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IN VITRO ANTIOXIDANT ACTIVITY OF DIFFERENT GASTROPODS, BIVALVES AND ECHINODERM BY SOLVENT EXTRACTION METHOD

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ABSTRACT: Antioxidants can protect the human body from free radicals and ROS effects. They retard the progress of many chronic diseases as well as lipid peroxidation. The commercially used synthetic antioxidants BHA and BHT have been suspected of being responsible for liver damage and carcinogenesis. Hence, a need for identifying alternative natural and safe sources of antioxidants has been created, and the search for natural antioxidants, especially of marine origin, has notably increased in recent years. The aim of this current study is to find the antioxidant effect of marine invertebrates of molluscan group comprising gastropods, bivalves and echinoderms methanolic extract using different *in-vitro* assays such as DPPH, reducing power and total antioxidant activity. The present findings imply that, bivalves possess higher antioxidant activity that formed as regular seafood for mankind than other groups investigated; hence, these methanolic extracts can be served as ironic agents of antioxidants and also used as pharmaceutical agents to prevent various degenerative diseases where it demands further research towards purification and mechanisms of action.

INTRODUCTION: Marine organisms are considered as a vast untapped resource of bioactive molecules having enormous therapeutic potential which led to the growing interest in investigation of natural products for the discovery of immuno-stimulatory activity such as antioxidant, anti-inflammatory and antimicrobial compounds [1]. Among marine organisms, molluscan group are widely distributed throughout the world and have many representatives in the marine and estuarine ecosystems namely slugs, whelks, clams, mussels, oyster, scallops, squids and octopus. This rich diversity to marine organisms assumes a great opportunity for the discovery of new bioactive compounds [2]. Recently, many studies on bioactive compounds from molluscs exhibiting antioxidant, antitumor, antibacterial andantiviral activities have been reported worldwide [3-5].

Antioxidants from natural sources play a paramount role in helping endogenous antioxidants to neutralize oxidative stress [6]. The generation of reactive oxygen species (ROS) is an unavoidable consequence of life in an aerobic environment. In which, the production of ROS is essential to many organisms for the production of energy to fuel biological processes [7].

On the other hand, ROS such as hydroxyl, superoxide and peroxyl radicals are formed in human cells by endogenous factors and

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exogenously result in extensive oxidative damage [8-10]. This uncontrolled generation of free radicals is associated with lipid and protein peroxidation, resulting in cell structural damage, tissue injury, or gene mutation and ultimately lead to the development of various health disorders such as Alzheimer's disease, cancer, atherosclerosis, diabetes mellitus, hypertension, and ageing [11]. These free radicals are naturally scavenged by antioxidant mechanism in mammalians.

Thus the free radicals and ROS are considered important because the human body constantly quenches excessive oxidants through various scavenging mechanisms such as use of antioxidant enzymes and molecules. These antioxidants refer to any substance that hinders the reaction of a substance with dioxygen or any substance that inhibits free radical reaction [12]. In certain circumstances, this in-situ capacity becomes inefficient, which makes mandatory dietary intake of antioxidant compounds as an alternative, suggesting that there is an inverse relationship between dietary intake of antioxidants and the incidence of diseases caused by the deficiency of these substances [13]. Hence antioxidants are called as free radical scavengers.

Generally, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butyl-hydroquinone (TBHQ) and propyl gallate (PG) have been in use to reduce the deleterious effect of oxidative-induced reactions in food and biological systems [14, 15]. However, the potential toxicity of these synthetic antioxidants has aroused an increased interest among scientists to focus on isolation and characterization of natural antioxidants from natural sources [16, 17].

Recently, published data indicated that natural polysaccharides and their conjugates, which are widely distributed in animals, plants, and microorganisms, in general possessed potential and potent antioxidant activities and could be explored as novel potential antioxidants [18- 22]. As marine natural products play a significant role since ancient times, we have attempted to highlight the most promising antioxidant activity of some gastropod, bivalve and echinoderm that have the greatest potential lead molecule to be clinically useful in treatments.

This is because ocean is the unique bio store of active compounds and also due to the preference among consumers for seafood. Hence, the search for new defensive antioxidants to reduce the oxidative stress of human body in a natural and safe way is a practical and reasonable approach. In view of the above, the present work was carried to investigate the in vitro antioxidant activities of 16 samples comprising11 gastropods, 4 bivalves and 1 echinoderm.

MATERIALS AND METHODS:

Sample collection: Species of molluscs (11 gastropods, 4 bivalves) and echinoderms (1) were collected from 4 different stations of east coast of Tamil Nadu, India. The collected samples were classified and grouped using taxonomy and tabulated with their availability (**Table 1**). The animals were immediately transferred to the laboratory and stored at -40°C until use.

Solvent extraction: The whole body muscle of the specimens was removed by breaking the shells. The weighed sample was then homogenized and cold steeped with methanol over night at -18^{0}C.The extracts were centrifuged at 15,000 rpm for 30 min and the supernatants was concentrated by rotary flash evaporator (VC100A Lark Rotavapor at 30^{0}C) with reduced pressure to give predominantly an aqueous suspension and freeze dried.

Protein and carbohydrate content: Amount of protein and carbohydrates in the samples was quantified by the method of Lowry *et al*. [23] with BSA as a standard and Ashwell [24] using D-glucose as standard.

Total antioxidant activity: Total antioxidant activity of crude methanolic extracts was determined according to the method of Prieto *et al*. [25]. Briefly, 0.3 ml of sample was mixed with 3.0 ml reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95ºC for 90 min under water bath. Absorbance of all the sample mixtures was measured at 695 nm. Total antioxidant activity is expressed as the number of equivalents of ascorbic acid in milligram per gram of extract.

TABLE 1: LIST OF MOLLUSCS AND ECHINODERM SAMPLES

S. No	Sample	Collected station	Taxonomy	Availability
1.		**Gastropod**		
a.	*Harpa conoidalis*	Mudasalodai	Lamarck	Frequent
b.	*Rapana rapiformis*	Mudasalodai	Born	Frequent
c.	*Hemifuses conchlidium*	Mudasalodai	Linne	Abundant
d.	*Babylonia spirata spirata*	Mudasalodai	Linne	Frequent
e.	*Telescopium telescopium*	Parangipettai	Linne	Rare
f.	*Turitella attenuata*	Pazhaiyar	Reeve	Rare
g.	*Turitella acutangular*	Mudasalodai	Linne	Rare
h.	*Murex trapa*	Rameshwaram	Roeding	Frequent
i.	*Murex virgineus*	Mudasalodai	Roeding	Frequent
j	*Ficus gracilis*	Mudasalodai	Sowerby	Rare
2.		**Bivalves**		
K	*Meretrix meretrix*	Pazhaiyar	Linne	Frequent
l	*Meretrix casta*	Pazhaiyar	Chemnitz	Frequent
m	*Perna viridis*	Pazhaiyar	Linne	Rare
n	*Crassostrea madrasensis*	Rameshwaram	Preston	Abundant
3.		**Echinoderm**		
o	*Salmasis bicolor*	Mudasalodai	Agassiz	Frequent

Free radical scavenging activity (DPPH Method): The radical scavenging abilities of crude methanolic extracts of samples was measured from bleaching of a purple-colored methanol solution of DPPH (2, 2-diphenyl-1-picrylhydrazyl) following Blois [26]. About 10-100µl of the sample extract was added with 2 ml of DPPH(Hi media Laboratories Pvt, Ltd, Mumbai) in methanol (0.33%) in a test tube and made up to 3 ml with distilled water. After incubation at 37˚C for 30 mins the absorbance of each solution was determined at 517 nm using spectrophotometer (Hwang et al., 2001). The corresponding blank reading was also taken and the remaining DPPH was calculated by using the following formula.

DPPH radical scavenging (%) = {1-(A sample or standard- A blank/A control)* 100}
Where, A control is the absorbance of the control (DPPH solution without sample), A sample or standard is the absorbance of the test sample (DPPH solution plus test sample or standard) and A blank is the absorbance of the methanol.

Reducing power: Reducing power of crude methanolic extract obtained from mollusk sample was determined by the method explained by Oyaizu [27]. Briefly, 1.0 ml of methanol containing different concentration of sample was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (1%). Reaction mixture was incubated at 50 °C for 20 min. After incubation, 2.5 ml of trichloroacetic acid (10%) was added and centrifuged (650g) for 10 min. From the upper layer, 2.5 ml solution was mixed with 2.5 ml distilled water and 0.5 ml FeCl3 (0.1%). Absorbance of all the sample solutions was measured at 700 nm. Increased absorbance indicates increased reducing power.

Statistical analysis: All the tests were conducted with three replicates. Data were presented as means ± standard deviations. The statistical analysis was performed by using SPSS 16.0 software (SPSS Inc. Chicago, IL, USA).

RESULTS:

Estimation of Protein: The amount of protein present in the methanol extract of molluscan groups is represented as graphical interpretation. Among the samples screened *M. casta* (762 µg/mg) and *P. virids* (761 µg/mg) possess higher amount of protein content where *T. telescopium* (41 µg/mg) possessed lower amount. On total the bivalve extracts possessed higher protein content than gastropods and echinoderm.

Estimation of Carbohydrates: Methanolic extract of mollusca *M. tribute* (421µg/mg) shows the highest carbohydrate content whereas *H. conoidalis* possess (71µg/mg) low value among gastropods. On overall, methanolic extract of *P. viridis* (620µg/mg) possess highest carbohydrate content and the echinoderm *S. bicolor* (23µg/mg) possess lowest concentration.

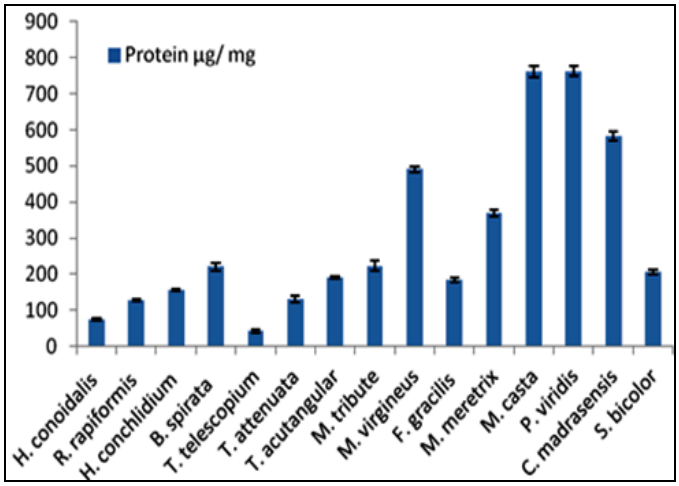

FIG. 1: SHOWS THE CONCENTRATION OF PROTEIN µg/ mg IN METHANOLIC EXTRACTS

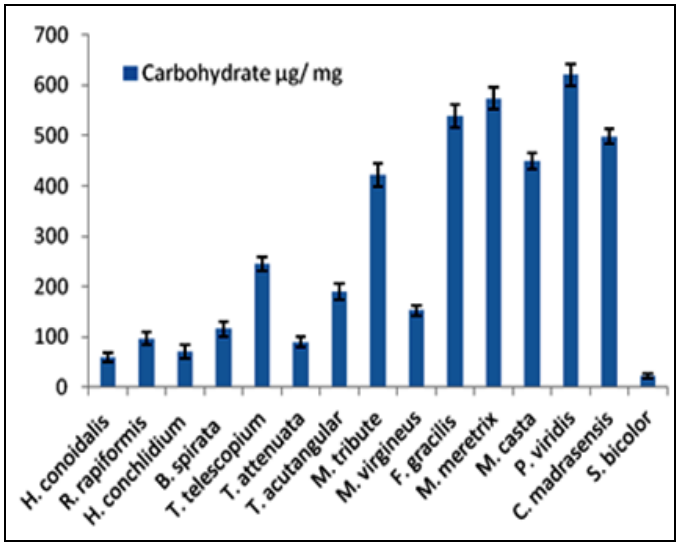

FIG. 2: SHOWS THE CONCENTRATION OF CARBOHYDRATE µg/mg IN METHANOLIC EXTRACTS

Total Antioxidant Activity: TAA mainly concentrates on the thermodynamic conversion and measures the number of electrons or radicals donated or quenched by a given antioxidant molecule and measure the capacity of biological samples under defined conditions. The phosphor-molybdenum method was based on the reduction of MO (VI) to MO (V) by the antioxidant compound and the formation of green phosphate/ MO (V) complex at acidic pH with a maximal absorption at 695 nm. In this assay *B. spirata* (620 µg/mg) extract was found to possess higher and *M. casta* (23 µg/mg) lower activities, as compared to the standard (Gallic acid) used for this study. The antioxidant activity of the samples was given in **Fig. 3**.

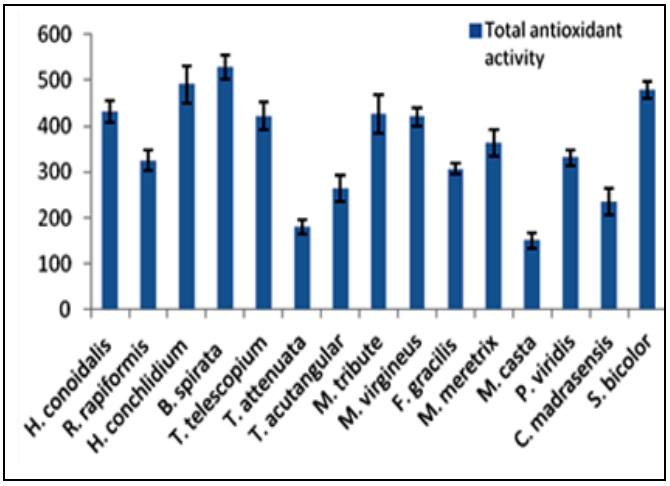

FIG. 3: SHOWS THE TOTAL ANTIOXIDANT ACTIVITY OF CRUDE METHANOLIC EXTRACTS

1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging: The DPPH assay constitutes a quick and low cost method, which has frequently been used for the evaluation of the antioxidative potential of various natural products [28]. DPPH is a compound that possesses a nitrogen free radical and is readily destroyed by a free radical scavenger. This assay was used to test the ability of the antioxidative compounds functioning as proton radical scavengers or hydrogen donors [29].

The percentage scavenging activity of molluscan and echinoderm extracts against DPPH was shown in the **Fig. 4**. Significant differences in the activities among different species were observed. Among all the samples, the scavenging effect was high in *M. meretrix* (74.52%) followed by *P. viridis* (73.64%) and lower for *T. attenuate* (23.54 %).

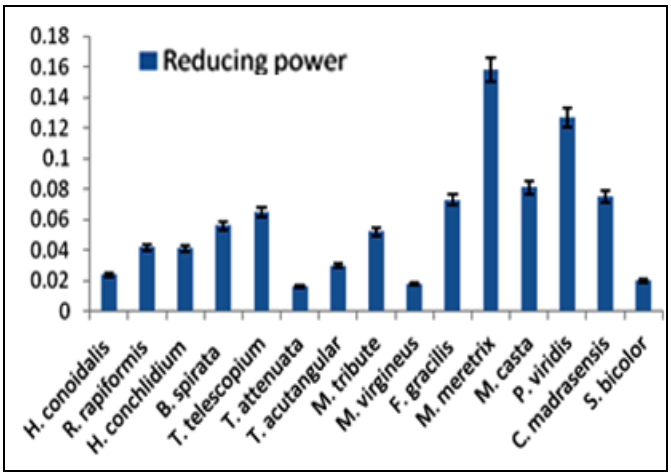

FIG. 4: SHOWS THE REDUCING POWER ACTIVITY OF CRUDE METHANOLIC EXTRACTS

Reducing power: The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity (Meir, Kanner, Akiri&Philosoph-Hadas, 1995). Fe (III) reduction is often used as an indicator of electron donating activity [30]. In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be then monitored by measuring the formation of Perl's Prussian blue at the absorbance (OD value) of 700 nm. **Fig. 5** shows the reducing powers of the extracts. The reducing properties are generally associated with the presence of reductones [31]. Gordon [32] reported that the antioxidant action of reductones was based on the breaking of the free-radical chain by donating a hydrogen atom. All of these molluscan extracts showed good activity. The maximum activity was absorbed in the extract of *M. casta* (0.158) and *P. viridis* (0.127) whereas the minimum activity was observed for *H. conchlidium* (0.024), *S. bicolor* (0.02) and M. virgins (0.018). 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.

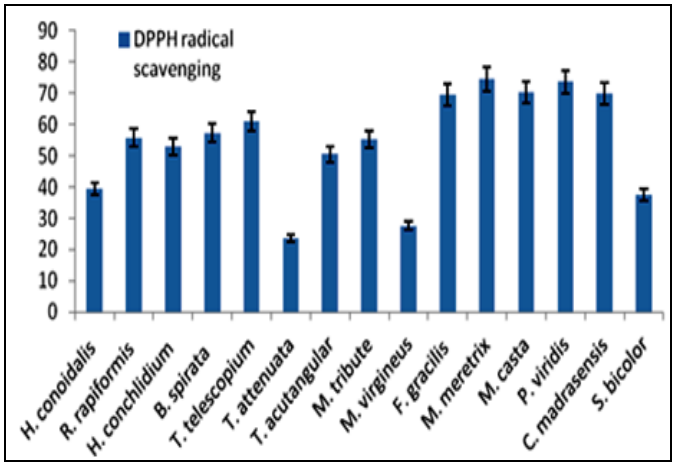

FIG. 5: SHOWS THE DPPH RADICAL SCAVENGING ACTIVITY OF CRUDE METHANOLIC EXTRACTS

DISCUSSION: Marine invertebrates have pronounced pharmacological activities or other bioactive properties which are useful in the biomedical arena. Since marine natural products are becoming increasingly attractive due to their potential applications in the pharmaceutical industries, the identification of new sources of these materials is extremely important.

Oxidative stress, the consequence of an imbalance of pro-oxidants and antioxidants in the organism, is rapidly gaining recognition as a key phenomenon in chronic diseases [33]. As a consequence of this reactivity of ROS and their potential to damage cells and tissues, marine and other organisms balance the production of these radicals with a wide variety of cellular antioxidant defenses [34]. Thus, antioxidants have gained more importance on account of their positive effects, as health promoters in the treatment of cardiovascular problems, atherosclerosis, many forms of cancer, ageing process, etc.

Hence the quest for natural antioxidant compounds has initiated the search towards marine organisms which serves as a reservoir of unique molecules. Among the molluscs screened here the total antioxidant activity was high in bivalve and echinoderm where other extract showed moderate activity. A number of studies have demonstrated potential for ROS generation, antioxidant enzyme, free radical scavenger responses and oxidative damage in species of invertebrates, mainly in molluscs [35 - 37].

DPPH radical (oil-soluble free radical) scavenging activity assay has been extensively used for screening antioxidant activity because it can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentrations [38]. The decrease in absorbance of the DPPH radical caused by antioxidant was due to the scavenging of the radical by hydrogen donation.

The DPPH radical scavenging abilities of the molluscan extracts when significantly comparable to those of ascorbic acid (100%), this study showed that the extracts have the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants. These considerable differences in DPPH radical scavenging effects of methanol extracts from gastropods, bivalves and cephalopod may have been due to species-specific differences resulting in considerable differences in their chemical compositions. Thus, the methanol extract of gastropod and bivalve species could be the better potential source as natural antioxidants.

Earlier report evidence that the antioxidant peptide isolated from *Conus betulinus* (body and viscera) shows 20-25% of scavenging effect [39]. Similarly, the gastropod *P.trapezium* meat possess natural antioxidant potential as its methanolic extract was found to exhibit a good scavenger of DPPH radical with an IC_{50} value of 4021 micro gram/ml. In our study, the scavenging activity was higher for bivalves than the other two groups.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activities of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging [40]. As shown in fig (5) the reducing power of the bivalve extract was compared with the standard and found to be superior. Fe (III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of antioxidant action [30].

All the bivalve extracts had shown good reducing power activity. Earlier, the reducing power of sea urchin gonad hydrolysates at the concentration of 1.25-10 mg/ml (protein basis) exhibited a dose dependent effect [41]. Sasikumar *et al* [6] reported that the reducing power of the samples might be due to the di and mono hydroxyl substitutions in the aromatic ring which possess potent hydrogen donating abilities. Similar results were represented for the in vitro antioxidant activity of solvent extracts prepared from two Indian molluscs viz., *Loligo duvauceli* and *Donax cuneatus* [42].

CONCLUSION: Reactive Oxygen species (ROS) generated endogenously or exogenously are associated with the pathogenesis of various diseases such as atherosclerosis, diabetes, cancer, arthritis and ageing process. Thus antioxidants which can scavenge ROS are expected to improve these disorders. The free radical scavenging activity of the extracts was evaluated based on the ability to scavenge the synthetic DPPH.

The results obtained in the present study indicate that bivalve and gastropod methanolic extracts exhibit potent free radical scavenging and antioxidant activity.

The findings of the present study suggest that bivalve and gastropod extract could be a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the progress of aging and age associated oxidative stress related degenerative diseases such as cancer and various other human ailments. Further it is reasonably concluded that isolation and characterization of the antioxidant components through in-vivo studies will help in understanding their mechanism of action as a better antioxidant.

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