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ANTIOXIDANT PROPERTIES OF TWO EDIBLE BIVALVE *MERETRIX MERETRIX* AND *M. CASTA*

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ABSTRACT

To evaluates *In vitro* antioxidant properties of two edible bivalves *Meretrix meretrix* and *M. casta.* Both bivalve extracts MME and MCE showed effective antioxidant activities, reducing power, hydrogen donor activity, free radical scavenging activity and ferrous iron chelating ability. Dose dependent radical scavenging activity was observed, increased concentration showed increased radical scavenging activities and results were compared to known antioxidant compound ascorbic acid and BHA. As compare to MCE, MME showed more significant antioxidant activities at *In vitro* modelling assays. FT-IR spectroscopy analysis of both extracts MME and MCE showed various levels of peaks, it meant for the various characteristic functional groups were present in the both bivalve extracts. The result indicates that both bivalve species had efficient antioxidant properties.

KEYWORDS: *Meretrix* sp., Total antioxidant activity, reducing power, DPPH, FT-IR.

INTRODUCTION

Enzymatic and non-enzymatic antioxidant system is protecting our human body against reactive oxygen species (ROS) induced damage (Anderson, 1999). Increased amount of ROS can damage the cellular Biomolecular systems such as, cell death, cell injury and it results multiple diseases like cancer, stroke, myocardial interaction and Alzheimer's diseases. There are many synthetic antioxidants are available butylated hydroxyl anisole (BHA) and Butylated hydroxyl toluene (BHT), but these are cause several toxic side effects and also it acts as a carcinogen (Anangnostopoulou *et al.*, 2006). On the consideration of the above fact we need safer and cheaper drug for human welfare. Every year more than hundred new compounds were discovered from the marine environment. Many bioactive compounds are reported from marine organisms are under preclinical and clinical trials. Among marine phylum marine invertebrates produce several structurally different bioactive metabolites with prominent pharmacological activities. e.g. Bryostatins (Schaufelberger e*t al.*, 1991) and didemnins (Rinehart *et al.*, 2000).

In marine invertebrates bivalve mollusc plays a key role in the production of bioactive compounds such as proteins, peptides, immune modulatory compounds, etc. Traditionally the bivalve mollusc sp. (*M. meretrix* and *M. Casta*) was used for remedy for many deadly diseases in tropical countries (Xie *et al.*, 2012). It widely distributed in south east coast of India (Jayabal, 1986). Quite a large number of bioactive compounds were reported from *M. meretrix,* anticancer (Sugesh *et al.*, 2014), antioxidant (Nazeer *et al.*, 2011), antimicrobials (Sugesh and Mayavu, 2013), etc. the very little amount of antioxidant reports are available on marine organisms. In the above fact present investigation was carried out to evaluate the antioxidant properties of two edible bivalve species *M. meretrix* and *M. casta.*

MATERIALS AND METHODS

Preparation of Molluscan Extracts

The live specimen of marine bivalves (*M. meretrix* and *M. casta*) was collected from Vellar estuary of Parangipettai south east coast of India (Lat 11^0 29' N; 79^0 46'E). The collected animals were brought into the laboratory and shells were washed with distilled water and broken by using hammer. The extraction procedure was followed by Ning *et al.*, (2009). The extracts obtained from *M. meretrix* and *M. casta*, shortly named as MME (*M. meretrix* Extract) and MCE (*M. casta* Extract).

Total antioxidant activity

The antioxidant activity was determined by the conjugated dine method of Lingnert *et al.*, (1979). The

molluscan extracts MME and MCE and standard BSA was mixed with linoleic acid emulsion in sodium phosphate buffer in test tubes and placed in darkness to accelerate oxidation after incubation, methanol in deionized water was added and the absorbance of the mixture was measured at 234nm against a blank.

Superoxide radical scavenging assay

The superoxide radical scavenging ability of molluscan extracts was assessed by Nishikimi *et al.,* (1972). The reaction mixture containing extracts of MME and MCE and standard BSA, PMS (30µm), NADH (338µm) and NBT (72µm) in phosphate buffer (0.1m pH7.4) was incubated at room temperature and the absorbance was read at 560nm against blank. The capability scavenging the superoxide radical was calculated by following equation.

Scavenging effect (%) = {1-Sample (560nm) /Control (560nm) x 100}

Hydroxyl radical scavenging assay

The hydroxyl scavenging assay was followed by Halliwell *et al.,* (1987). The reaction mixture containing molluscan extract MME and MCE, standard BSA was incubated with deoxyribose (3.75mM), H_2O_2 (1mM), $Fecl_3$ (1mM, pH7.4) at 37^0C. The reaction was terminated by adding TBA (1%, w/v) and TCA (2%, w/w) and then heating the tubes. The contents were cooled and the absorbance of the mixture was measured at 535nm against reagent blank. The decreased absorbance of the reaction mixture was indicated decreased oxidation of deoxyribose.

Scavenging ability of 1, 1 diphenyl-2-picrylhydroxyl radicals

The scavenging ability of molluscan extracts was assessed by using Shimada *et al.,* (1992). The molluscan extracts MME, MCE and standard BSA was mixed with methanolic solution containing DPPH radicals, resulting in a final concentration of 10mM/1DPPH. The mixture was shaken vigorously and left to stand for 30 minutes in the dark and the absorbance was measured at 517nm against blank.

Reducing power

The reducing power of the molluscan extracts was quantified by method described earlier by Yen and Chen (1995). The reaction mixture containing different concentrations of molluscan extract of MME and MCE, standard BSA in phosphate saline buffer (0.2M, pH6.6) was incubated with potassium ferric cyanide (1%, w/v) at 50^0C. The reaction was terminated by adding TCA solution (10%, w/v) and the mixture was centrifuged. The supernatant was mixed with distilled water and ferric chloride (0.1%, w/v), solution and the absorbance of the reaction was measured at 700nm. The increased absorbance of the reaction mixture indicated increased reducing power.

Ferrous iron chelating assay

The ferrous ion-chelating potential of molluscan extracts was investigated according to the method of Decker and Welch (1990). The Fe^{2+} chelating ability of extracts was monitored by measuring ferrozine complex at 562nm. The reaction mixture containing molluscan extracts and standard (EDTA) of different concentrations, Fecl2 (2mM) and ferrozine (5mM) was adjusted to a total volume of 0.8ml with water, shaken well and incubated for 10min. The absorbance of the mixture was measured at 562nm against the blank. EDTA was used as positive control. The ability of protein to chelate ferrous iron was calculated by following formula.

Chelating effect (%) = {1-Sample (562nm) / Control (562nm) x Control (562nm)} x 100

Statistical analysis

The experimental results were performed triplicate. The data were recorded as Mean±SD and analyzed by SPSS and followed by one way ANOVA. The difference was considered to be statistically significant at $P<0.05$ level.

FT-IR analysis

AVATAR 330, FT-IR spectrophotometer 10µg of each sample was mixed with 100µg of dried potassium bromide (KBr) and compressed to prepare the salt disc (10mm diameter) for further reading the spectrum further.

RESULTS

Total antioxidant activity

The marine bivalve molluscan extract MME showed maximum total antioxidant activity (%) of 72.13±0.15 at 100µg/ml and minimum activity was observed in at 25µg/ml, whereas in MCE extract highest activity was observed in 65.14±0.10 in 100 µg/ml and lowest total antioxidant activity was recorded in 25µg/ml. Similarly the standard (ascorbic acid) showed maximum activity in 60.13±0.12 to 80.19±0.15 at 50 µg/ml concentration. In comparison both marine bivalve mollusc the *M. meretrix* extract MME showed increased antioxidant activities. The total antioxidant activity was found to increase with increased concentration of the marine bivalve extracts of MME and MCE (fig.1 (a)). The MME and MCE showed equal of amount of antioxidant activity compared to that of standards (BHA and ascorbic acid).

Superoxide radical scavenging activity

The bivalve mollusc extracts MME and MCE was showing significant scavenging activities when compared to the standard (Ascorbic acid, 83.15±0.15). The maximum scavenging activity was recorded in extract MME 78.19±0.19 at 100µg/ml and the minimum was 33.13±0.29 at 25µg/ml concentration. Likewise, MCE showed highest scavenging activity in 66.54±0.63 at 100µg/ml and lowest activity in 18.25±0.52 at 25µg/ml concentration (fig. 1 (b)).

Hydroxyl radical scavenging assay

The effect of the extracts MME and MCE, standard (ascorbic acid, 60.15±0.25 at 100µg/ml) an oxidative damage, induced by Fe^{3+}/H_2O_2 on deoxyribose, as in plotting in fig. 2 (a). An inhibition (%) of 50.63±0.69µg/ml was observed in the MME extract at the highest concentration of 100µg/ml and the minimum was 12.15±0.25µg/ml. At MCE maximum radical scavenging activity was observed in 28.12±0.14 at 100µg/ml and the minimum was in 5.23±15 at 25µg/ml.

Scavenging ability on 1, 1-diphenyl-2-picrylhydrazyl radicals (DPPH)

The maximum scavenging ability (%) of 34.56±0.152, 32.45±0.56 and 60.45±0.25 at 100µg/ml was recorded in bivalve molluscan extracts MME, MCE and standard (Ascorbic acid). The minimum scavenging ability (%) of 11.45±0.69, 4.5±0.56 was observed in the molluscan extracts at the concentration of 25µg/ml. As compared to standards, the molluscan extracts showed exceptionally good scavenging activities (fig.2 (b)).

Measurement of reducing power

The reducing power of the bivalve molluscan extracts MME and MCE was reported to increase with increasing concentration (fig.3 (a)). The reducing power (%) of bivalve extracts MME and MCE showed the highest activity in 12.32±0.25, 12.12±0.56 at 100µg/ml concentration and lowest reducing power was recorded, 5.12±0.85, 3.5±0.95 on 25µg/ml concentration. The standard (ascorbic acid) were showed maximum reducing activities 22.32±0.56 respectively at100µg/ml concentration.

Ferrous ion chelating assay

The ferrous ion-chelating effect of bivalve extracts MME and MCE were found concentration dependent manner as shown in fig.3 (b). The chelating effect (%) of bivalve molluscan extracts MME showed highest in 65.13±0.25 at 100µg/ml and lowest 5.5±.68 at a 25µg / ml concentration. Whereas, in MCE the maximum chelating activity was observed in 36.12±0.52 at 100µg/ml and minimum was recorded in 4.5±0.58 at 25µg/ml concentration. The standard showed chelating activity (%) of 83.13±0.56µg/ml.

FT-IR spectrophotometer analysis

FT-IR spectral details of MME and MCE were shown in figure 4. In FT-IR analysis the molluscan extracts (MME and MCE) characteristics were revealed at absorption bands. The peaks were attributed in 3894.28 and 3988.79 cm-1. The presence of peak meant that the characteristic of functional groups present in the both two bivalve extracts MME and MCE.

Fig.1. Total antioxidant and Superoxide radical scavenging activities of molluscan extracts

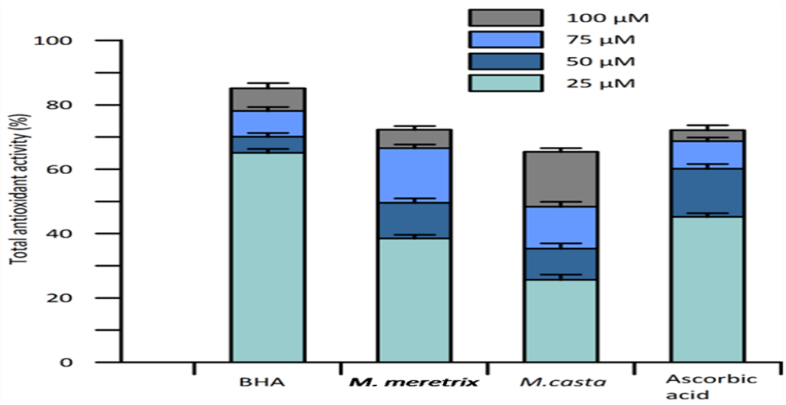

Fig.1. (a). Total antioxidant activities of molluscan extracts

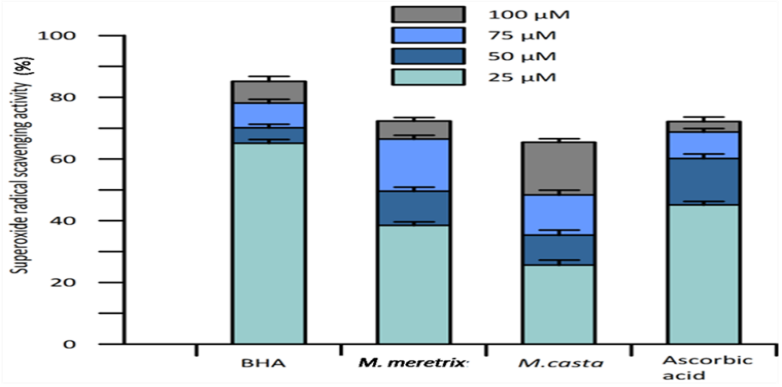

Fig.1. (b) Superoxide radical scavenging activities of MME and MCE

Fig.2. Hydroxyl radical scavenging and DPPH of molluscan extracts

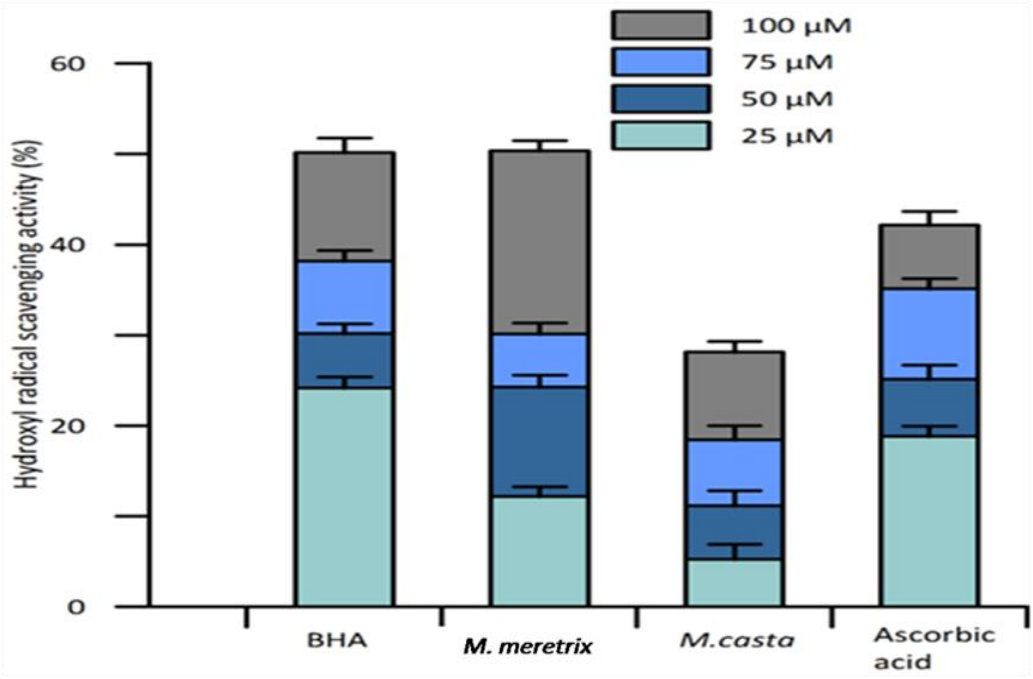

Fig.2.(a). Hydroxyl radical scavenging activities of molluscan extract MME and MCE

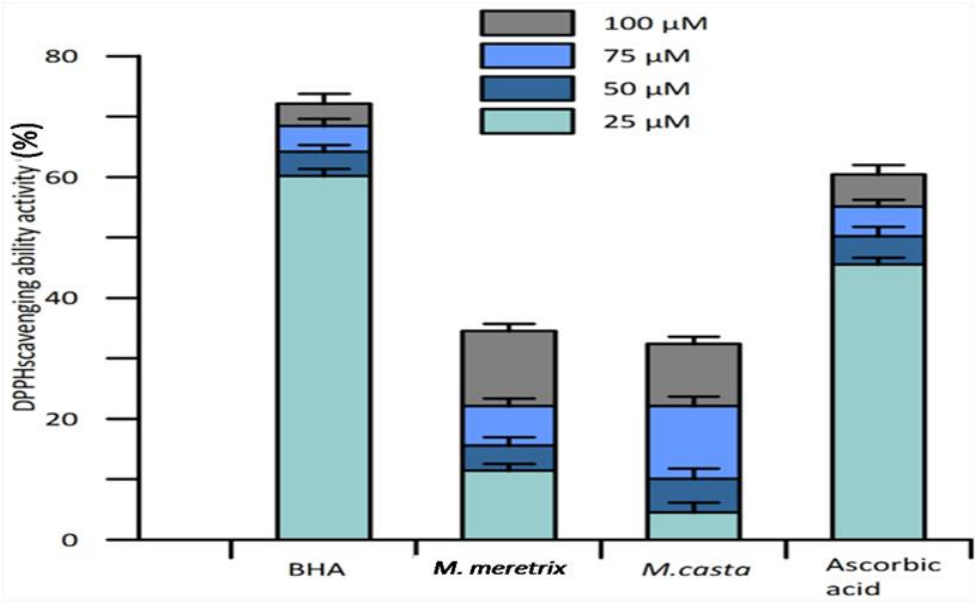

Fig.2. (b). Scavenging ability of MME and MCE on 1, 1-diphenyl-2-picrylhydrazyl radicals (DPPH)

Fig.3. Reducing power and ferrous iron chelating ability of molluscan extracts

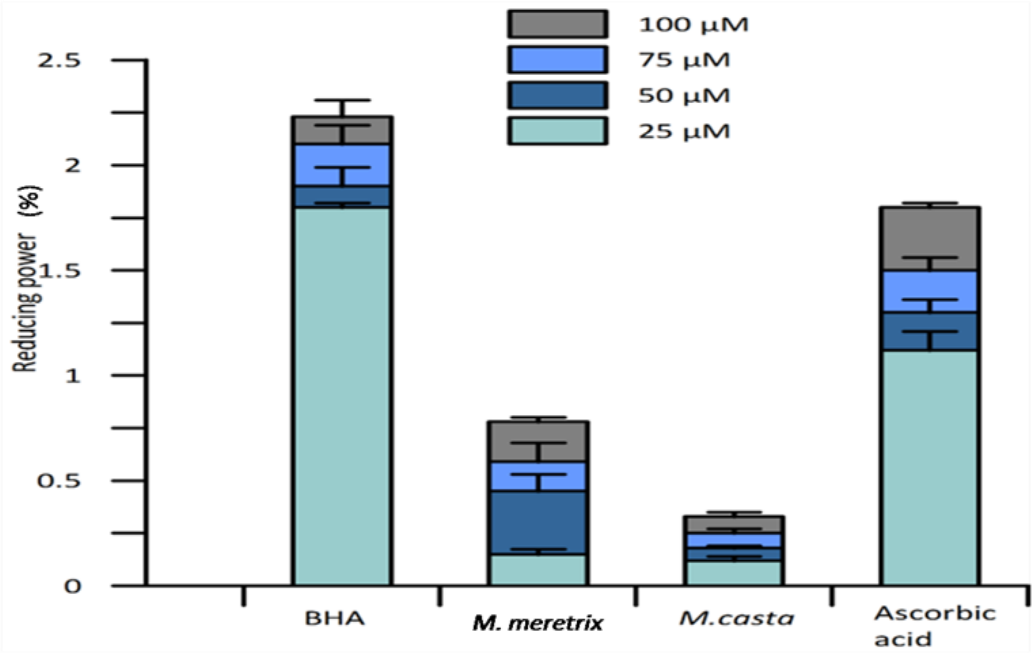

Fig.3. (a) Reducing the power of bivalve extracts MME and MCE

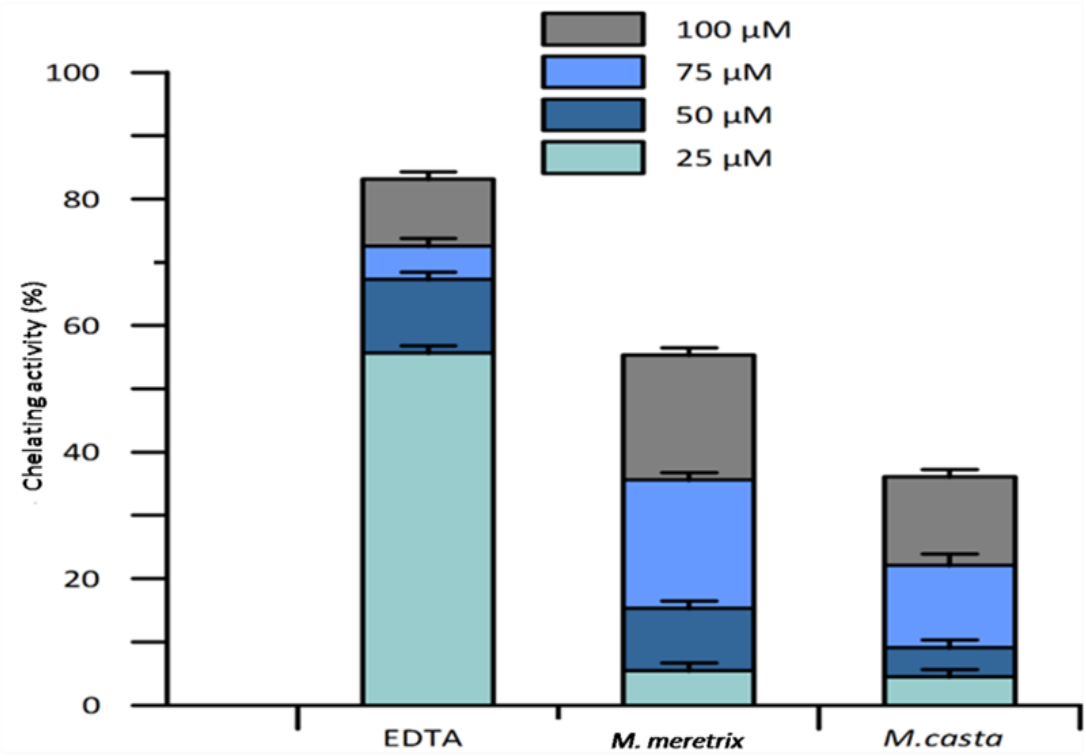

Fig.3. (b) Ferrous iron chelating ability of molluscan extracts MME and MCE

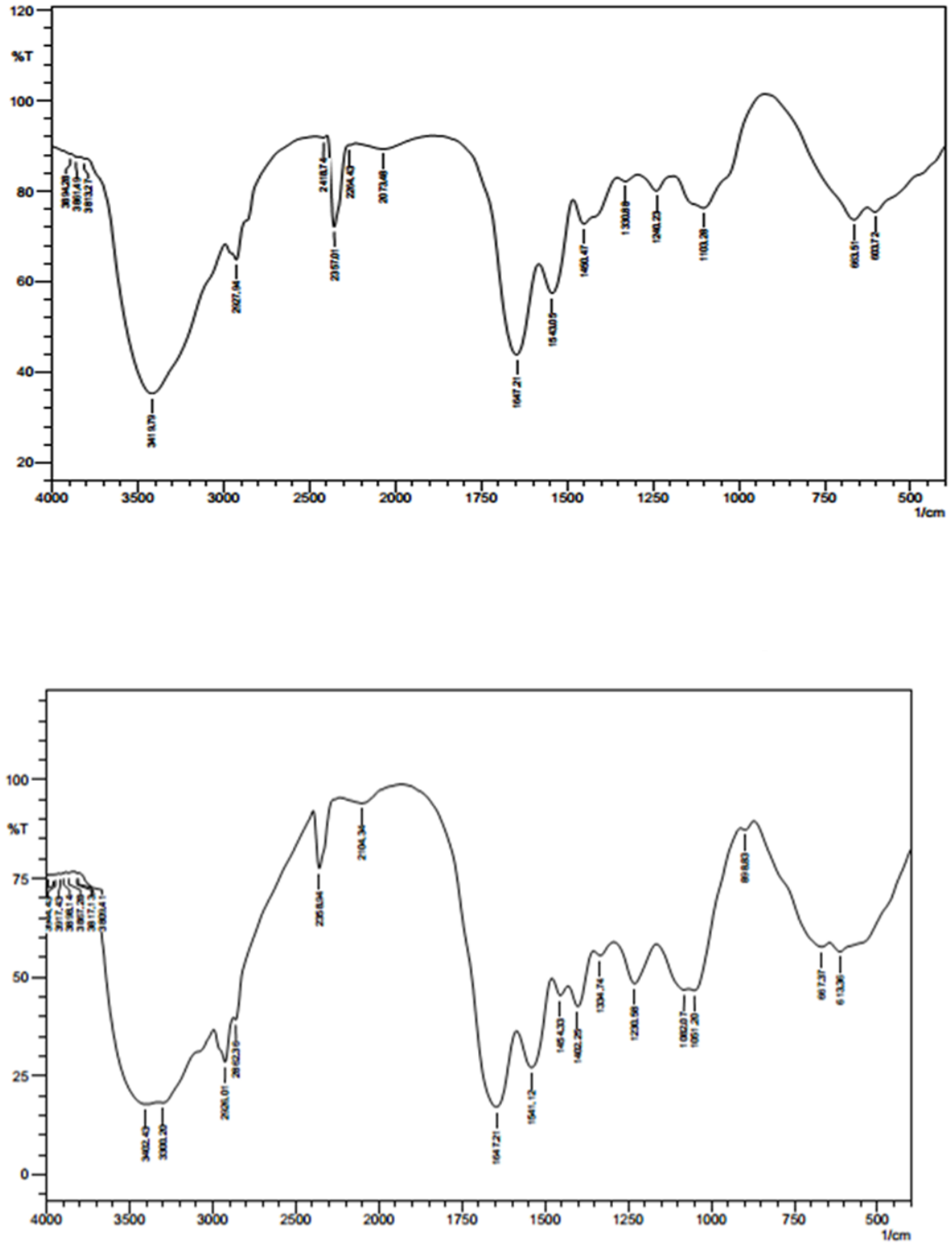

Fig. 4: FT-IR analysis of bivalve extracts MME and MCE.

DISCUSSION

Oxidative stress is potentially harmful to cells and ROS are implicated in the etiology and progression of many diseases. In normal condition antioxidant defense systems are able to detoxify ROS and prevent the damage to cellular macromolecules and organelles. During excessive oxidative stress, antioxidants are depleted and ROS can damage cellular components and interfere with critical cellular damaging activities such as membrane lipids, proteins and DNA can lead to many health disorders such as hypertension, cardiovascular, cancer, diabetes mellitus and neurodegenerative and inflammatory diseases with severe injuries (Moskowitz *et al.*, 2000). Antioxidant may have a positive effect on

human health as they can protect the human body against damage by ROS.

Free radicals are highly reactive molecules with an unpaired electron and produced by radiation or as byproducts of metabolic processes. They initiate chain reactions which lead to the disintegration of cell membranes and cell compounds, including lipids, proteins and nucleic acids. Antioxidants compounds scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus reduce the level of oxidative slow/prevent the development of complication associated with stress-related diseases (Wu and Hensen, 2008). Many synthetic antioxidants have shown toxic and mutagenic effects, which have attention towards naturally occurring antioxidants (Formica and Regelson, 1995). Free radicals and reactive oxygen species have been implicated in a large number of diseases and have a deleterious effect on heart functioning. Various experimental and clinical studies have shown that enormous amount of reactive oxygen species such as superoxide, hydrogen peroxide and hydrogen radicals are generated in failing myocardium (Rajadurai and prince, 2006). Therefore, therapeutic interventions having antioxidants or free radical scavenging activity may be useful against oxidative stress associated with various cardiovascular diseases including myocardial infarction.

Linoleic acid, an unsaturated fatty acid is usually used as a model compound in lipid peroxidation and antioxidation related assays in which carbon centered peroxy radicals and hydroperoxides, etc., Involved in the oxidation process. During the linoleic acid oxidation peroxides are formed. These compounds oxidize Fe^{2+} to Fe^{3+}. The Fe^{3+} ions form a complex SCN-, which has a maximum absorbance at 500nm. Therefore high absorbance indicates high linoleic acid oxidation (Feng *et al.,* 2008). In the consideration of the above conjugated dine method was implemented to assess the total antioxidant property of the protein extract derived from *M. meretrix* and *M. casta*. The extract MME and MCE showed a consistent amount of antioxidant activities. In MME at 50µg/ml concentration 49.6% and high at 100 µg/ml, 72.3% of total antioxidant was observed. Whereas MCE 50µg/ml concentration 35.3% and high at 100µg/ml, 65.43% total antioxidant activities. Equal amount antioxidant where shown by standard ascorbic acid (49.65%-72.12%) and BHA (70.14%-85.17%). Barvin vino (2010) isolated a glycosaminoglycon (GAG) from the cuttlefish *Sepia brevimana* Steenstrup, 1875, which showed significant antioxidant activities at 0.1mg/ml concentration 82.1%. Shanmugam *et al.,* 2012 reported a biopolymer 'chitosen' isolated from the shell of Donacid clam *Donax scortum* (Linnaeus, 1758) showed potent antioxidant activities.

Superoxide anion is very harmful to cellular components. Numerous biological reactions generate superoxide anions which are highly toxic species. In PMS/NADH-NBT system, the superoxide anion derived from PMS/NADH coupling reactions reduces NBT. The decrease of absorbance at 560nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. In the present investigation, superoxide radical scavenging activity of MME, 33.26% at 25µg/ml and 78.19% at 100 µg/ml and MCE, 18.25% at 25µg/ml and 66.54% at100 µg/ml. In reference compounds BHA, 48.12% at 25µg/ml and 83.12% at 100µg/ml and ascorbic acid, 40.25% at 25µg/ml and 71.14% at 100µg/ml. Superoxide radical scavenging activity of molluscan bivalve extracts MME, MCE and standards are increased when increasing the concentration levels. In the above observation it concluded that MME and MCE have a more potent scavenger of superoxide. Likewise Barvin vino (2012) observed the cuttlefish extracts GAG scavenge the superoxide in a concentration dependent manner.

The hydroxyl radical is one of the more potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and cause damage to cell (Halliwell and Gatteridge, 1984). In our study the scavenging effect of molluscan extracts MME, 24.23% at 50µg/ml, MCE, 11.14 at 50µg/ml and same concentration standards (BHA and ascorbic acid) 58.28 and 50.35%. Respectively Barvin Vino *et al.,* (2012) reported a chitosen from cuttlefish showed high level of hydroxyl scavenging activity (72.1%). In all ROS, hydroxyl radical cause severe damage in biomolecules (Yan *et al.,* 1998). Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage (Aurand *et al.,* 1977). They were produced by incubating ferric-EDTA with ascorbic acid and H2O2 at pH 7.4 and reacted with 2-deoxy-2-Ribose to generate a malondialdehyde (MDA) like product and the compound form a pink chromogen upon heating with TBA at low pH (Halliwell *et al.,* 1987). The addition of molluscan extract MME and MCE in the reaction mixture, it removed the hydroxyl radicals from the sugar and prevented the reaction. It indicates that molluscan extracts MME and MCE are better hydroxyl radical scavenger.

DPPH was one of the compounds that possessed proton free radicals with a characteristic absorbance, which decrease significantly on exposure to proton radical scavengers. Further, it is well accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen donating ability. Thus scavenging of DPPH free radicals was directly affected by the amount of attractable atoms in a protein molecule. The free radical scavenging ability using the DPPH method was assessed to identify the active ingredients at low concentration (Yu *et al.,* 2002). The ability of molluscan extracts MME and MCE to scavenge the radicals was quantified by the decrease in its 517nm. In the present investigation the bivalve molluscan extracts MME and MCE reported a DPPH scavenging activity of 15.63% and 10.12 % at

50µg/ml concentration, it indicates the hydrogen donating ability of molluscan extracts, the effects of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability (Conforti *et al.*, 2007).

Reducing power assay has also been used to evaluate the ability of natural antioxidants to donate electrons. Reducing the capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Dorman *et al.*, 2003). The bivalve molluscan extracts MME and MCE showed very low levels of reducing capacity, 5.12±0.85 and 3.5±0.95 at 25µg/ml. Reducing the capacity of a protein compound extracted from bivalve mollusk *M. meretrix* and *M. casta* was assessed based on the measurement of Fe^{3+}, Fe^{2+} transformation. The reducing properties are generally associated with the presence of reductions, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Reducing the capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir *et al.*, 1995). The antioxidant activity of putative antioxidants has been attributed to various mechanisms, including the prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Yildirim *et al.*, 2000)

Iron chelating agents are thus expected to inhibit the metal dependent oxidative processes and have potential in combating reactive oxygen species mediated diseases (Finfrock *et al.*, 2003). Chelating agents may serve as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ions (Gordon, 1990). In the present study show the bivalve molluscan extracts MME and MCE reported a marked for iron binding and suggesting that their action as peroxidation protectors may be revealed to the iron binding capacity.

The chelating ability of MME was 15.13% at 50µg/ml concentration and at same dose MCE showed 9.12%. In the reference compound EDTA, 67.3% at 50µg/ml respectively. Metal chelating capacity was significant, since it reduced the concentration of the catalyzing transition metal in lipid peroxidation. It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of meal iron (Gulcin *et al.*, 2007).

ROS play key role in much physiologic and pathogenic process. In fact, many ophthalmologic and neurodegenerative diseases seem to be mediated, at least in part, by oxidative stress (Finkel and Halbrook, 2000). Excess ROS generation has damage to various cell components and triggering of the activation of specific signaling pathways. Both of these effects can influence numerous cellular processes linked to aging and the development of age-related disease. H_2O_2, O^{2-} and HO are the best known ROS and they can be generated either exogenously (Ultraviolet light, ionizing radiation and chemotherapeutic) or intracellularly (mitochondria, peroxisomes, lipoxygenase, NADPH oxidase and cytochrome p450) from several different sources (Finkel, 2000).

FT-IR spectral peaks revealed the presence of amine salt, ionized compounds, carboxyl and hydroxyl groups in the molluscan extracts MME and MCE. Likewise, Meenakshi *et al.*, (2011) reported *In vitro* antioxidant and FT-IR analysis of two seaweeds from Gulf of Mannar.

CONCLUSION

In this study reveals that the both two bivalve extracts MME and MCE of *M. meretrix* and *M. casta* showed potential antioxidant, radical scavenging, reducing power and metal chelating abilities. Further FT-IR analysis showed various ranges of peaks, it concluded that many functional groups were present in the bivalve extracts MME and MCE.

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