

Antioxidative Potential of *Perna viridis* and its Protective role against ROS Induced Lipidperoxidation and Protein Carbonyl

K.B. Jena, T.G. Jagtap and X.N. Verlecar*

Biological Oceanography Division, National Institute of Oceanography,
Dona-Paula, Goa-403004

*For correspondence - verlecar@nio.org

Abstract

The antioxidant potential of methanol extracts of green-lipped mussel (*Perna viridis*) were evaluated using tests such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, reducing potential, scavenging capacity of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^\cdot), and inhibition of lipid peroxidation (LPX) and protein carbonyl (PC). The scavenging activity of DPPH radical, H_2O_2 and OH^\cdot radicals increased in dose dependent manner. The reducing power also increased significantly with increasing concentration but the effect was less sharp as compared to ascorbic acid as standard. Inhibition of LPX and PC levels shown by extract indicates its potential to protect the cell damage from ROS. Present investigation forms a first comprehensive report on the nutraceutical property of *P. viridis*.

Key words: *Perna viridis*; antioxidant potential; lipid peroxidation; protein carbonyl

Introduction

Reactive oxygen species (ROS), which constitute free radicals such as superoxide anion ($O_2^{\cdot-}$), OH^\cdot and non free-radicals such as H_2O_2 and singlet oxygen (1O_2) represent various forms of oxygen centered molecules (1). In living beings, ROS are formed endogenously during normal cellular metabolism. Exogenously ROS is obtained during exposure to ionization radiations and many

xenobiotic substances (1). In cellular systems, ROS are balanced at physiological concentrations by varieties of antioxidants, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferase (GST), reduced glutathione (GSH) and ascorbic acids (ASA) (1). Under pathological conditions ROS are overproduced (2, 3). The imbalance between ROS and antioxidant defence mechanisms leads oxidative damages to biomolecules like lipids, proteins and nucleic acids leading to disruption of cellular functions (1,4). The cellular damage results in several human diseases, such as atherosclerosis (5), cancer (6), gastric ulcer (7), aging and etc. (8). Their broad range of effects on biological and medicinal systems has aroused considerable interest among scientists to control these effects.

Much attention has recently been focused to search naturally occurring antioxidant for use in foods or medicinal purposes to replace synthetic antioxidants, which are being restricted due to their side effects of carcinogenicity (9, 10). Antioxidants acquired through diet are of great interest as possible protective agent against mutagen and oxidative damage (11). Hence, the studies on natural antioxidant have gained increasingly greater importance.

Perna viridis, an estuarine bivalve, is an excellent source of proteins, carbohydrates, lipids,

essential vitamins and serves as important sea food for human consumption (12). Earlier investigations are limited only to biological (13) and nutritional (12) status of this sea food. However, the present studies are intended to explore the medicinal properties of this bivalve *P. viridis* by understanding its antioxidant status and ROS scavenging potential.

Materials and methods

Collection and maintenance of mussels : Green lipped mussels (*Perna viridis*) were obtained from unpolluted areas of Goa coast and acclimatized for one week under laboratory condition (salinity 30 ‰, pH 8.0 and temperature range from 23-25°C). Mussels were fed with unicellular algal species (*Chlorella* sp.) twice daily at a constant initial density (2x10⁶cells/l) during acclimatization. To avoid faecal contamination, the water was exchanged every 24 hours and oxygen provided by continuous air-bubbling system.

Preparation of crude extract : After acclimatization period whole body tissue was carefully excised, thoroughly washed with distilled water and surface dried with tissue paper. A 10% (w/v) tissue homogenate was prepared in methanol, and homogenate was centrifuged at 8000 rpm for ten minutes. The supernatant was filtered through Whatman paper No 1, and the extract was concentrated through Rota evaporator. The condensed methanol extract containing 400 mg / ml of total dissolved solid (after blotted dry), was preserved at -20 °C until further use. All the experiments were conducted in triplicate.

Free radical scavenging activity : Free radical scavenging potential was measured by 2, 2-diphenyl-1-picryl-hydrazil (DPPH) by the method of Blois (14). The reaction mixture containing 2.5 ml of DPPH solution (0.1mM in methanol) and

extract (0.1-0.4 ml) was adjusted to a total volume of 3 ml by adding methanol. The absorbance was measured at 0 min and after 30 min at 517 nm. Butylated hydroxytoluene (BHT) was used as the control. Scavenging effect was calculated as below and expressed as percent

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

A₀ was absorbance at 0 minutes

A₁ was absorbance at 30 minutes

Measurement of reducing power : The reducing power of extract was determined by standard protocol, with a slight modification (15). The reaction mixture containing 2.5 ml phosphate buffer (0.2 M, pH 6.6), 2.5 ml potassium ferricyanide (1%) and different volumes of extracts (0.1 - 0.5 ml), was adjusted with distilled water to a total volume of 6.0 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, allowed to cool at room temperature. Later 2.5 ml trichloroacetic acid (TCA, 10 %) was added to the reaction mixture and centrifuged at 2000 rpm for ten minutes. The upper layer of 2.5 ml of solution was separated in another test tube, 2.5 ml distilled water and 0.5 ml FeCl₃ (1.0 %) were added to it and allowed to react for ten minutes at room temperature. The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing power.

Hydrogen peroxide scavenging assay : The H₂O₂ scavenging power of extracts was estimated using the method of Ruch *et al.* (16) with some variations. A solution of H₂O₂ (10mM) was prepared in phosphate buffer (pH 7.4). Reaction mixture containing 2.5 ml of H₂O₂ solution and varying concentration of test samples (0.1-0.5 ml) was adjusted to 3 ml by adding phosphate buffer solution (0.1M, pH 7.4). The absorbance was measured at 0 min and after 60

min at 240nm. Ascorbic acid (ASA) was used as the control. The percentage of scavenging effect (%) was calculated as

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

A₀ was absorbance at 0 minutes

A₁ was absorbance at 60 minutes

Hydroxyl scavenging assay : Hydroxyl radical scavenging ability was measured according to procedure by Kaur and Saini (11), with few modifications. The degradation of deoxyribose by OH[·] was measured colorimetrically in presence and absence of test samples. The reaction mixture contained 100mM of FeSO₄, 2mM of H₂O₂, 3mM of deoxyribose and varying concentration of test sample. The test tubes were then incubated for 30 min at 37 °C. After incubation, TCA (0.5 ml, 5%) and TBA (0.5 ml, 1%) were added to reaction mixture and kept in boiling water bath for 45 minutes. The absorbance was measured at 532nm. The scavenging effect (%) was calculated as

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

Where A₀ is the absorbance in the presence of extract, A₁ - absorbance without FeSO₄, and H₂O₂ and A₂ - absorbance of the control (without extract)

Preparation of sheep liver fractions : Sheep liver was obtained from slaughter house (Panaji, Goa), washed with ice cold KCl (1.15%) and homogenized (10% w/v) with Teflon Potter-Elvehjem homogenizer. Homogenate was filtrated through cheese cloth and centrifuged at 10000 rpm for 10 min, at 4°C. Supernatant was used for LPX assay.

LPX inhibition assay : Peroxidation of liver homogenate was induced by FeSO₄ solution. Liver homogenate was incubated with 100 mM of FeSO₄ for 30 minutes at 37 °C, the formation of thiobarbituric acid reactive substances

(TBARS) in the incubation mixture was measured at 532nm (17). The percentage inhibitory effect was calculated as

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

A₀ was the absorbance in the presence of extract, A₁ - absorbance without sheep liver homogenate and A₂ - absorbance of the control (without extract).

Inhibition of protein carbonyl assay : Protein oxidations were carried as described earlier with minor modifications (18). Bovine serum albumin (BSA) was oxidized by a Fenton-type reaction. The reaction mixture (1.5 ml) containing Bovine serum albumin (BSA, 2mg), FeCl₃ (50 μM), H₂O₂ (1 mM) and ascorbic acid (100 mM) and varying of sample extracts (0.1-0.5 ml) was incubated for 30 minutes at 37°C. After incubation, 1ml of 10mM 2, 4-dinitrophenylhydrazine (DNPH) in 2 M HCl was added to the reaction mixture. Samples were incubated for 30 min at room temperature.

Then, 1 ml of cold TCA (10%, w/v) was added to the mixture and centrifuged at 8000 rpm for 10 minutes. The protein pellet was washed three times with 2 ml of ethanol/ethyl acetate (1:1, v / v) and dissolved in 1 ml of guanidine hydrochloride (6 M). The carbonyl content was calculated from the absorbance measurement at 366 nm with the use of molar absorption coefficient of 22 000 mol cm⁻¹ and expressed as nmol / mg protein.

Statistical analysis : All experiments were conducted with three replicate and results were expressed as mean ± standard deviation (SD). Differences among the means were analyzed by one-way analysis of variance (ANOVA) and post hoc tests (Newman-Keuls). Differences were considered statistically significant when P < 0.05.

Results and discussion

Free radical scavenging activity : DPPH is a stable free radical and this radical scavenging assay was introduced as a antioxidant property within the test samples (19). This free radical reacts with reducing agents, pairing electrons, and discolouring stoichiometrically, depending upon the number of electrons utilized (14). The reducing capability of DPPH radical was monitored by decrease in its absorbance at 517 nm. BHT was used as standard, which is a known antioxidant. DPPH scavenging potential of the extract and standard depicted in Fig-1A reveals that increasing extract concentrations significantly enhanced the free radical scavenging capacity ($P < 0.05$). The action of an antioxidant is to scavenge free radicals by donating hydrogen to a free radical and reducing to an unreactive species (20). The decrease in the absorbance of DPPH solution caused by test samples is indicative of its reducing action. This suggests that scavenging potential of extract is directly proportional to extract concentration ($P < 0.05$).

Reducing power : The reducing power of the test compound, which is a major indicator of antioxidant potential (21), is involved with various mechanisms, such as prevention of chain initiation, binding of metal ions, decomposition of peroxides and radical scavenging (22,23). Methanolic extract of *P. viridis* showed significant increase in reducing power with higher sample concentration (Fig-1B, $P < 0.05$). The reducing power of extracts is generally associated with presence of reductones, which react with certain precursors of peroxide and provide protection against peroxide damage (24).

Hydrogen peroxide scavenging assay : Although H_2O_2 is not a free radical, it can react with metals such as Fe^{2+} or Cu^{2+} as well as superoxide anions in the Haber-Weiss reaction, producing highly reactive hydroxyl radicals (25).

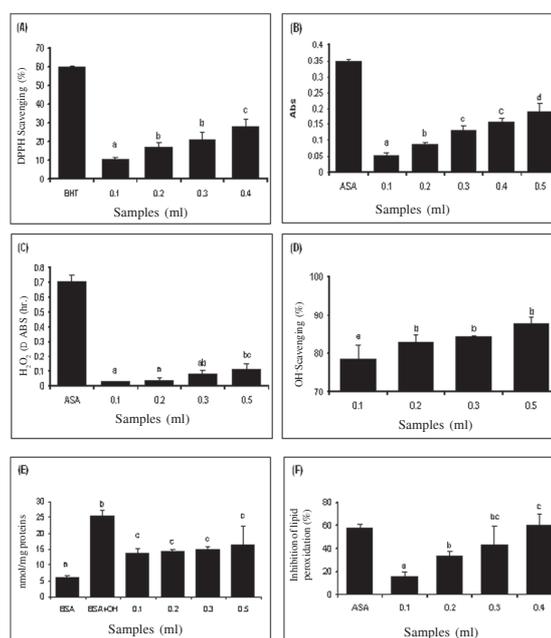


Fig. 1. (A) The DPPH radical-scavenging activity, (B) the reducing power, (C) the H_2O_2 scavenging activity, (D) the OH^\cdot scavenging potential, (E) protein carbonyl (nmol / mg protein) and (F) inhibition of lipid peroxidation. Values are mean of triplicate determination \pm SD ($n = 3$). Superscripts of different letters are significantly different from each other at $P < 0.05$.

Removal of H_2O_2 is therefore of utmost importance for cell or food systems to enable prevention of several diseases. As shown in Fig-1C, methanolic extract demonstrated H_2O_2 scavenging activity in a dose dependent manner. However as compared with extracts, ascorbic acid showed more effective capacity for scavenging H_2O_2 . It has been reported that electron donors may accelerate the conversion of H_2O_2 to H_2O (26), which could possibly scavenge H_2O_2 . A significant correlation observed between reducing power and H_2O_2 scavenging action (Fig-2A, $P < 0.01$) could support this statement.

Hydroxyl radical scavenging assay : Hydroxyl radical is most reactive with a wide range of molecules found in living cells, such as sugars, amino acids, lipids and nucleotides. The resultant effect is lipid peroxidation (27), protein oxidation (28), DNA damage (29), and all other damages. Such consequences can either be prevented and /or reversed by increasing the cellular antioxidant capacity. It is vital therefore to explore for OH[·]

radical scavengers in food materials for avoidance of several dreadful diseases which are resultant of these oxyradicals.

In biological systems formation of OH[·] radicals occur in several pathways, the most important mechanism being the Fenton reaction (30). In the present study, the scavenging potential was measured by Fenton reaction. Our results reveal that methanolic extract of *P.viridis* has tremendous scavenging potential for OH[·] radicals (Fig-1D), which could be due to reducing action and / or scavenging capacity for H₂O₂ or metals. Similarly the significant correlation between OH[·] radicals v/s reducing action ($r = 0.965$, $P < 0.01$, Fig-2B) and OH[·] radicals v/s H₂O₂ ($r = 0.95$, $P < 0.05$, Fig-2C) further strengthens our views on scavenging potential of OH[·] radicals by *P.viridis* extract.

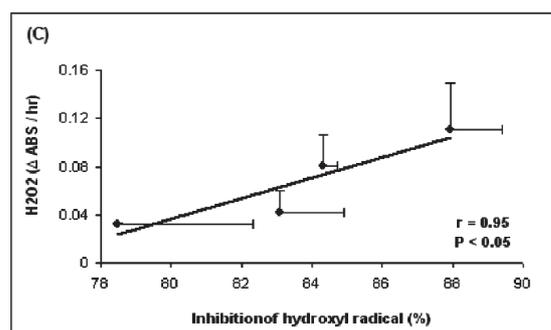
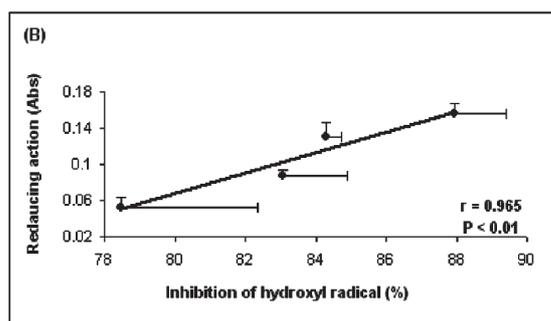
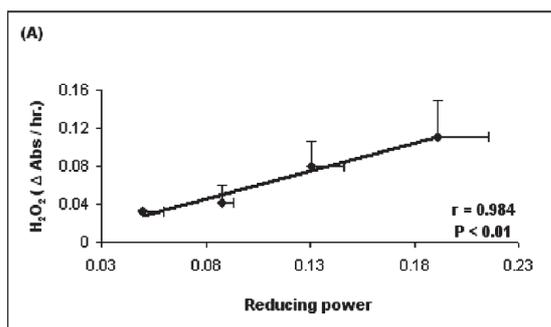


Fig. 2. Correlation between reducing action vs H₂O₂ scavenging potential (A), reducing action vs OH[·] scavenging potential (B), H₂O₂ and OH[·] scavenging potential (C).

Inhibition of protein carbonyl assay : Oxidation of proteins and carbonyl derivatives of proteins may result from oxidative modification of amino acids (31), which are responsible in aging and several pathological events (32). Carbonyl compounds possess toxic and carcinogenic properties and therefore their presence is of great concern to ill effects on health and food (33). Quantitative estimate of protein carbonyl derivatives is used as a sensitive assay for oxidative damage of proteins (34). Methanolic extract of *P.viridis* showed significant inhibitory effects on protein oxidation (Fig-1E, $P < 0.05$), indicating its effective use in pharmacology and food industry.

LPX inhibition assay : Macromolecules such as carbohydrates, lipids, proteins and DNA, can undergo oxidative damage in presence of ROS. Among these, lipids are more susceptible to oxidative damage in physiological process (35). Oxidation of lipids generates various degradation products, such as malondialdehyde (MDA) which

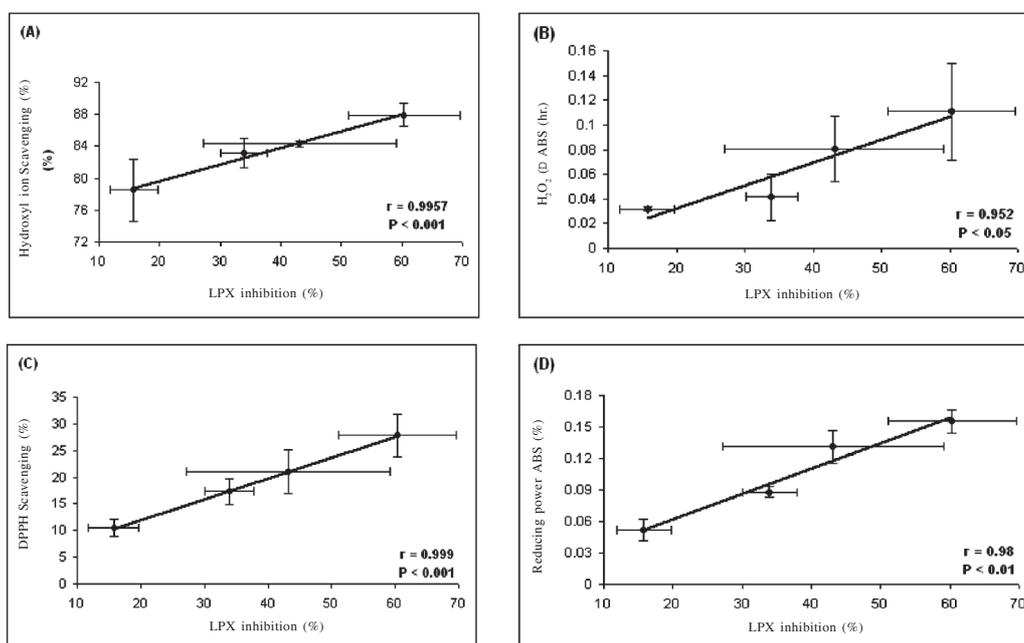


Fig. 3. Correlation between lipid peroxidation vs and OH[•] scavenging potential(A), lipid peroxidation vs H₂O₂ (B), lipid peroxidation vs DPPH scavenging potential (C) and lipid peroxidation vs reducing action (D).

forms a major cause of cell damage (36). Inhibition of LPX in cells and food materials is therefore of significant importance in preventing several diseases. *In vitro* induction of lipid peroxidation by Ferrous ions is a tool for measuring antioxidant potential of extracts. Ferrous ion stimulates lipid peroxidation and supports decomposition of lipid peroxides, generating highly reactive intermediates such as hydroxyl radicals, perferryl and ferryl species (37). Our investigations indicated that, methanolic extracts of *P. viridis* significantly inhibited lipid peroxidation (Fig-1F, P < 0.05). Decrease in LPX by test sample is probably the evidence of the scavenging action of OH[•] radicals, H₂O₂, other free radicals and / or the reducing potential of the extract. Similarly, a significant correlation between OH[•] radical scavenging capacity vs LPX (r = 0.9957, P < 0.01, Fig-3A), H₂O₂ scavenging action vs LPX (r =

0.952, P < 0.05, Fig-3B), DPPH scavenging potential v/s LPX (r = 0.999, P < 0.001, Fig-3C) and reducing action vs LPX (r = 0.98, P < 0.01, Fig-3D) are all in support of this hypothesis.

In conclusion, the present study determines that *P. viridis* is a rich source of natural antioxidants and has highly efficient protecting action against lipid peroxidation and protein oxidation. Hence *P. viridis* can be considered as the most suitable candidate for conducting further studies on purification, characterization and identification of antioxidant biomolecules for application in food industry and pharmaceutical investigations.

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