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In vitro antioxidant activity of tissue extracts of green mussel *Perna viridis*

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Original Article

Abstract

Molluscs have provided medicinally useful products for many cultures around the world and their utility has been known for centuries. There are even reports on mussels used for therapy in ancient Crete. The present work was intended to assess the antioxidant properties of Indian green mussel *Perna viridis* by employing various *in vitro* assays. Antioxidant activities of ethyl acetate, methanol and aqueous/ethanol extracts of the mussel was evaluated using different *in vitro* assays (DPPH, hydroxyl, superoxide radical scavenging assays, reducing power and beta carotene bleaching activity) and compared with the standard compound BHT (Butylated Hydroxy Toluene). In the range of concentrations tested (0.15–6 mg/ml), the three extracts showed a dose dependent pattern in scavenging the DPPH radical and higher activity was detected for ethyl acetate extract with an IC_{50} value of 0.616 mg/ml. The *P. viridis* extracts showed stronger scavenging activity for hydroxyl radicals compared to the other free radicals tested. For superoxide anion, methanol extract displayed better scavenging activity than aqueous/ethanol extract (IC_{50} 2.79 and 3.88 mg/ml respectively). β-carotene-linoleic acid assay was found to be dose dependent for all the three extracts and they inhibited β-carotene oxidation with significant difference in absorbance at 492 nm compared to control. The results of the reducing power and hydroxyl radical scavenging activities implies that these extracts can also act as electron-donors, and as such can terminate radical chain reactions. Although ethyl acetate extract showed an overall better activity, (IC_{50} for OH-—0.782; DPPH- 0.616) it proved to be slightly pro-oxidant against superoxide and hydroxyl radicals towards higher concentrations. It may be concluded that the extracts from *P. viridis* possess nontoxic antioxidant components, which render them suitable as potential therapeutics and good candidates for more detailed investigation. Further study is necessary to isolate active principles and elucidate the molecular structure of these compounds.

Keywords*: Perna viridis, DPPH, hydroxyl radical, superoxide radical scavenging assay.*

Introduction

The mussel *Perna viridis*, commonly known as the Indian green mussel, is a widely distributed edible mytilid bivalve seen all along both east and west coasts of India. *P. viridis* which is abundantly found in most Asian region is an inexpensive source of protein with high biological value, essential minerals and vitamins (Ismail *et al.*, 2004; Fuentes *et al.*, 2009). Mussels represent one of the important and economically useful organisms suited for cultivation (Indumathi *et al.*, 2015). In recent years the importance of mussels as a food resource and its health and nutritional benefits have been

reported (Chakraborty *et al.*, 2011). *P. viridis* are found to be a good source of proteins, minerals like Zn, Mn and Cu, vitamins like tocophcrol, vitamin C & D etc (Chakraborty *et al.*, 2011). There are also reports on the anti-inflammatory properties of *P. viridis* (Sreejamole *et al.*, 2011; Chakraborty *et al.*, 2010).

Although marine invertebrates particularly molluscs, have been explored for numerous biological and physiological functions, they are seldom studied for their antioxidant property. A few to mention here are studies on the free radical scavenging activity of the mussel hydrolysate prepared from *P. viridis* whole tissue against radiation and chemically induced ROS (Sreekumar, 2007). There are also reports on antioxidant peptides derived from *Theragra chalcogramma* (Jae *et al.*, 2005), squid (Rajapakse *et al.*, 2005b) and fermented mussel sauce (Rajapakse *et al.*, 2005a) and antioxidant activity of a low molecular weight peptide from blue mussel, *Mytilus edulis* (Jung *et al.*, 2005) and *Mytilus coruscus* muscle protein (Jung *et al.*, 2007). DPPH radical scavenging activity of methanolic extract of gastropod, *Pleuroploca trapezium* was also been reported (Anand *et al.*, 2010). Similarly studies on a peptide from *Crassostrea gigas* demonstrated free radical scavenging activity against linoleic acid, hydroxyl and superoxide radical scavenging models (Qian *et al.*, 2008). More recently Chakraborty *et al.* (2016) reported high oxyradical scavenging capacity (24-32%) of *P. virdis*.

Apart from the above mentioned studies very little is known about antioxidant properties of crude extracts from molluscan tissues and organs, nevertheless, molluscs deserve much more attention from marine natural product researchers, in terms of their nutritional value and status as an unavoidable food commodity next to fishes. Therefore, current study attempts in evaluating the antioxidant/ free radical scavenging properties of the three extracts (EtOAc, MeOH and aqueous/EtOH) of *P. viridis* using different *in vitro* antioxidant assays depicting various radical scavenging systems.

Material and methods

Sample collection

The mussels were collected from its natural bed at Anthakaranazhi, Alappuzha Dist Kerala, India and brought to the laboratory in live condition in aerated plastic containers filled with seawater of ambient salinity. Mussels of size range 7-9 cm were selected followed by thorough washing in running water to get rid of the debris. The identification of the mussel was done at Zoological survey of India, Chennai.

Preparation of the extracts

For the extraction, the procedure mentioned in the extraction of invertebrates by Cannell (1998) was adopted. The whole mussel tissue (300 g) was macerated in a blender and extracted twice with ethyl acetate (EtOAc) by mechanical stirring overnight. The suspension was centrifuged at 8,000 rpm for 20 min at 4°C. The resultant residue was successively extracted in the same way with methanol (MeOH) and water:ethanol, 7:3 (aqueous/EtOH). The three supernatants were evaporated to dryness in a rotary evaporator (35-55°C) under reduced pressure with a yield of 1.8, 6.4 and 3.2%, respectively, for ethyl acetate, methanol and aqueous/ethanol. The extracts were stored in airtight glass vials at -25° C until use.

In vitro antioxidant assays

The antioxidant property of the three extracts of *P. viridis* namely EtOAc, MeOH and aqueous/EtOH were evaluated using various *in vitro* assays. All the assays were carried out in six sample replications, and values are represented as the average of six replicates.

DPPH radical scavenging assay

Scavenging of DPPH radical by the EtOAc, MeOH and aqueous/EtOH extracts of *P. viridis* was measured according to the method of Hou *et al.* (2002). The absorbance read on a UV-VIS spectrophotometer (Hitachi, U-2001) at 517 nm against blank. Butylated Hydroxy Toluene (BHT) was used as the positive control and the percentage inhibition of test and the standard were calculated.

Hydroxyl radical scavenging assay

The TBARS formed by the deoxyribose degradation of hydroxyl radical generated by Fentons reaction (Fe^{2+}-ascorbate-EDTA-H_2O_2) was estimated by the method of Ohkawa *et al.* (1979). The hydroxyl radical scavenging activity was determined by comparing the absorbance of control with that of treatments at 530 nm.

Superoxide radical scavenging assay

The ability of *P. viridis* extracts to inhibit the auto-oxidation of pyrogallol was measured according to the modified method of Marklund and Marklund (1974). The inhibition of pyrogallol auto oxidation was measured at 325 nm. BHT was used as the standard for comparison.

Evaluation of reducing power

Reducing power of three extracts of *P. viridis* was investigated using the method developed by Oyaizu (1986). Absorbance of the test samples were measured at 700 nm.

β-carotene-linoleic acid assay

Antioxidant activity of *P. viridis* was evaluated using β-carotene bleaching assay as suggested by Siddhuraju and Becker (2003). Antioxidant capacities of the extracts were compared with that of BHT and the blank.

Statistical analysis

The IC_{50} values of extracts for each antioxidant assays were calculated using linear regression Probit analysis using SPSS version 14.

Results

DPPH radical scavenging activity

The DPPH scavenging property of three extracts of *P. viridis* is shown in Fig. 1. As depicted in graph in the range of concentrations (0.15–6 mg/ml) tested, the three extracts showed a dose dependent pattern in DPPH radical scavenging. The higher scavenging activity was detected for EtOAc extract (IC_{50} 0.616 mg/ml) followed by aqueous/EtOH (IC_{50} 0.784) and then by MeOH extract (Table 1). The activity was significantly higher for BHT (IC_{50} 0.343 mg/ml) compared to *P. viridis* extracts. At higher dose, the EtOAc, MeOH and aqueous/EtOH extract (6 mg/ml), showed a percentage of inhibition of 88.2, 76.5 and 82.2 % respectively.

Hydroxyl radical scavenging activity

The results showed a more or less stronger scavenging activity of *P. viridis* extracts towards the hydroxyl than for other free radicals, indicated by the reduction in pink chromogen formation (Fig. 2 and 3). In the case of aqueous/EtOH and MeOH extracts,

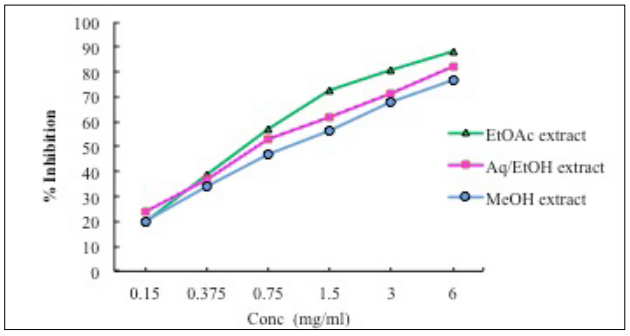

Fig. 1. Effect of *P. viridis* extracts on DPPH radical scavenging activity

the activities varied considerably at the lower concentration but were almost similar towards the higher concentrations. The two extracts were capable of scavenging hydroxyl radical in a dose dependent manner. By contrast, for EtOAc extract, the activity seems to be independent of concentration. IC_{50} values for MeOH and aqueous/EtOH extract of *P. viridis* were 0.536 mg/ml and 0.876 mg/ml respectively (Table 1).

Superoxide radical scavenging activity

The superoxide radical scavenging activity of *P. viridis* extracts were tested using pyrogallol auto oxidation method and the results are shown in Fig. 4. Comparatively better superoxide anion scavenging activity was displayed by MeOH extract than

Table 1. IC_{50} values of *P. viridis* extracts and standard BHT for different *in vitro* antioxidant assays (lower and upper bound at 95% confidence interval are given in parentheses.

P. viridis extracts/ STD	IC_{50} (mg/ml)		
	DPPH radical	Hydroxyl radical	Superoxide radical
EtOAc extract	0.616 (0.495 - 0.750)	0.782 (0.994 - 0.602)	NA
MeOH extract	1.04 (0.804 - 1.349)	0.536 (0.388 - 0.694)	2.789 (2.133 - 3.923)
Aq/EtOH extract	0.784 (0.605 - 0.996)	0.876 (0.698 - 1.089)	3.878 (2.953 - 5.551)
BHT (STD)	0.343 (0.308 - 0.384)	0.02 (0.018 - 0.023)	0.171 (0.145 - 0.211)

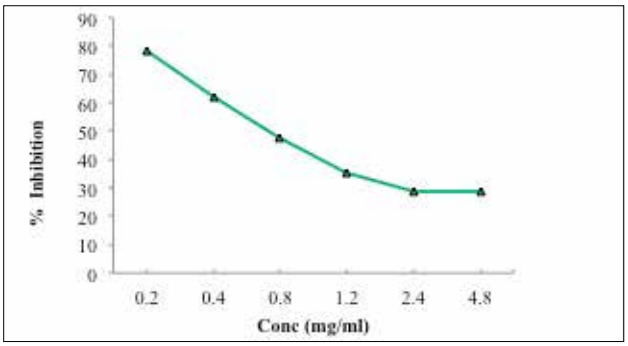

Fig. 2. Effect of EtOAc extract of *P. viridis* on hydroxyl radical scavenging activity

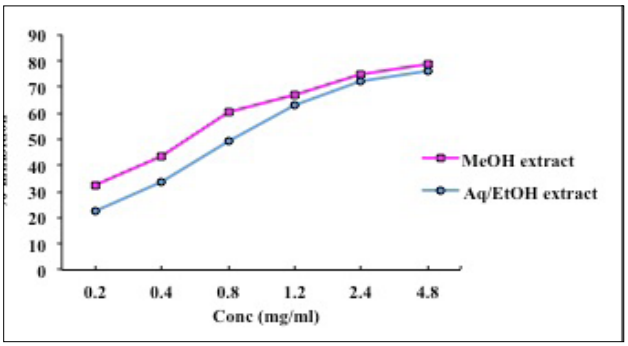

Fig.3. Effect of MeOH and aqueous/EtOH extracts of *P. viridis* on hydroxyl radical scavenging activity

aqueous/EtOH extract (IC_{50} 2.79 and 3.88 mg/ml respectively). EtOAc extract on the contrary showed remarkable scavenging property at lower concentrations while the activity decreased eventually as the concentration increased with a percentage inhibition of 85.1% at 0.15 mg/ml.

Reducing power property

Fig. 5 illustrates the reducing capabilities of extracts of *P. viridis* at different concentrations. The result shows that all the three extracts of *P. viridis* exhibited a dose dependent increase in Perl's Prussian blue formation at 700 nm. However, the activity was

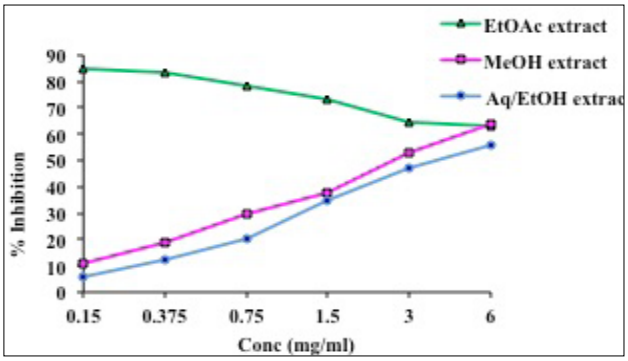

Fig. 4. Effect of *P. viridis* extracts on superoxide radical scavenging activity

much higher for BHT compared to the *P. viridis* extracts, which showed only a moderate increase in absorbance. Reducing power of *P. viridis* extracts and standard followed the order: BHT > MeOH extract > aqueous/EtOH extract > EtOAc extract.

β-carotene bleaching activity

The effect of different concentration of *P. viridis* extracts (0.6– 1.5 mg/ml) on β-carotene- linoleic acid assay are shown in Fig. 6, 7 and 8. The activity was found to be dose dependent and inhibited β-carotene oxidation with significant difference in absorbance at 492 nm compared to control. Higher concentrations (1.5 mg/ml) of EtOAc and aqueous/EtOH extracts showed inhibition of beta-carotene bleaching comparable to that of BHT.

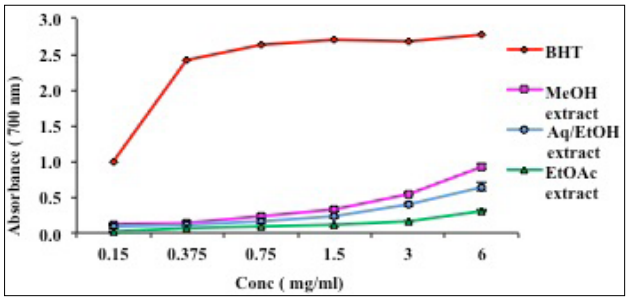

Fig. 5. Effect of *P. viridis* extracts on reducing power

Discussion

The results of the present study showed a significant concentration dependent activity of *P. viridis* extracts in scavenging DPPH radicals. Similar findings were also reported by Pachaiyappan *et al.* (2014) and Jena *et al.* (2010) on the DPPH radical scavenging activity of methanolic extract of *P. viridis*. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule, 1, 1-diphenyl-2-picryl hydrazine (Sreeyan and Rao, 1996; Soares *et al.*, 1997). The radical react with suitable reducing agents, the electrons become paired off, and the solution loses its colour stoichiometrically depending on the number of electrons taken up (Blois, 1958). The extracts were able to reduce the stable radical

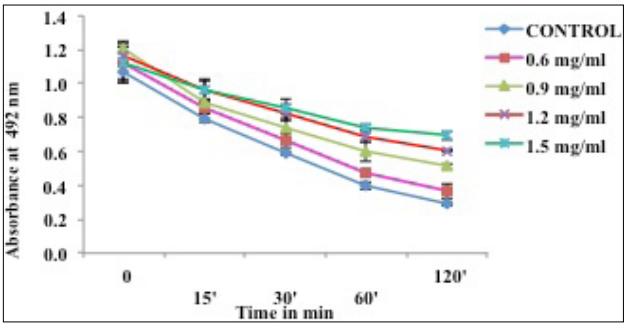

Fig. 6. Effect of EtOAc extract of *P. viridis* extracts on β-carotene – linoleic acid assay

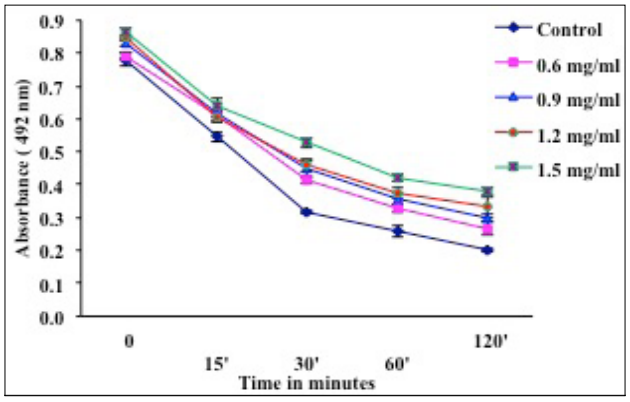

Fig. 7. Effect of MeOH extract of *P. viridis* extracts on β-carotene- linoleic acid assay

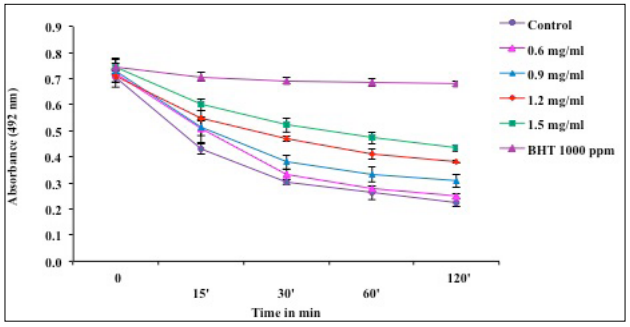

Fig. 8. Effect of aqueous/EtOH extract of *P. viridis* extracts and BHT on β-carotene – linoleic acid assay

DPPH to the yellow coloured di-phenyl picryl hydrazine, which was visually noticeable by the discolouration of test samples from purple to yellow. The decrease in absorbance caused by the antioxidants in the extracts might be due to the hydrogen donating ability resulted in the scavenging of the radical.

The hydroxyl radicals formed by Fentons reaction degrade the deoxyribose into thiobarbituric acid reactive substances, indicated by the pink chromogen formation. Antioxidants added to the reaction mixture may compete with the deoxyribose for hydroxyl radicals and inhibit sugar degradation. Addition of *P. viridis* extracts caused a significant decrease in pink chromogen formation on heating with TBA,

indicating the removal of hydroxyl radicals thus preventing the degradation of deoxyribose. The ability of a compound to inhibit deoxyribose degradation under these conditions reflects its iron chelating ability and the ability of its iron complex to participate in Fenton chemistry (Halliwell *et al.*, 1987).

The results also showed a pro-oxidant tendency of EtOAc extract towards higher concentration for hydroxyl radical scavenging assay. Similar observations were reported for the antioxidant assay on grape extracts (Stagos *et al.*, 2005) and ethanol extract of *Opuntia ficus-indica* (*Lee et al.*, 2002). The detection of phenolic compounds in EtOAc extract of *P. viridis* by Sreejamole and Radhakrishnan (2010) and more recently the presence of phenolics by Chakraborty *et al.* (2016) can be correlated with the present findings.

In the current study *P. viridis* extracts displayed considerable scavenging activity for superoxide anion radicals in pyrogallol autoxidation system. Pyrogallol can autoxidate fast in alkali conditions and release superoxide anions, and in return, the anions can accelerate the autoxidation (Zhonggao *et al.*, 2005). However, the superoxide anions are scavenged by adding some scavenger or antioxidant, whereby the autoxidation would be depressed. The decrease in the increment of absorbance in test samples compared to control indicates the superoxide anion scavenging activity of the *P. viridis* extracts. The probable mechanism of scavenging superoxide anions in this case may be the inhibitory effect of these extracts against the generation of superoxides in the reaction mixture. It has been reported earlier that phenolic compounds exhibits superoxide radical scavenging activity (Taubert *et al.*, 2003; Kubo *et al.*, 2002). Consequently, such an observation can be linked with the presence of phenolics in extracts.

All the three extracts of *P. viridis* showed an increase in Perl's Prussian blue formation with increase in concentration suggesting their reductive ability. The presence of reductants (i.e. antioxidants) in the extract causes the reduction of the Fe^{3+}/ferricyanide complex to ferrous form (Leskovar *et al.*, 2004), which can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Reducing power of the *P. viridis* extracts indicate that it can act as electron donors, convert the free radicals to more stable products and thereby terminate radical chain reactions. Similar observations were also made by Pachaiyappan *et al.* (2014) for methanolic extract of *P. viridis*.

Addition of *P. viridis* extract caused a reduction in β-carotene bleaching indicative of its antioxidant activity. In this model system, the linoleic acid free radical formed attacks the unsaturated β-carotene and oxidizes them. Thus, the solution loses its characteristic orange colour, which can be monitored spectrophotometrically. However, the extent of β-carotene bleaching can be inhibited by the antioxidants, which neutralizes the linoleate free radical (Jayaprakasha *et al.*, 2001). Thus, it is feasible to assume that the antioxidant property of the *P. viridis* extracts lies in scavenging the linoleic acid free radical thereby inhibiting the discolouration of β-carotene.

The preliminary chemical analysis of EtOAc, MeOH and aqueous/EtOH extracts ofP. viridis indicated the presence of alkaloids, polyphenols, terpenes, saponins and steroids (Sreejamole and Radhakrishnan, 2010). Phenol and phenolic compounds have been shown to possess significant antioxidant activities. Owing to their ability to scavenge free radicals and reactive oxygen species (such as singlet oxygen, superoxide free radicals and hydroxyl radicals), they have been described as effective antioxidants (Hall and Cuppett, 1997). Moreover, antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors, the ability of the polyphenol derived radical to stabilize and delocalize the unpaired electron (chain-breaking function), and their potential to chelate metal ions (termination of the Fenton reaction) (Rice-Evans *et al.*, 1997).

Although it is known that polyphenols contribute to the antioxidant properties of most of the plant derived natural products, they are least reported in molluscs. However, Mamelona *et al.* (2007) and Zhong *et al.* (2007) have reported the presence of polyphenols in sea cucumber *Cucumaria frondosa* showing antioxidant activity, which they suggest to have derived from algal diet.

Studies have also shown marine-derived sugars (Ruperez *et al.*, 2002) and carotenoids (Tsushima *et al.*, 1995) exhibiting antioxidant activity in vitro. In addition to phenolic compounds, some of the alkaloids, saponins and triterpenoids have shown to possess antioxidant activity. Similar findings were reported by Rai *et al.* (2006) in their study on *Nelumbo nucifera* seeds. Presence of these chemical constituents in the extracts of *P. viridis* further substantiates this. Hence, the observed *in vitro* antioxidant activity may be because of these constituents, which needs further investigation.

The results of the radical scavenging tests revealed that *P. viridis* contained some active antioxidant substances. Though slightly pro-oxidant, LC_{50} values of EtOAc extract were much lesser compared to MeOH and aqueous/EtOH extracts. Comparable observation was also cited by Ekanayake *et al.* (2004) on fish tissue. The results of the present investigation on *P. viridis*, being an earnest attempt on a highly commercially important species, yielded interesting informations and which may give an ample basis for future studies in this area. Therefore, considering their food value, it can be concluded that *P. viridis* proved to be a useful source of antioxidants for human consumption.

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