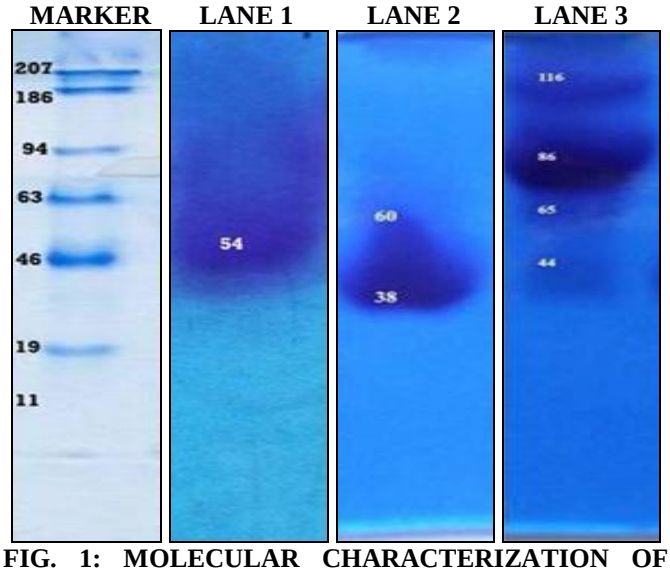

FIG. 1: MOLECULAR CHARACTERIZATION OF PROTEIN BY SDS-PAGE Lane 1-*turbo brunneus* lane 2-*cypraea annulus* lane 3-*babylonia spirata*

The stained gel revealed that experimental organisms contained different types of proteins. The size of the protein was determined by comparing the electrophoresis mobility of marker proteins with known molecular weight. The electrophoretic profile of the gastropod samples showed the presence of low to high molecular weight proteins. The molecular weight of *T. brunneus* revealed the presence of 54 kDa protein band, in *C. annulus*, the molecular weight ranged from 38 kDa to 60 kDa and in *B. spirata* it was found from 44 kDa to 116 kDa subunits **Fig. 1**.

Antioxidant Activity:

DPPH Radical Scavenging Activity: Effect of chloroform extract of three gastropods *Turbo brunneus*, *Cypraea annulus* and *Babylonia spirata* on DPPH free radical scavenging activity has been observed at various concentrations (20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml) respectively.

The results of the DPPH scavenging activity of three gastropod extracts are shown in **Fig. 2**.

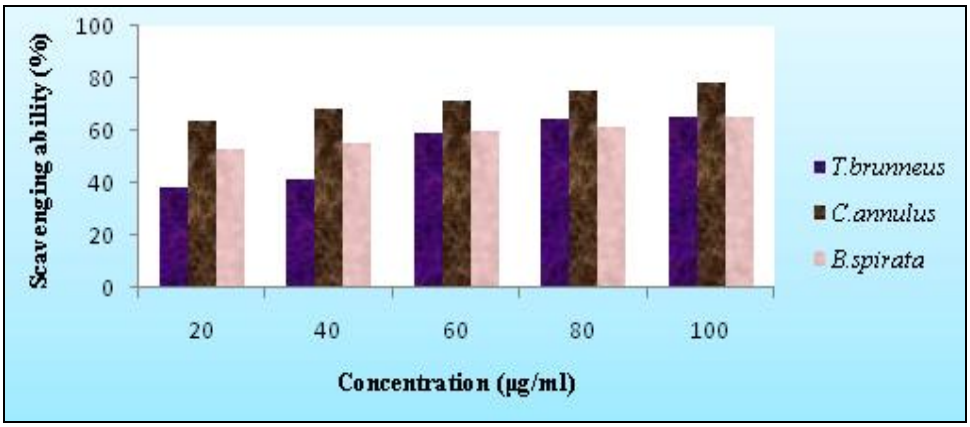

FIG. 2: DPPH RADICAL SCAVENGING ACTIVITY OF *TURBO BRUNNEUS*, *CYPRAEA ANNULUS* AND *BABYLONIA SPIRATA*

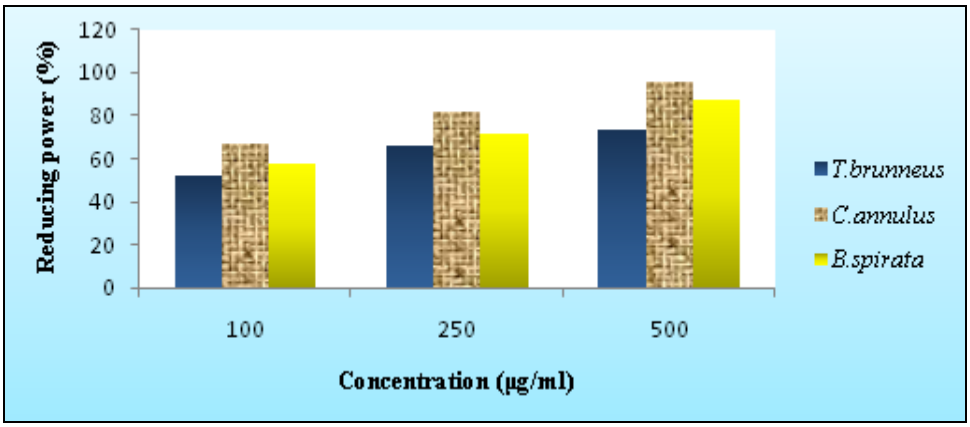

FIG. 3: REDUCING POWER ACTIVITY OF *TURBO BRUNNEUS*, *CYPRAEA ANNULUS* AND *BABYLONIA SPIRATA*

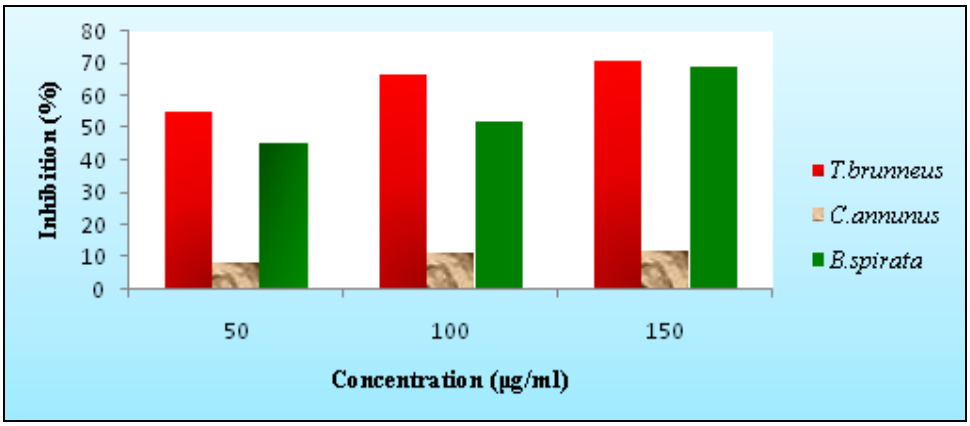

FIG. 4: H_2O_2 RADICAL SCAVENGING ACTIVITY OF *TURBO BRUNNEUS*, *CYPRAEA ANNULUS* AND *BABYLONIA SPIRATA*

The chloroform extracts of *T. brunneus* showed maximum scavenging activity of 64.89% at 100 µg/ml, 64.36% at 80 µg/ml, 59.04% at 60 µg/ml and 41.31% at 40 µg/ml and minimum activity of 38.45% at 20 µg/ml concentrations. In *Cypraea annulus* highest scavenging activity of 78.30% was observed at 100 µg/ml followed by 75.08% at 80 µg/ml, 71.39% at 60 µg/ml, 68.11% at 40 µg/ml and lowest scavenging activity of 64% were recorded at 20 µg/ml concentration. *Babylonia spirata* showed scavenging ability of 65.20% at 100 µg/ml followed by 61.22% at 80 µg/ml, 59.69% 60 µg/ml, 55.04% at 40 µg/ml and 52.58% at 20 µg/ml concentrations respectively. Among all the three samples, the scavenging effect was high in *Cypraea annulus* (78.30%) followed by *Babylonia spirata* (65.20%) and *Turbo brunneus* (64.89%). The maximum scavenging ability was observed at 100 µg/ml concentration and minimum scavenging activity at 20 µg/ml concentration. The percentage of scavenging activity was found to increase with the increase in concentration.

Reducing Power Activity: In the reducing power activity, the presence of antioxidants in the samples would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. The chloroform extracts of three gastropods *Turbo brunneus*, *Cypraea annulus,* and *Babylonia spirata* showed good reducing power activity. In *T. brunneus* the maximum activity of 73.35% was observed at 500 µg/ml concentration followed by 65.89% at 250 µg/ml concentration, and minimum activity was observed at 52.07% at 100 µg/ml concentration.

In *C. annulus* at 500 µg/ml concentration, the reducing power was 95.36%, at 250 µg/ml concentration was 81.67%, and at 100 µg/ml concentration, reducing power was 67.21%. In *B. spirata* the highest reducing power of 87.5% was recorded at 500 µg/ml followed by 71.34% at 250 µg/ml concentration and lowest reducing the power of 57.51% at 100 µg/ml concentration respectively **Fig. 3**. The reducing power of chloroform extracts of the three samples was reported to increase with increasing concentration. Of all the three species, *C. annulus* showed the highest reducing power of 95.36% at 500 µg/ml concentrations, and the lowest reducing power of 52.07% at 100 µg/ml concentrations was observed in the extract of *T. brunneus*.

H_2O_2 Radical Scavenging Activity: The percentage radical scavenging activity of three samples is shown in **Fig. 4**.

In *T. brunneus* the radical scavenging activity of 71% was observed at 150 µg/ml concentrations, 66.32% at 100 µg/ml concentration, and 54.76% at 50 µg/ml concentrations. In *C. annulus* percentage inhibition of 12% was reported at 150 µg/ml, 11.25% at 100 µg/ml and 8.34% at 50 µg/ml concentration. In *B. spirata* the scavenging activity was 69% at 150 µg/ml, 52.39% at 100 µg/ml and 45.34% at 50 µg/ml concentration. Among all the three samples maximum radical scavenging activity was reported in *T. brunneus* (71% at 150 µg/ml concentration), and minimum activity was reported in *C. annulus* (8.34% at 50 µg/ml concentration).

DISCUSSION: Marine invertebrates, which develop in a different environment, are the source of a broad range of pharmacological substances [16]. Among the invertebrates, the molluscs found to possess good biomedically important products and have developed very effective mechanisms that are part of their innate immunity [17]. They are considered as one of the important sources to derive bioactive compounds that exhibit antitumor, antimicrobial, anti-inflammatory, and antioxidant properties [4]. Marine molluscan extracts are usually complex mixtures of bioactive molecules, mainly proteins, peptides, and sterols. Cyclic and linear peptides obtained from marine animals have increased our knowledge about new potent cytotoxic, antimicrobial, ion channels specific blockers and many other properties with novel chemical structures associated with original mechanisms of pharmacological activity [18].

Proteins with different effects have been reported by various workers. Naraoka T *et al.,* (2002) [19] reported a protein that occurred in the ink of ilex argentinus migrated as a 94 kDa protein on polyacrylamide gel electrophoresis [20].

Isolated the partially purified echotoxins extracted from *Monoplex echo*, had the molecular mass of 7 kDa by gel filtration on Sephadex G-75 column. Monastyrnaya MM *et al.,* (2002) [21] have isolated 20 kDa protein from the sea anemone *R. macrodactylus* [22] isolated 19 kDa proteins from the globular vesicles of the sea anemone *A. villosa*.

Zandi K *et al., (*2007) [23] isolated 60 kDa protein from the purple fluid of *Aplysia dactylomela*. Saravanan R *et al., (*2009) [24] isolated 14 kDa protein from the *Conus figulinus*. More or less similar molecular weight protein 14 kDa and 29 kDa were also isolated from marine bivalves *Meretrix casta* and *Perna viridis*. Sumita S *at al.,* (2009) and Sugesh S *et al.,* (2010) [25, 26] reported the crude protein bands ranging from 45 to 261 kDa on *Meretrix meretrix* and *Meretrix casta*. In *Melo melo,* the methanol extract of mucus, nerve tissue, body tissue, and kidney was subjected to TLC to determine the presence of peptides and amide groups and also subjected to SDS-PAGE.

After electrophoresis, the clear band was detected in the gel, which represented proteins of molecular weight of 14, 17, 22, 45 kDa. Sivasubramanian K *et al.,* (2011) and *Vennila R et al.,* (2011) [27, 28] studied the molecular weight profiling of 82 to 248 kDa protein bands from an ink sample of *Octopus sp*. Periyasamy N (2012) *et al.,*[29] detected various protein bands of 97, 63, 61, 42 kDa in *Conus inscriptus* and 93, 61, 42, 40 kDa in *Conus betulinus* [30] revealed the presence of 47 to 106 kDa proteins in the tissue extracts of *Cantharus tranquebaricus* which may be responsible for various biological activities. Thangaraj S and Bragadeeswaran S (2012) [31] reported three types of neurotoxins with molecular weights between 45 kDa and 95 kDa in sea anemones *S. mertensii* and *S. gigantea.* In *Octopus aegina* and *Octopus dolfusii* the molecular weight, determination was done using SDS-PAGE, the protein bands existed from 32.83 kDa to 72.36 kDa for both the species [32].

In *Conus lentiginosus,* the molecular weight of purified toxins was determined by SDS-PAGE on a 12% gel system using standard protein markers and yielded 3 bands 40 kDa, 71 kDa, and 120 kDa [33].

The molecular weight determination of crude tissue extract of *Perna viridis* ranged from 63 kDa to 29 kDa. These revealed proteins may be responsible for various biological activities in the tissue extracts [34, 35] isolated 9 kDa and 110 kDa protein bands from *Pomacea insularum* and in *Callinectes sapidus* the molecular weight of peptide molecule ranges within 40 to 100 kDa which is in agreement with the present work.

In the present study, the protein isolated from *T. brunneus, C. annulus* and *B. spirata* showed the molecular weight of 54 kDa, 38 kDa to 60 kDa, and 44 kDa to 116 kDa subunits **Fig. 1**. The results of the present study coincide with the findings of the above authors. Oxidation reactions are a necessary part of life; unfortunately, they can also get damaged because of the production of reactive oxygen species. The reactive oxygen species (ROS) formations such as superoxide anion, hydroxyl radical, and hydrogen peroxide are natural byproducts of the normal metabolism of oxygen that have crucial roles in homeostasis and cell signaling in human body [36]. Accumulation of ROS in the body can result in oxidative damage to cellular components leading to cell death and tissue injury. Antioxidants from natural sources are preferred by consumers due to the concerns about the toxic and carcinogenic effects of synthetic antioxidants. The antioxidants are responsible for prevention of chain initiation, binding of transition metal ion-catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [37].

The use of DPPH radical provides an easy, rapid and convenient method to evaluate the antioxidants and radical scavengers [38]. DPPH is a stable free radical assay which is widely used to evaluate the free radical-scavenging activity of various natural products and some pure synthetic compounds. The decrease in absorbance of DPPH radical caused by antioxidants is visually noticeable as a change in color from purple to yellow when measured at 517 nm [39]. The degree of discoloration indicates the scavenging potential of the antioxidant compound in the extracts. In the present study, chloroform extracts of *T. brunneus, C. annulus,* and *B. spirata* showed potential antioxidant activities.

The extracts were able to reduce the stable radical $DPPH^{-}$ to the yellow-colored diphenyl picryl hydrazine. The scavenging effect was high in *C. annulus* (78.30%) followed by *B. spirata* (65.20%) and *T. brunneus* (64.89%) **Fig. 2**. The maximum scavenging ability was observed at 100 µg/ml concentration and minimum scavenging activity at 20 µg/ml concentration. The percentage of scavenging activity was found to be increased with increasing concentration.

The methanolic extract of gastropod *Pleuroploca trapezium* was found to exhibit a good scavenger of DPPH radical with an IC_{50} value of 4021 µg/ml [2]. Fahmy R and Soliman M (2013) [40] also reported good antioxidant activity in *Sepia officinalis* ink and *Coelatura aegyptiaca* extracts. The scavenging ability in *S. officinalis* ink and *C. aegyptiaca* extracts varied from 86.14% to 95.19% at various concentrations (10, 20, 30, 40, and 50 µg/ml). Subhapradha N *et al.,* (2013) [10] reported the methanolic extract of *B. spinosa* with scavenging ability of 39.43% at 10 mg/ml. Madhu VN *et al.,* (2014) [34] studied the crude protein with significant DPPH scavenging activity of 76.9% at 100 µg/ml in the methanolic extract of *P. viridis*. Ramesh S *et al.,* (2014) [41] reported that purified extract of conotoxin from *C. amadis* showed a scavenging activity of 46.2 ± 0.2% at 120 µg/ml. Borquaye LS *et al.,* (2015) [42] described the antioxidant potential of the crude peptide extracts of *Galatea paradoxa* and *Patella rustica*. Borquaye LS *et al.,* (2016) [43] reported antioxidant activities of ethyl acetate and methanol extracts of *Littorina littorea* and *Galatea paradoxa*. At the higher concentration, *P. rustica* showed good scavenging ability. The present study agrees well with the above findings.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing power ability greatly depends on the presence of reductones, which exhibit antioxidant potential by breaking the free radical chain by donating a hydrogen atom [44]. The amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at the absorbance of 700 nm.

The antioxidant action of reductones was based on the breaking of the free-radical chain by donating a hydrogen atom [45]. In the present study, reducing the power of chloroform extracts of three samples *T. brunneus*, *C. annulus,* and *B. spirata* was found to be increased with increasing concentration. In *T. brunneus* the maximum activity of 73.35% was observed at 500 µg/ml concentration followed by 65.89% at 250 µg/ml concentration, and minimum activity was observed at 52.07% at 100 µg/ml concentration. In *C. annulus* at 500 µg/ml concentration, the reducing power was 95.36%, at 250 µg/ml concentration was 81.67%, and at 100 µg/ml concentration, reducing power was 67.21%. In *B. spirata* the highest reducing power of 87.5% was recorded at 500 µg/ml followed by 71.34% at 250 µg/ml concentration and lowest reducing the power of 57.51% at 100 µg/ml concentration respectively **Fig. 3**. The compounds from these extracts may act in a similar fashion as reductones by donating electrons and reacting with free-radicals to convert them to more stable products and terminating the free-radical chain reaction.

Very similar to the present study [46] reported that the *O. macrocera* showed higher reducing ability at highest concentration (100 µg/ml). Nazeer RA and Naqash ASY (2013) [47] described the *in-vitro* antioxidant activity of solvent extracts from two Indian molluscs *Loilgo duvauceli* and *Donax cuneatus*. Pachaiyappan A *et al.,* (2014) [48] showed the maximum reducing power in the extract of *M. casta* (0.158) and *P. viridis* (0.127) and minimum activity was observed in the extract of *H. conchlidium* (0.024), *S. bicolor* (0.02) and *M. virgins* (0.018) [34] showed the reducing capacity of 27.8% at 100 µg/ml in green mussel. Pachaiyappan A *et al.,* (2014) [49] reported the reducing power of the samples might be due to the di and mono hydroxyl substitutions in the aromatic ring which possesses potent hydrogen donating activities.

Hydrogen peroxide is a weak oxidizing agent [50]. In *T. brunneus* the H_2O_2 radical scavenging activity of 71% was observed at 150 µg/ml concentrations, 66.32% at 100 µg/ml concentration, and 54.76% at 50 µg/ml concentration. In *C. annulus*, scavenging activity of 12% was reported at 150 µg/ml, 11.25% at 100 µg/ml, and 8.34% at 50 µg/ml concentration. In *B. spirata* the scavenging activity was 69% at 150 µg/ml, 52.39% at 100 µg/ml and 45.34% at 50 µg/ml concentration **Fig. 4.** Sivaperumal P *et al.,* (2013) [46] reported the hydrogen peroxide radical scavenging activity in *O. macrocera* with the highest inhibition of about 91.68% at the concentration of 100 µg/ml. Similar works of hydrogen peroxide radical scavenging activity on molluscs were also observed by Kumar VS *et al.,* (2014) [51] and Subhapradha N *et al.,* (2013) [10], Kumar VS *et al.,* (2014) [51] studied antioxidant activity in methanolic extract of *B. zeylanica* with maximum percentage inhibition of 65.4% at highest concentration 3.2 mg/ml and minimum inhibition of 4.2% at 0.1 mg/ml. Ponnusamy K *et al.,* (2016) [52] evaluated antioxidant properties from

tissue extract of cephalopods. Arumugasamy K and Cyril R (2017) [53] examined antioxidant activities of the tissue extracts of marine gastropod *Hemifusus pugilinus*. Gayathri M *et al.,* (2017) [54] reported *in-vitro* antioxidant properties from tissue extract of gastropods. Gayathri M *et al.,* (2017) [55] studied antioxidant activities of protein hydrolysate from *Cryptozona bistrialis*. These findings corroborate with the results of the present study. In view of the previous reports, it is felt that the gastropod *Turbo brunneus*, *Cypraea annulus*, and *Babylonia spirata* possess pharmaceutically important proteins that are responsible for antioxidant activity.

CONCLUSION: The present study demonstrates the effect of gastropod extracts on SDS-PAGE characterization of the protein responsible for the bioactivity. The proteins were found to possess antioxidant activities, as determined by DPPH, reducing power and hydrogen peroxide scavenging activities.

The results of the present study reveal that marine organisms are a rich source of antioxidant compounds with a remarkable impact in the field of pharmaceutical, industrial, and biotechnological product developments.

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CONFLICTS OF INTEREST: No conflicts of interest.

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