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Comparative *in vitro* study on free radical scavenging potential of selected bivalve species

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TITOLO
Studio comparativo *in vitro* sulla potenziale attività di smaltimento dei radicali liberi di specie selezionate di bivalvi

KEY WORDS
Bivalve, free radical, *in vitro*, antioxidant

PAROLE CHIAVE
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Summary
Bivalves such as, *Crassostrea* spp., *Meretrix casta*, *Placuna placenta* and *Polymesoda erosa* are largely consumed as edible seafood. It forms natural source of nutrition in coastal and worldwide population. Free radical scavenging activities of these species were tested against 2, 2–diphenyl–1–picryl–hydrazyl (DPPH), hydroxyl radical (˙OH), reducing power and *in vitro* inhibition of lipid peroxidation (LPX). Methanolic extracts exhibited dose dependency with increasing concentration (3 to 9 mg/ml) of sample. On comparing DPPH activities, *M. casta* and *P. placenta* statistically exhibited significant radical scavenging properties ($p<0.01$). However, scavenging effect of *Crassostrea* spp. was higher irrespective of dose dependency. Stable hydroxyl scavenging effects and increasing reducing actions with increase in absorbance were also seen to be concentration dependant. Superior activities against *in vitro* induced LPX were observed in the order of *P. placenta*> *M. casta*> *P. erosa* > *Crassostrea* spp. This indicates protective role against deterioration cell membrane lipids during oxidative stress. Moreover, correlation of variables ($p<0.001$) in the present study, further supports our views on, *M. casta* and *Crassostrea* spp. as natural source of antioxidative seafood.

Riassunto
Bivalvi come, *Crassostrea* spp. *Meretrix casta*, *Placuna placenta* e *Polymesoda erosa* sono largamente consumati come prodotti ittici commestibili. Costituiscono una fonte naturale di nutrimento per la popolazione costiera e di tutto il mondo. L'attività di smaltimento dei radicali liberi (scavenging) di queste specie è stata testata verso il 2,2-difenil-1-picrylhydrazyl (DPPH), idrossi radicale (•OH), potente riducente ed inibitore della perossidazione lipidica (LPX) *in vitro*. Gli estratti metanolici hanno mostrato una dose-dipendenza con l'aumentare della concentrazione del campione (da 3 a 9 mg/ml). Confrontando le attività DPPH, *M. casta* e *P. placenta* hanno mostrato proprietà statisticamente significative di scavenging radicalico ($p<0,01$). Tuttavia, l'effetto di scavenging di *Crassostrea* spp. era più alto a prescindere dalla dose-dipendenza. Anche gli effetti di scavenging degli idrossili stabili e l'aumentare delle azioni riducenti con aumento di assorbanza sono stati osservati essere dipendenti

dalla concentrazione. Per contro attività superiori *in vitro* contro LPX indotta sono state osservate nel seguente ordine: *P. placenta*> *M. casta*> *P. erosa*> *Crassostrea* spp. Questo dimostra il ruolo protettivo contro il deterioramento dei lipidi della membrana cellulare durante lo stress ossidativo. Inoltre, la correlazione delle variabili (p <0,001) nel presente studio, conferma ulteriormente le nostre opinioni su *M. casta* e *Crassostrea* spp. come fonti naturali di antiossidanti prodotti dall'ambiente ittico.

Introduction

Reactive oxygen species (ROS) and Reactive nitrogen species (RNS) are the major groups of free radicals formed during normal reactions of cell metabolism (1) as well as due to exposure of xenobiotics (2). Excess accumulation of free radicals in the cell exerts debilitating oxidative stress in an individual (3). Once formed, these highly reactive radicals start a chain reactions and affects functioning of cellular components like lipids, proteins, DNA and carbohydrates leading to number of diseases especially cancer, aging, arthritis, inflammation, diabetes, parkinson's disease and atherosclerosis (4, 5). In addition, radical-mediated lipid peroxidation negatively impacts flavour, texture, nutritive value and shelf life of food products (6). To withstand with such adverse effects, living organisms have their own antioxidant defense mechanisms.

Antioxidants play an important role in regulating various oxidative reactions by scavenging free radicals, chelating free catalytic metals and by acting as electron donors (7). The regulation takes place by enzymatic and non-enzymatic antioxidant systems. Beside several enzyme systems including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) within the body that scavenge free radicals, the principle micronutrient antioxidants such as vitamin E, beta-carotene, and vitamin C also known to have antioxidant potential (8). The body cannot manufacture these micronutrients and need to be supplied from external food sources. Recently naturally occurring antioxidants are preferred for their use in diet or medicines than synthetic antioxidant to avoid their adverse effects. For example, Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are suspected to be responsible for carcinogenicity and liver cancer (9, 10). Hence, the studies on natural antioxidant have gained increasingly greater importance.

Studies on different plant components such as fruits, leaves, barks as well as various food products are well known for presence of antioxidant compounds (11). However, animal sources are being less explored for their antioxidant properties (12). Seafood is being globally used as one of the important food sources with high nutritional values (vitamins and minerals). Antioxidant compounds isolated from animal sources like new chlorophyll-A related compounds (13), Proteins (14), lipids (PUFA) (15) and carotenoids (16) from mollusks exhibited prominent antioxidant properties. Bivalves including clams, mussels, and oysters are dominant components of shell fishery (17). *Crassostrea* spp., *Meretrix casta* (Chemnitz 1782), *Placuna placenta* (Linnaeus 1758) and *Polymesoda erosa* (Solander 1876) are largely consumed as important source of nutrition in India (18), and also exported worldwide as a sea food. However, limited amount of information is available on their nutritional proper-

ties including antioxidant potentials.

Efforts have been made to explore antioxidant potentials and free radical scavenging activities of a methanolic extracts of *Crassostrea* spp., *M. casta*, *P. placenta* and *P. erosa*. The extracts were examined by different free radical scavenging activities including DPPH radical scavenging, reducing power, hydroxyl radical scavenging and inhibition of *in vitro* lipid peroxidation. The data provides first comparative and comprehensive report on importance of edible bivalve species in scavenging of ROS.

Materials and methods

Chemicals

All chemicals and solvents used in experiments were of analytical grades. 2, 2–diphenyl–1–picryl–hydrazyl (DPPH), trichloroacetic acid (TCA) and ascorbic acid were obtained from Himedia Laboratories Pvt. Ltd., Mumbai. Potassium ferricyanide, ferric chloride ($FeCl_3$) was obtained from Qualigen fine Chemicals, Mumbai. Methanol and hydrogen peroxide (H_2O_2) were obtained from Sd-Fine chemical limited, Mumbai, ethylene diamine tetra acetic acid (EDTA), butylated hydroxy toluene (BHT), potassium chloride (KCL), sodium dodecyl sulfate (SDS) and Ferrous sulfate ($FeSO_4.7H_2O$) were obtained from SRL, Mumbai. 2-Deoxy-D-ribose was obtained from Sigma–Aldrich Inc., Germany.

Preparation of crude extracts

Four bivalve species (*Crassostrea* spp., *M. casta, P. placenta* and *P. erosa*) were brought to the laboratory from the consumer fish market of Goa, India (January and February, 2010) and dissected immediately. The whole body tissue was collected, washed with distilled water and surface dried with tissue paper. Tissue homogenate (10% w/v) was prepared in 90% methanol and kept in shaker for 48 hrs. All procedures were carried out in ice cold conditions. Then crude extracts were filtered through Whatman paper No 1. and concentrated using Rota evaporator (Buchi Rotavapor R-200). Extracts were stored in dark bottles at 4°C until further analysis. All analysis experiments were conducted in triplicate.

Yield estimation

The yield estimation was carried out by evaporating 1 ml of extract in pre-weighted aluminium dish at room temperature (27°C) until complete dryness and was expressed in mg (crude dry weight extract)/ml (12) and finally adjusted to 30 mg/ml with same solvent.

DPPH free radical scavenging activity

Free radical scavenging potential was measured against 2, 2–diphenyl–1–picryl–hydrazyl (DPPH) by standard method described by Blois (19). The reaction mixture containing 2.4 ml of DPPH solution (10 mM in methanol) and extracts of different concentration was adjusted to 3 ml by adding methanol. Initial and final absorbance was measured at 517 nm after 20 min of time interval. The (DPPH) scavenging activity of the sample was compared with standard compound Butylated hydroxytoluene (BHT). Scavenging effect was calculated using formula given below, and expressed as percent value.

DPPH radical scavenging activity (%) = $(A_0 - A_1 / A_0) \times 100$

Whereas, A_0 was absorbance at 0 minutes and A_1 was absorbance at 20 minutes.

Measurement of reducing power

The reducing power of the extract was determined by the method followed by Oyaizu (20), with minor modifications. The reaction mixture containing 2.5 ml phosphate buffer (0.2 M, pH 6.6), 2.5 ml potassium ferricyanide (1%)

and different concentrations of extracts, was adjusted with distilled water to final reaction volume 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 min and allowed to cool at room temperature. Later 2.5 ml of 10% trichloroacetic acid (TCA) was added to the reaction mixture and centrifuged at 2000 x g for 10 min. Supernatant (2.5 ml) was transferred to another test tube, to which 2.5 ml of distilled water and 0.5 ml $FeCl_3$ (0.1%) were added. The reaction mixture was allowed to react at room temperature for 10 min. Absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing power.

Determination of in vitro inhibition of lipid peroxidation

Preparation of ship liver fraction

Sheep liver was obtained from slaughter house (Panaji, Goa) washed with ice cold potassium chloride (1.15%) and homogenized (10% w/v) with homogenizer (Teflon Potter Elvejhem). Homogenate was filtered through cheese cloth and centrifuged at 10000 x g for 10 minutes at 4^0C. Supernatant was used for LPX assay.

Induction of LPX and determination of inhibition

Lipid peroxidation of tissue samples were assayed according to Ohkawa et al. (21) method with minor modifications. Peroxidation of liver homogenate was induced with $FeSO_4$ (100 µM) solution. To this, sample extract was added and further treated with thiobarbituric acid (TBA) for 60 min at 95°C. The formation of TBA-reactive species (TBARS) was measured at 532 nm. Ascorbic acid was used as a standard compound.

The percentage inhibitory effect was calculated as,

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

Where, A_0 was the absorbance in the presence of extract, A_1 was the absorbance without sheep liver homogenate and A_2 was the absorbance of the control (without extract).

Protein concentrations in the samples were analyzed by using Folin-Ciocalteu reagent and BSA as a standard (22).

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging ability of extracts was measured by quantifying Fenton reaction (23). Reaction mixture containing 200 µl each of $FeSO_4.7H_2O$ (10 mM), EDTA (10 mM), 2–Deoxy-D- ribose (10 mM) and sample extract of different concentrations was adjusted to 1.0 µl with phosphate buffer (0.1 mM, pH 7.4). Later, 200 µL of H_2O_2 (10 mM) was used to initiate the reaction. 1 ml of TCA (2.8%) and TBA (0.1%) were added after incubation at 37°C for 4 hrs. and placed in boiling water bath for 10 min. The absorbance was measured at 532 nm. The scavenging effect of hydroxyl radical was calculated in percentage as,

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

Where, A_0 was the absorbance in the presence of the tested samples, A_1 is the absorbance of the control and A_2 was the absorbance without sample.

Statistical analysis

All data were expressed as mean value ± SD of the number of experiments (n=3). Results were processed using Microsoft Excel 2007 and differences among the mean were tested using post hoc tests (Newman–Keuls) and one–way analysis of variance (ANOVA). Differences were considered statistically significant when $P < 0.05$.

Results and Discussion

Antioxidant efficacies depend on the type of antioxidant, their composition, concentration and antioxidant assays used for its screening (24). The scavenging effect are

due to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging activity (25). The results of present investigations are described as below.

DPPH free radical scavenging activity

DPPH oftenly used as a substrate to evaluate antioxidant activity, which undergoes reduction by accepting electrons or hydrogen atoms. This results in decolorization which can be quantitatively measured from the changes in absorbance (26). The methanolic extracts exhibited scavenging effect in the order of 44.12%, 42.76%, 32.46% and 43.64% by *Crassostrea* spp., *M. casta*, *P. placenta* and *P. erosa* respectively, at higher concentration of sample extract tested (Fig. 1a). *Meretrix casta* and *P. placenta* were observed to be statistically significant in their scavenging abilities ($P<0.01$). Overall, stable free radical DPPH scavenger was higher in *Crassosstrea* spp. whereas other species showed significant ($P<0.05$) concentration dependency. Moreover, antioxidant activities of protein hydrolysates from oyster and polyphenol fractions from Black mussel (*Mytilus galloprovincialis*) were observed to be prominent DPPH scavengers (14). Earlier studies on Metallothioneins (cysteine-rich proteins) isolated from sea mussel and fish rainbow trout, showed higher DPPH scavenging activity in mussel (27). This indicates protein constitution of bivalve tissue might be responsible for scavenging properties.

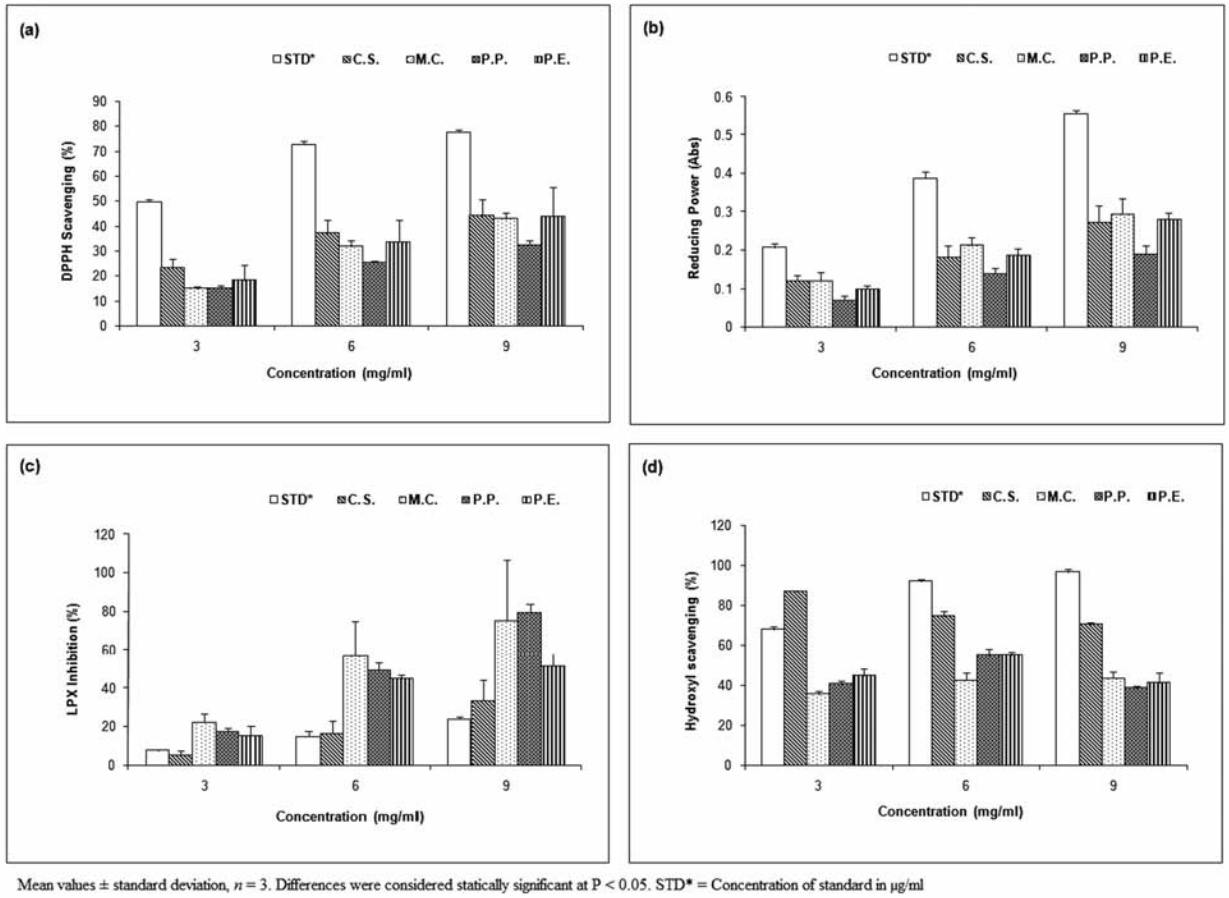
Reducing power

Presence of reductants in the extracts responsible for reduction of Fe^{3+} complex to the Fe^{2+} was monitored by measuring the formation of Perl's Prussian blue. All four organisms in the present investigation, showed increased reducing power with increase in concentrations of their methanolic extracts (Fig. 1b). Furthermore, dose dependency was supported by significant reducing properties at $P<0.01$ level. Methanolic extract of *P. placenta* exerts absorbance of 0.189 at highest concentration, which was less than other three species with almost same in their reducing ability. Oyster *C. talienwhannensis* (14) and mussel *Perna viridis* (28) have been reported for their significant role towards reducing action. The reducing ability of compound depends on the presence of reductones, which have antioxidative potential by providing protection against peroxide damage (29). Prominent reducing activities observed in bivalve extracts could help in protecting cell damage mediated by singlet oxygen radicals. Moreover, it can be a supplementary source of antioxidative rich meat in seafood system.

In vitro inhibition of lipid peroxidation

Oxidation of lipid generates various degradation products such as malonaldehyde (MDA) which forms a major cause of cell damage (30). Inhibition of ferrous ion induced LPX was quantified by measuring MDA-TBA adduct. Decreased production of MDA due to inhibition of LPX by extract of studied organisms shown decrease in colour intensity. Methanolic extracts of bivalve species except *M. casta* significantly inhibited lipid peroxidation ($p<0.01$). The order of observed inhibition of LPX at highest concentration (9 mg/ml) were *P. placenta*> *M. casta*> *P. erosa* > *Crassostrea* spp. (Fig. 1c). Higher inhibitory values of samples than standard compound suggests superior antioxidant activity. It has been reported that fat-containing food is protected by natural antioxidants (31). Also, other biomolecules like polyphenols from *M. galloprovincialis* (32) and purified peptide from *C. gigas* (33) showed inhibition against lipid peroxidation.

Figure 1 - Radical scavenging efficacies of species *Crassostrea* spp. (C.S.), *Meretrix casta* (M.C.), *Placuna placenta* (P.P.) and *Polymedosa erosa* (P.E.) against (a) DPPH scavenging, (b) Reducing power, (c) Inhibition of lipid peroxidation, (d) Hydroxyl radical scavenging.



Mean values ± standard deviation, $n = 3$. Differences were considered statically significant at $P < 0.05$. STD* = Concentration of standard in µg/ml

Hydroxyl radical scavenging assay

Hydroxyl radical mediated Deoxyribose degradation in a Fe^{3+}-EDTA-ascorbic acid and H_2O_2 reaction mixture, results in formation of pink chromogen product when heated with TBA (34). Bivalve extracts under study, were found to prevent degradation of sugar by scavenging ·OH radicals, and it increased with elevated concentrations. Scavenging effect of extract was either stabilized or decreased after certain concentration (Fig 1d), which may due to over dose of substrate or shortest half life of ·OH radical. *Crassostrea* spp., *P. placenta*, *P. erosa* extracts showed strongly significant inhibitory activities ($p<0.01$). In this context, purified peptide from oyster significantly scavenges cellular radicals and shown protective effect on

Table 1 - Linear regression equations of correlation between antioxidant parameters

Species	Correlation variables	Regression equation	R^2 value
Crassostrea spp.	DPPH vs Reducing power	y = 0.0056x - 0.0032	0.651[b]
	DPPH vs LPX	y = 0.9339x - 14.199	0.485[a]
	DPPH vs ·OH scavenging	y = -0.5893x + 97.756	0.655[b]
	Reducing power vs LPX	y = 131.92x - 7.0644	0.469[a]
	Reducing power vs ·OH scavenging	y = -91.267x + 94.797	0.761[c]
	LPX vs ·OH scavenging	y = -91.267x + 94.797	0.610[b]
M. Casta	DPPH vs Reducing power	y = 0.0059x + 0.0346	0.818[c]
	DPPH vs LPX	y = 1.9418x - 6.4477	0.651[b]
	DPPH vs ·OH scavenging	y = 0.2933x + 31.731	0.622[b]
	Reducing power vs LPX	y = 309.71x - 13.44	0.697[b]
	Reducing power vs ·OH scavenging	y = 44.44x + 31.165	0.602[b]
	LPX vs ·OH scavenging	y = 0.1019x + 35.234	0.435[a]
P. placenta	DPPH vs Reducing power	y = 0.0062x - 0.0155	0.895[c]
	DPPH vs LPX	y = 3.5227x - 36.765	0.988[c]
	DPPH vs ·OH scavenging	y = -0.0408x + 45.826	0.002[NS]
	Reducing power vs LPX	y = 510.91x - 20.515	0.900[c]
	Reducing power vs ·OH scavenging	y = -9.3102x + 46.097	0.004[NS]
	LPX vs ·OH scavenging	y = -0.0367x + 46.625	0.016[NS]
P. erosa	DPPH vs Reducing power	y = 0.0051x + 0.0267	0.775[c]
	DPPH vs LPX	y = 1.018x + 4.8862	0.645[b]
	DPPH vs ·OH scavenging	y = -0.0205x + 47.691	0.002[NS]
	Reducing power vs LPX	y = 194.51x + 0.3976	0.797[c]
	Reducing power vs ·OH scavenging	y = -20.588x + 50.945	0.053[NS]
	LPX vs ·OH scavenging	y = 0.0175x + 46.389	0.002[NS]

P values: < [a]0.05, [b]0.01, [c]0.001, [NS] Non significant

DNA damage caused by hydroxyl radicals (33).

Antioxidant variables applied in the present study were correlated for accessing significance level (Tab. 1). Correlation between DPPH and reducing power (R^2=0.818), reducing power and hydroxyl scavenging (R^2=0.761), DPPH and inhibition of LPX (R^2=0.988) and reducing power and inhibition of LPX (R^2=0.797) at P<0.001 observed in *M. casta*, *Crassostrea* spp., *P. placenta*, *P. erosa* respectively, are suggestive of rich antioxidant potential of these bivalve species. This supports our views, on importance of bivalve meat as an antioxidant rich source of seafood.

Conclusion

Results derived from four tests suggest that methanolic extracts of selected bivalve species have more

or less similar scavenging potential. However, *M. casta* and *Crassostrea* spp. showed highest antioxidant properties whereas, *P. erosa* and *P. placenta* were found with moderate activities. It is suggested that *M. casta* and *Crassostrea* spp. could be used as potent natural antioxidant sources as they exhibited highest DPPH scavenging, efficient reducing action and protecting role against lipid peroxidation. However, further isolation and characterization of specific antioxidant compounds from bivalves is necessary for new drug designing.

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