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Protective role of edible clam *Paphia malabarica* (Chemnitz) against lipid peroxidation and free radicals

[Lipid peroksidasyonu ve serbest radikal oluşumuna karşı yenilebilir istiridye *Paphia malabarica*nın (Chemnitz) rolü]*

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ABSTRACT

Objective: To investigate in vitro inhibition of lipid peroxidation and free radical scavenging properties of a seafood *Paphia malabarica* (Chemnitz) as a natural source of antioxidants.

Methods: Antioxidant activities of *Paphia malabarica* extracts were tested spectrophotometrically against 2,2-diphenyl-2-picryl-hydrazyl, hydroxyl radical scavenging, in vitro inhibitions of lipid peroxidation and results were presented as percentage relative activities by comparing with standard synthetic antioxidant compounds. In addition, reducing potentials and presence of antioxidant compound in sample extract was tested with exponential increase in absorbance and ferric reducing antioxidant power values respectively.

Results: The scavenging potential of 2,2-diphenyl-2-picryl-hydrazyl radical and reducing action increased in a dose dependent manner. Inhibition of lipid peroxidation shown by methanol extract and its significant correlation with •OH scavenging activity implies its potential to protect the cell damage against reactive oxygen species. Increasing antioxidant equivalents of ascorbic acid in ferric reducing antioxidant power assay further supported its role in reducing reactions.

Conclusion: Dose dependant antioxidant responses by methanolic extracts of clam P. malabarica and their role in breaking chain reactions of lipid peroxidation highlights its potentials against reactive oxygen species mediated radical reactions. Present investigation forms a first comprehensive report on the nutraceutical property of a seafood P. malabarica as a natural source of antioxidants.

Key Words: *Paphia malabarica*, antioxidant, free radicals, DPPH, lipid peroxidation (LPX)

Conflict of interest: The authors declare that there are no conflicts of interest.

ÖZET

Amaç: Doğal bir antioksidan olarak *Paphia Malabarica*nın (kemnitz) lipid peroksidasyonu ve serbest radikal oluşumuna karşı etkilerinin invitro ortamda araştırılması.

Gereç ve Yöntemler: *Paphia malabarica* ekstraklarının antioksidan aktivitesi 2,2-difenil-2-pikiril-hidrazil ile hidroksi radikallerinin temizlenmesi ve lipid peroksidasyonunun baskılanması fonksiyonu olarak spektrofotometrik yöntemle ölçülmüş ve sonuçlar standart sentetik antioksidan bileşiklerden elde edilen değerlere karşı yüzde nispi aktivite olarak sunulmuştur. Ek olarak örnek ekstraktlardan absorbansda eksponansiyel artış ve ferrik indirgeyici antioksidan güç olarak sırasıyla indirgeme potansiyeli ve antioksidan bileşen mevcudiyeti ölçülmüştür.

Bulgular: 2,2-difenil-2-pikiril-hidrazil radikalinin radikal temizleme potansiyeli ve indirgeyici gücü doza bağımlı olarak artıs gösterdi. Metanollü ekstrakt ile lipid peroksidasyonunun inhibisyonu ve hidroksi radikali temizlenmesi ile olan direkt korelasyonu hücreyi reaktif oksijen türlerine karşı koruyucu potansiyeline işaret etmektedir. Ferrik indirgeyici antioksidan güç deneyinde artan askorbik asit eşdeğerlikleri indirgeyici reaksiyonlardaki rolünü ayrıca desteklemektedir.

Sonuç: İstiridye P. Malabaricanın metanollü ekstraktlarının doza bağımlı antioksidan cevabı ve lipid peroksidasyon reaksiyon zincirlerini kırmadaki rolü reaktif oksijen türlerine bağlı radikal reaksiyonlarına karşı potansiyalini vurgulamaktadır. Bu araştırma bir deniz besini P. Malabaricanın doğal antioksidan kaynağı olarak nutrasötik özelliği üzerine kapsamlı ilk çalışmadır.

Anahtar Kelimeler: *Paphia malabarica*, antioksidan, serbest radikaller, DPPH, lipid peroksidasyonu (LPX)

Çıkar Çatışması: Yazarların çıkar çatışması bulunmamaktadır.

Introduction

Clams, mussels, and oysters are dominant components of bivalve fishery [1] and forms important source of nutrition in coastal states [2, 3]. *Paphia malabarica* (Chemnitz) (a clam), is a commonly consumed as seafood in its cooked form by the coastal populations of India [1]. It is also widely distributed along the coastal and estuarine areas in Vietnam, China and Bangladesh. They are filter feeding burrowing bivalves, belong to Phylum Mollusca and alone contribute 90% of India's clam meat with bulk of its catch comes from Ashtamudi estuary in the south west coast of India [4]. They are also exploited widely for both meat as food and shell as an important source of raw material for industrial applications. The raw as well as processed *P. malabarica* has great market demand in Japan and other European countries. Moreover, this species has high nutritional value and percentage edibility [5] yet they are to be explored for its nutritional and antioxidative properties.

In a living organism, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are major forms of free radicals formed during normal metabolic activities and their overproduction may leads to debilitating oxidative stress and cell death [6]. To cope up with such adverse effects living organism utilizes self antioxidant defense systems. However, some of the essential antioxidants are also need to be supplied through external diet. In context to the same, various products used for consumption are being continuously evaluated using in vitro antioxidant determination methods. The most commonly used 2,2-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging has been found to have direct correlation with free radical scavenging whereas, reducing power and ferric reducing antioxidant power (FRAP) activities potentially equates total antioxidant capacities and presence of related bioactive compounds [7]. Moreover, evaluating hydroxyl radical scavenging and inhibition of lipid peroxidation helps to overcome the deteriorative effects on cell membrane damages and food quality [8].

Applications of naturally occurring antioxidants in food or medicinal materials have been increased significantly owing to their levels side effects, such as carcinogenicity as compared to that of synthetic antioxidants [9]. The antioxidant properties of food material like spices, herbs, hulls [10], wheat, alfalfa [11], and meat samples [12] as well as some of the industrial byproducts like Kraft black liquor [13] and wine [14] have been studied. Some of the nutritional compounds like Glycosaminoglycans [15] and Glycoprotein [16] have been isolated from bivalves to study its free radical scavenging activities. Therefore, in order to support the nutritional value of bivalves it is necessary to assess its antioxidant activities.

Evaluating the role of *P. malabarica* extracts in inhibition of free radical mediated oxidation of lipids as well as its reducing potential and radical scavenging properties could reveal their antioxidant properties.

Materials and Methods

Chemicals and instrumentation

All the analytical grade chemical reagents required were obtained from Sigma-Aldrich (Sigma-Aldrich Inc., St Louis, Missouri, Germany, USA). Whereas, Methanol (HPLC grade), Ferric chloride and Potassium ferricyanide were acquired from S.D. Fine-Chemicals Ltd., Mumbai, India. Sample concentration was carried out with Rota evaporator (Buchi Rotavapor R-200, Sand Hutton, United Kingdom) and spectrophotometric analysis was performed with UV–visible spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan).

Sample preparation and extraction

Paphia malabarica were handpicked from the intertidal regions during low tide and immediately transferred to laboratory in live condition. Samples were then washed with distilled water to remove associated debris. Animals were further dissected and the whole body tissue was blot dried with tissue paper to remove extraneous water content. The whole body tissue (10% w/v) was then homogenized and extracted in methanol (90% v/v) by agitation in rotary shaker for 24 hours. All the extraction procedures were carried out at 4 °C in order to maintain the stability of bioactive compounds [17]. The stepwise methanolic extraction procedure included repeated extractions at every 6 hours of time interval. Initially, the whole extract contents were centrifuged (8000 × g for 10 minutes at 4 °C) and supernatant was collected in separate vial. The tissue pellet obtained in consequent steps was further treated similarly with methanol to achieve maximum extraction and recovery of the bioactive compounds. All the fractions were finally pooled together, filtered through Whatman paper No. 1 and concentrated through Rota evaporator (Buchi Rotavapor R–200).

The yield estimation was carried out [17] by evaporating 1 mL extract in pre-weighted aluminium dish at room temperature (27 ºC) until complete dryness and was expressed as mg(crude dry weight extract)/mL. The condensed methanol extracts were adjusted to 10 mg/mL either by diluting or by concentrating with the same solvent. Sample extracts were then preserved at -20 °C until further use, and all parameters were repeated with triplicate analysis. As synthetic antioxidants exhibits higher activities at the same concentration of the sample extract, all standards were maintained at lower concentration (40 µg/mL).

DPPH radical scavenging assay

Free radical scavenging potential was measured against 2,2-diphenyl-1-picryl-hydrazyl (DPPH) by colorimetric reduction assay [18]. The reaction mixture containing 2.5 mL of DPPH solution (0.1 mM in methanol) and extract (0.1, 0.2 and 0.3 mL) was adjusted to 3 mL by adding methanol. The absorbance was measured at

0 minute and after 30 minutes at 517 nm. Butlylated hydroxytoluene (BHT) was used as the standard. Scavenging effect was calculated using formula given below, and expressed in terms of relative percent activity by comparing with standard.

$[A_0 - A_1 / A_0] \times 100$

Whereas, A_0 - absorbance at 0 minute and A_1 - absorbance at 30 minutes

Reducing power assay

The reducing power of the extract was determined by using standard protocol [19]. The reaction mixture containing 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide (1%) was added to aliquots of extract (0.1, 0.2 and 0.3 mL). Ascorbic acid was used as standard solution while blank was maintained with same reaction mixture without sample. The mixtures were incubated at 50 °C in water bath for 30 minutes and allowed to cool at room temperature. Later 2.5 mL of 10% trichloro actetic acid (TCA) was added to the reaction mixture and centrifuged at 2000 × g for 10 minutes. 2.5 mL of supernatant was transferred to another test tube, and to this 2.5 mL of distilled water and 0.5 mL $FeCl_3$ (1%) were added and allowed to react for 10 minutes at room temperature. The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing power.

Inhibition of in vitro lipid peroxidation

Lipid peroxidation of tissue samples were assayed according to the method reported in literature [20]. The sheep liver was washed with ice-cold potassium chloride (1.15%) and 20% tissue homogenate was prepared in KCl and filtered. The filtrate was centrifuged at 10,000 × g for 10 minutes at 4 °C and post mitochondrial fractions (PMF) were obtained along with most of the other organelles. In vitro lipid peroxidation was induced with $FeSO_4$ (100 µM) in PMF of sheep liver extract. To this, sample extract (0.1, 0.2 and 0.3 mL) was added and further treated with thiobarbituric acid (TBA) for 60 minutes at 95 °C. The formation of TBA-reactive species (TBARS) was measured at 532 nm. Samples and standard (Ascorbic acid) were analyzed in triplicates. The inhibition of lipid peroxidation was expressed in relative percentage (%) activity and calculated by following formula,

$[1- (A_0 - A_1 / A_2)] \times 100$

Where, A_0 - absorbance in the presence of extract (i.e. reaction mixture + sheep liver homogenate + sample extract of *P. malabarica*), A1 - absorbance without sheep liver homogenate (i.e. reaction mixture + sample extract of *P. malabarica*) and A2 - absorbance of the control without *P. malabarica* extract (i.e. reaction mixture + sheep liver homogenate).

Protein concentrations of ship liver homogenate were estimated by using Folin-Ciocalteu reagent and BSA as a standard [21]. 1 mg of final protein concentration was used for induction of each lipid peroxidation (LPX) reaction.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging ability of *P. malabarica* extracts were tested by quantifying Fenton reaction [22]. Reaction mixture containing 200 µL each of $FeSO_4$. H_2O (10 mM), EDTA (10 mM), 2-deoxyribose (10 mM) was added with sample (0.1, 0.2 and 0.3 mL), 1 mL of phosphate buffer (0.1 mM, 7.4) and 200 µL of H_2O_2 (10 mM) to initiate the reaction. Later, 1 mL of trichloroacetic acid (TCA - 2.8%) and TBA (0.1%) were added after incubation at 37 ºC for 4 hours and placed in boiling water bath for 10 minutes. The absorbance was measured at 532 nm.

The scavenging effect of hydroxyl radical was calculated as follow,

$[1- (A_{sample} / A_{control})] \times 100$

Whereas, A_{sample} is the absorbance in the presence of the tested samples while $A_{control}$ is the absorbance of the control. Results were expressed as relative activity (%).

Ferric Reducing Antioxidant Power (FRAP) assay

FRAP was evaluated by the measuring Fe^{2+}/TPTZ-complex by colorimetric method [23]. The FRAP reagent containing 2.5 mL of 10 mM TPTZ in 40 mM HCl solution, 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ and 25mL of acetate buffer (300 mM, pH 3.6) was prepared. Different concentrations of sample extract were added in 3 ml of FRAP reagent and incubated for 15 min at 37 °C. The antioxidant potential or the antioxidant content of the extract is expressed in terms of µg/mL of ascorbic acid (AsA) equivalents.

Final reaction volume for each biochemical assay was kept constant by adjusting either with same solvent or phosphate buffer.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Changes in biochemical parameters were tested using one-way ANOVA and post hoc tests (Newman-Keuls) to discriminate between means of values. Differences were considered statistically significant when $P < 0.05$.

Results

Data obtained in the present investigation was compared with standard synthetic antioxidant and presented as relative activity in case of DPPH, in vitro inhibition of LPX and hydroxyl radical scavenging assays. Whereas, reducing powers and FRAP values are expressed in terms of increasing absorbance and µg mL^{-1} of AsA equivalents, respectively.

The DPPH scavenging potential was measured by decrease in its absorbance. A significant dose dependent

DPPH scavenging was observed (Figure 1A; $P < 0.01$) which was higher for standard as compared with sample extract. The relative percent scavenging activity for initial 0.1 ml was 18.72 ± 3.94% followed by 27.68 ± 2.39% and ± 36.80% 3.18 for 0.2 and 0.3 ml of sample respectively as compared to that of standard compound (BHT) used. Similarly the significant correlation ($r = 0.952$; $P < 0.01$) was observed between DPPH radicals and reducing action by methanolic extracts of *P. malabarica*.

The antioxidant reducing action was evaluated by observing the ability of extract to reduce the Fe^{3+}/ ferricyanide complex to ferrous form and measuring formation of Perls Prussian blue. A similar dose dependency in absorbance values was exhibited by methanolic extracts (0.06 ± 0.01 < 0.13 ± 0.02 < 0.17 ± 0.02) in the present study (Figure 1B; $P < 0.01$). Correlation between DPPH scavenging potential and reducing action ($r = 0.952$; $P < 0.01$) and LPX and reducing action ($r = 0.843$; $P < 0.001$) strengthens our views on radical scavenging.

The MDA-TBA adduct was measured to find the levels of lipid peroxidation. The inhibition of LPX levels in terms of relative activities were 30.20 ± 14.11 % for 0.1ml, 67.82 ± 5.92 for 0.2ml, 69.3 ± 16.42% for 0.3ml of samples respectively. Figure 1C indicates approximately similar inhibitory activities for 0.2 and 0.3 ml of sample concentrations. A positive correlation with DPPH radical scavenging ($r = 0.729$; $P < 0.01$) and Reducing power ($r = 0.843$; $P < 0.001$) as well as significant inhibition of LPX was observed when compared to control (Figure 1C; $P < 0.01$).

On the other hand, methanolic extracts of *P. malabarica* also exhibited prominent hydroxyl radical scavenging activities as compared to commercial antioxidants like BHT (Figure 1D; $P < 0.01$). The relative scavenging abilities for 0.1ml, 0.2 ml and 0.3ml of samples were 24.30 ± 8.38%, 44.27 ± 8.65%, and 56.96 ± 8.44% respectively. Significant correlation between LPX and ·OH radical scavenging ($r = 0.774$; $P < 0.01$) were also observed.

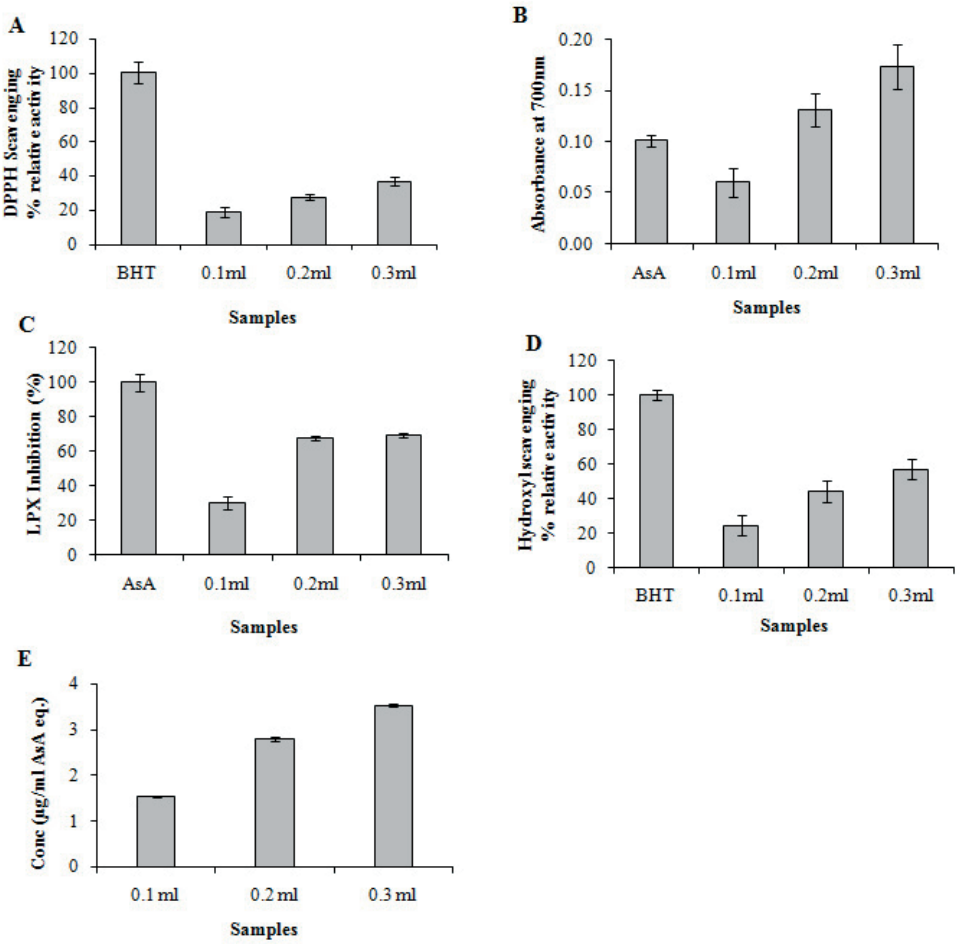

Figure 1. DPPH scavenging activity (A), reducing power (B), in vitro inhibition of lipid peroxidation (C), hydroxyl scavenging activity (D) and FRAP activity (E) of the extract of *P. malabarica.*
Values are the means of triplicate determination ± SD (N = 3).

Presence of antioxidant compounds in the sample extracts was evidenced with determination of standard equivalent compounds in terms of FRAP values. The observed antioxidant values of the sample extracts were found to be in the order of $1.54 \pm 0.021 < 2.8 \pm 0.046 < 3.53 \pm 0.039$ µg/ml of AsA equivalent for 0.1, 0.2 and 0.3 ml respectively (Figure 1E). Increasing FRAP values shows presence of active antioxidant compounds in sample extracts. Correlation of these values with DPPH scavenging potential ($r = 0.880$; $P < 0.01$), reducing power ($r = 0.945$; $P < 0.01$), LPX ($r = 0.758$; $P < 0.01$) and OH radical scavenging ($r = 0.769$; $P < 0.01$) further significantly supports our hypothesis.

Discussion

The exponential decrease in the absorbance of DPPH solution caused by test samples in present investigations is may be due to its reducing capability. Some of the fat-soluble antioxidant compounds from animal tissue have been reported for their efficient solubility in methanol [17], in concern to which fatty acids i.e. lipophilic antioxidants from muscle tissue of a related species *Tapes decussatus* (grooved carpet shell) observed to have prominent antioxidant properties [24]. Our recent findings on bivalves such as *Perna viridis* [25], *Crassostrea* spp., *Placuna placenta* and *Polymesoda erosa* had revealed their potency towards radical scavenging activities [26]. Moreover, antioxidant peptides from *Meretrix casta* (Chemnitz) [27], Glycosaminoglycans [15] and Glycoprotein [16] from *Paphia undulate* were shown to have radical scavenging activity. Presencce of similar types of bioactive compounds in studied organisms (*P. malabarica*) might be the reason behind observed radical scavenging effects.

The compounds, which are able to exhibit an increase in absorbance, can be considered as iron reductants and such reducing action serves as a significant indicator of potential antioxidant activity [28,29]. The observed exponential increase in reducing potential in the present study is due to the donation of electrons by active compounds of sample extracts (Figure1B). Methanolic extracts of bivalve species has shown similar type of trend in their reducing abilities [25,26]. Moreover, different types of reactions such as binding of transition metal ion catalysts, decomposition of peroxides, and prevention of continued hydrogen abstraction are also known to take part in reducing actions [30]. Thus, the overall reducing activitiy in the present investigation is due to the quenching effect of reductants of *P. malabarica* which might have possibly extracted in methanolic extracts.

In vitro studies on animal cell cultures showed that α-tocopherol has inhibitory activities on LPX reactions and supportive role in cell proliferation [31]. Protein, carbohydrate and vitamin content of the bivalves, *Perna viridis, Donax cuneatus, Meretrix meretrix* [32] and *Paphia malabarica* [33] has been attributed to various nutritional and bioactive properties. Such biomolecules may take part in the inhibiting reactions of lipid peroxidation. A positive correlation with DPPH radical scavenging and reducing power as well as significant inhibition of LPX (Figure 1C) emphasize role of *P. malabarica* extracts in inhibiting chain reactions of lipid peroxidation. In addition, xenobiotics produced by carbohydrate detoxification reactions, organochlorinated compounds, and trace metals are able to generate ROS and free radicals in vivo which consequently cause induction of lipid peroxidation and oxidative stress [34]. Results of the present study indicated studied organism by its nature has the ability to nullify adverse effects of LPX, which could also help in its survival and stability in various environmental conditions.

Hydroxyl radical is one of the most commonly involved ROS in biological damages of lipids, proteins and DNA [35]. In vivo, it may be produced from irradiation (X-ray) or from H_2O_2 in metal catalyzed reactions and due to its high reactivity, it acts on surrounding target molecules which leads to chain reactions e.g. initiation of lipid peroxidation due to abstraction of hydrogen atoms from polyunsaturated fatty acids (PUFA) [36,37]. Increasingly scavenging effects on hydroxyl radicals in the present study indicates bioactive molecules from bivalves [15,16] could prevent degradation of PUFAs. It is well known that food with high fat content is protected by natural antioxidants. Previous research reports that conjugated linoleic acid and phospholipids possess antioxidant potential [38]. In the present study the related fat content was not studied which may be, one of the important factor involved in antioxidant potential. However, significant correlation between LPX and OH radical scavenging by *P. malabarica* extracts supports inhibitory activities on LPX induced due to reactive oxygen species (ROS). Similarly, our earliar findings also revealed prominent hydroxyl scavenging in various types of bivalve species [25,26] which further confirms potential role of *P. malabarica* extract in deterring ROS induced free radical reactions.

The increasingly exponential values of FRAP measurement represents higher content of antioxidants in the sample extract [39]. A recent study has shown that, carotenoproteins expressed by cloned bivalve species possess significant FRAP or total antioxidant capacity values which also help for in vivo stabilization of free radicals [40]. Amino acids, oligosaccharides and unsaturated fatty acids of aqueous and alcoholic extracts from clam *Mactra veneriformis* have contributed significant antioxidant activities [41]. Synergistic effects of carotenoids, vitamins and minerals are also found to impart antioxidant properties to biological substances [42]. One or more of these types of compounds might have contributed the observed activities for studied organism. Results of correlation analysis obtained with antioxidant parameters in order to view level of significance and interdependency further supported our hypothesis.

In addition to the efficient in vitro radical scavenging activities, bivalve species are also found exhibit in vivo antioxidant responses when exposed to natural or artificial stressors. These determinations heavily rely on surrogate measurements of elevated antioxidant defenses (e.g. enzymes) and indicators of oxidative damage (e.g. DNA strand breaks) [43]. Studies of bivalves like *Mytilus galloprovincialis* [44], *Laternula elliptica* [45] and *Scrobicularia plana* [46] challenged with either chemicals, temperature or metals shown to express elevated levels of antioxidative enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione (GSH). The enzymatic reactions between free radicals and antioxidantive enzymes results in conversion of ROS to the less harmful or reactive forms and helps to prevent cellular damages. Despite the involvement of various cellular (in vivo) defense pathways against ROS, the biochemical data (in vitro) obtained in the present study further strengthens protective role of *P. malabarica* against free radicals.

In the present investigation, we have demonstrated the importance of seafood clam *P. malabarica*, in terms of radical scavenging, reducing capabilities and inhibition of lipid peroxidation. Our findings suggest its role in balancing normal metabolic functions of living organism when supplied through diet. This forms a first comprehensive report on the nutraceutical property of a seafood *P. malabarica* as a natural source of antioxidants.

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