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Original Article

ANTIOXIDANT AND FREE RADICAL SCAVENGING CAPACITY OF RED SEAWEED *HYPNEA VALENTIAE* FROM RAMESHWARAM COAST TAMIL NADU, INDIA

REVATHI. D, BASKARAN. K, SUBASHINI. R*

Department of Biochemistry and Biotechnology, Annamalai University, Annamalai Nagar, Tamil Nadu, India
Email: dr.r.subashini@gmail.com

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ABSTRACT

Objectives: In this study, *In-vitro* antioxidant and free radical scavenging activity of *Hypnea valentiae* was evaluated in a series of *in vitro* assays. Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress.

Methods: The evaluation of antioxidant properties was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis 3ethylbenzthiazoline-6-sulphonic acid (ABTS), ferric reducing antioxidant power (FRAP), nitric oxide scavenging assay (NO), reducing power, superoxide, hydroxyl radical scavenging and hydrogen peroxide radical assay, the metal chelating activity as well as phosphomolypdenum assay.

Results: Among the tested red algae, the maximum antioxidant activity was recorded in the methanol extract of *Hypnea valentiae*. Whereas methanol crude extract of red algae and diatom showed good antioxidant potential.

Conclusions: The findings of the present study suggest that all seaweed extracts could be a potential source of natural antioxidant that could have great importance as therapeutic agents.

Keywords: *Hypnea valentiae*, Free radical scavenging, Antioxidant activity.

INTRODUCTION

Red algae have attracted on the emerging interest mainly for their proactive substances which have great chances to be used as antioxidant [1, 2]. The marine environment in which seaweed exists possess great taxonomic diversity and synthesis metabolites with varied structure with interesting biological activities for food material and medical applications [3]. Seaweeds contain different varieties of inorganic and organic substances which can be used for human health for examples poly phenols, carotenoids and tocopherols, terpenes, ascorbic acid, alkaloids [4].

Free radicals are produced as a part of normal metabolic processes. Reactive oxygen species (ROS) include free radical for example, hydroxyl radical ($OH^{.}$), superoxide anion (O_2) and non free radical species such as hydrogen peroxide (H_2O_2), nitric oxide (NO) are various forms of activated oxygen and are destructive to various physiologically important molecules including protein, lipids, cell membrane, DNA and other cellular constituents [5]. Fresh and dry seaweeds are extensively consumed by people, especially living in coastal areas. Seaweeds are classified as rhodophyta (red algae) or phaeophyta (brown algae) or chlorophyta (green algae) depending on their nutrient and chemical composition [6].

The use of marine algae as potential sources of cosmetic and pharmaceutical agents as well as an important source of food has been increasing recently since they contain bioactive compounds rich in carotenoids, essential fatty acids, polysaccharides (e. g. Alginates, carrageenan and agar) and antioxidants with potential to replace the synthetic compounds such as BHA (Butylated hydroxy anisole) and TBHQ (tert-Butylhydroquinone) considered less healthy for people [7].

Many of these metabolites have been found to possess a variety of biological activities such as anti feedant (diterpenes), anthelmintic (β-chamigrane-type sesquiterpenes), antimalarial (brominated sesquiterpenes), antifouling (sesquiterpes) and antimicrobial (Chlorellin derivatives, halogenated compounds such as haloforms, halogenated alkanes and alkenes, alcohols, aldehydes, hydroquinones, sterols, ketones, allolaurinterol) activities [8]. In our previous studies, antifouling, anticoagulant, antioxidant and haemagglutination [9], glucosidase are inhibitory properties [10], antimicrobial activities of aqueous extract of macroalgae, growth responses of microalgae and selective cytotoxic activities of macroalgae from the Indian coast have already been reported [11]. Based on that sea weeds and their extracts are beneficial to health and some even have been reported to retain biological activity of potential medicinal value. Hence, the present study investigated on the antioxidant activity of seaweed *Hypnea valentiae.*

MATERIALS AND METHODS

Chemicals

DPPH, ABTS and FRAP were obtained from Sigma Aldrich (Steinheim, Germany). Methanol was of HPLC grade (Lab-Scan, Dublin, Ireland). All the other reagents were of analytical grade and obtained from Merck (Darmstadt, Germany).

Collection of algae samples

Marine algae samples were collected from the coastal region of Rameshwaram area on January 5th, 2015. Algae was washed with sea water to remove extraneous materials and brought to the laboratory in a plastic bag containing sea water to prevent evaporation.

Preparation of sample extract

5 g of *Hypnea valentiae* powdered seaweed was extracted overnight with 100 ml methanol at room temperature and centrifuged at 2800 rpm for 10 mins. The supernatant was collected in a separate bottle after passing through a filter paper and the residue was re-extracted three times under the same conditions as mentioned above. The combined extracts were frozen, dried. These extracts were kept at 80 °C until analysis. The freeze dried extracts were re dissolved in methanol and used for the analysis.

DPPH radical scavenging activity

Various concentrations of *Hypnea valentiae* of the sample (4.0 ml) were mixed with 1.0 ml of methanol solution containing DPPH radicals, resulting the final concentration of DPPH being 0.2 mM [12]. The mixture was shaken vigorously and left to stand for 30 minutes, and the absorbance was measured at 517 nm. BHT was used as a control. The percentage of DPPH decolorization of the sample was calculated according to the equation

$$\% \text{ decolorization} = [1\text{-}(ABS_{sample}/ABS_{control})] \times 100$$

IC_{50} values (mg extract/ml) were the inhibitory concentration at which DPPH radicals were scavenged by (50%) BHT was used for comparison.

$ABTS^{+}$scavenging activity

Samples were diluted to the produce various concentrations 5-50 mg/ml. The reaction was initiated by the addition of 1.0 ml of diluted $ABTS^{+}$to 10 ml of different concentrations of *Hypnea valentiae* of the sample or 10 ml methanol as a control [13]. The absorbance was read at 734 nm and the percentage inhibition was calculated. The inhibition was calculated according to the equation.

$$I= A_1/A_0 \times 100$$

Where A_0 is the observance of control reaction and A_1 was the observance of test compound.

Ferric-reducing antioxidant power assay (FRAP)

A stock solution of 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) in 40 mM HCL, 20Mm $FeCl_3.6H_2O$ and 0.3M acetate buffer (pH 3.6) was prepared [14]. The FRAP reagent contained 2.5 ml TPTZ solutions, 2.5 ml ferric chloride solution, and 25 ml acetate buffer. It was freshly prepared and warmed to 37°C. FRAP reagent (900 ml) was mixed with 90 ml water and 30 ml of *Hypnea valentiae* sample and standard antioxidant solution. The reaction mixture was incubated at 37 °C for 30 minutes and the absorbance was recorded at 595 nm. An intense blue color complex was formed when ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex was reduced to ferrous (Fe^{2+}) form. The absorption at 540 nm was recorded.

Nitric oxide radical activity

Nitric oxide radical generated from sodium nitroprusside was measured [15]. Briefly, the reaction with a mixture (5.0 ml) containing sodium nitroprusside (5 mM) in phosphate-buffered saline (pH 7.3), with *Hypnea valentiae* sample at different concentration was incubated at 25 °C for 3 hours. The nitric oxide radical thus generated interacted with oxygen to produce the nitrite ion which was assayed at 30 minute intervals by mixing 1.0 ml of the incubation mixture with an equal amount of Griess reagent. The absorbance of the chromophore (purple azo dye) formed during the digitization of nitrite ions with sulfanilamide and subsequent coupling with naphthyl ethylene diamine dihydro chloride was measured at 546 nm.

Reducing power assay

The reducing power was determined as described by Yen and Chen [16]. Briefly, 0.13 ml of *Hypnea valentiae* different concentration (5-50 mg/ml) in phosphate buffer (0.2 M, pH 6.6) were mixed with 0.125 ml of potassium ferricyanide (1%, w/v) and incubated at 50° C for 20 min. Afterwards, 0.125 ml of TCA (10%, w/v) was added to the mixture to terminate the reaction. Then, the solution was mixed with 1.5 ml of ferric chloride (0.1%, w/v) and the absorbance was measured at 700 nm.

Superoxide anion radical scavenging activity

This assay was based on the reduction of nitro blue tetrazolium (NBT) in the presence of nicotinamide adenine dinucleotide (NADH) and phenazine metho sulfate (PMS) under aerobic condition [17]. The 3 ml reaction mixture contained 50 ml of 1M NBT, 150 ml of 1M NADH with or without sample, and Tris buffer (0.02M, pH 8.0). The reaction was started by adding 15 ml of 1M PMS to the mixture and the absorbance change was recorded at 560 nm after 2 minutes. Percent inhibition was calculated against a control without the extract.

Hydroxy radical activity

The reaction mixture 3.0 ml contained 1.0 ml of 1.5 mM $FeSO_4$, 0.7 ml of 6 mM hydrogen peroxide, 0.3 ml of 20 mM sodium salicylate, and varying concentrations of *Hypnea valentiae* sample [18]. After incubation for 1 hour at 37°C, the absence of the hydroxylated salicylate complex was measured at 562 nm. The percentage scavenging effect was calculated as

$$\text{Scavenging activity} = [1\text{-}(A_1\text{-}A_2)/A_0] \times 100\%$$

Where A_0 was the observance of the control (without extract), A_1 was the observance in the presence of the extract, and A_2 was the observance without sodium salicylate.

Hydrogen peroxide radical

Hypnea valentiae against H_2O_2 was measured according to the method [19]. A solution of 40 Mm H_2O_2 was prepared in phosphate buffer (pH-7.4) and 1.4 ml of different concentrations (5-50 mg/ml) of the *Hypnea valentiae* was added to 0.6 ml of the H_2O_2 solution. The assay mixture was allowed to stand for 10 minutes at 25 °C and the absorbance was measured against a blank solution at λ max =230 nm. The *Hypnea valentiae* H_2O_2 scavenging capacity index was calculated as follows

$$= \frac{\text{Absorbance}_{Blank} - \text{Absorbance}_{Test}}{\text{Absorbance}_{Blank}} \times 100$$

Hypnea valentiae was expressed as IC_{50}, which is defined as the concentration (mg/ml) of the *Hypnea valentiae* required to scavenge 50 % of H_2O_2. BHT was used as a control.

Metal chelating activity

The reaction mixture contained 1.0 ml of various concentrations of the *Hypnea valentiae* sample, 0.1 ml of 2 mM $FeCl_2$, and 3.7 ml methanol [20]. The control contained all the reaction reagents except the sample. The reaction was initiated by the addition of 2.0 ml of 5 mM ferrozine. After 10 minutes at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. A lower absorbance of the reaction mixture indicated a higher metal chelating ability. The capacity to chelate the ferrous ion was calculated by.

$$\% \text{ Metal Chelation} = [1\text{-}(ABS_{sample}/ABS_{control})] \times 100.$$

Phosphomolybdenum assay

Phosphomolybdenum assay used for determining the antioxidant capacity is based on the reduction of M_0 (VI)–M_0 (V) by the antioxidants and subsequent formation of a green phosphate/M_0 (V) complex at acid pH. 0.3 ml *Hypnea valentiae* sample is taken in a tube and mixed with 3 ml of reagent solution containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate and incubated at 95 °C for 90 min. BHT is utilized as a reference the standard. The absorbance of the mixture was measured at 695 nm with methanol blank. The antioxidant activity is expressed as the number of gram equivalents of BHT [21].

Statistical analysis

All the assays were carried out in triplicate. Experimental results are expressed as mean±standard deviation. The results were analyzed using one-way analysis of variance and the group means were compared using Duncan's multiple range tests using SPSS version 16.

RESULTS AND DISCUSSION

The DPPH method measures the radical-scavenging capacity of antioxidants to ward DPPH radical in organic systems and has been used extensively as a prescribing method for new antioxidants from natural resources due to its stability, simplicity, rapidity and reproducibility. The DPPH radical in fact may be neutralized by either direct reduction via single electron transfer (SET) or by radical quenching via hydrogen atom transfer (HAT). Upon reduction, the color of the solution fades from purple to yellow and the reaction progress is conveniently monitored by a spectrophotometer [22, 23]. The effect of seaweed extracts and standard on DPPH radical was compared and shown in Fig.1. The scavenging effect increases with the concentration of standard and samples. At 50 mg/ml concentration, *Hypnea valentiae* possessed 41.22% scavenging activity on DPPH. All the concentration of

Hypnea valentiae showed higher activity than the standard BHT (37.74%).

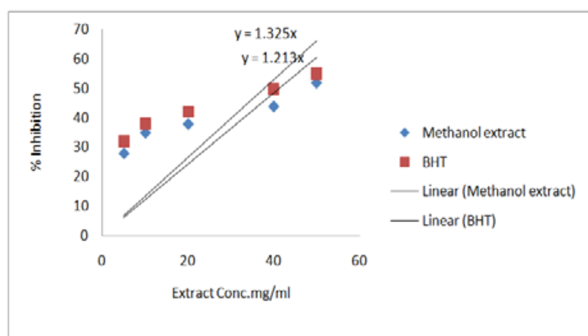

Fig. 1: DPPH radical scavenging activity of *Hypnea Valentiae* Each value is expressed as mean±standard deviation (n=3)

The ABTS scavenging activity was determined by differential extraction methods. This has chain breaking antioxidant property [24]. The percentage efficiency of ABTS scavenged by seaweed extract was found to increase with increasing concentration Fig.2. The IC_{50} values of $ABTS^{+}$radical scavenging activity of *Hypnea valentiae* extracts of experimental alga were 50 mg/ml (44.76 %) and its IC_{50} values were higher than that of BHT (37.74 %).

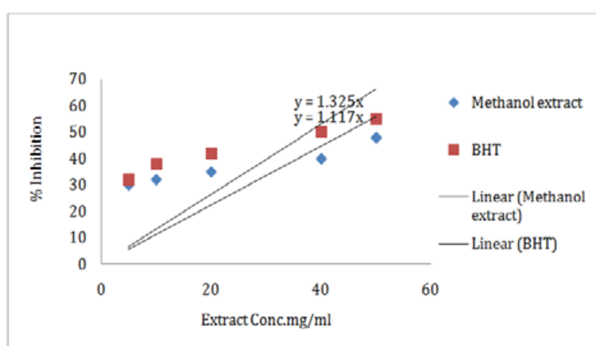

Fig. 2: ABTS radical scavenging ability of *Hypnea Valentiae* Each value is expressed as mean±standard deviation (n=3)

The FRAP mechanism is electron transfer rather than mixed SET and HAT; thus, FRAP cannot detect compounds that act by radical quenching (HAT) [25]. The reducing activity of the green algae *Hypnea valentiae* as determined by reducing power assay varied as seen in Fig.3. The antioxidant activity of the *Hypnea valentiae* extract determined by reducing power assay was as follows: The reducing powers were found to be higher in *Hypnea valentiae* extract. At concentration of 50 mg/ml of *Hypnea valentiae* 50% of FRAP generated by incubation was scavenged (41.42%). The IC_{50} value of BHT was 37.74%.

Active oxygen species and free radicals are involved in a variety of pathological events nitric oxide radicals play an important role in inducing an inflammatory response and their toxicity multiplies only when they react with O_2-radicals to form peroxynitrite, which damages biomolecules like proteins, lipids and nucleic acids [26]. Nitric oxide is generated when sodium nitroprusside reacts with oxygen to form nitrite. Suppression of (NO)release may be attributed to a direct (NO). Scavenging effect as the seaweed extracts decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro* as shown in Fig.4.

The IC_{50} values of the nitric oxide radical assay were compared to the standard antioxidants BHT (50 mg/ml). The IC_{50} values of methanol extracts of brown alga *Hypnea valentiae* was 50 mg/ml (40.92%). It was also found that the IC_{50} value of the algal extracts was lower than that of BHT (37.74%).

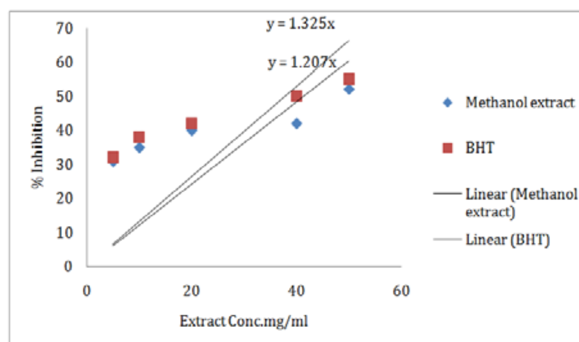

Fig. 3: FRAP radical scavenging ability of *Hypnea Valentiae* Each value is expressed as mean±standard deviation (n=3)

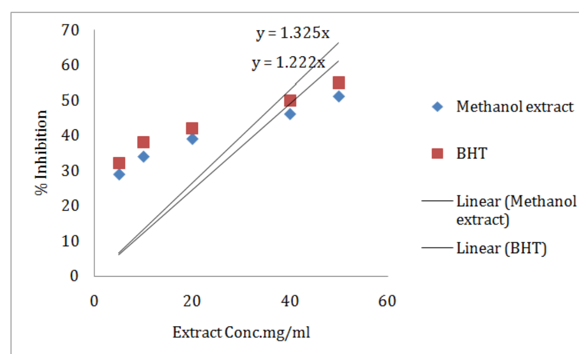

Fig. 4: Nitric oxide radical scavenging ability of *Hypnea Valentiae*. Each value is expressed as mean±standard deviation (n=3)

Antioxidants with reducing power are those that can act as electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, allowing them to act as primary and secondary antioxidants [27]. Such as antioxidants react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride (Fe^{3+}) to form ferrous complexes (Fe^{2+}) that have a maximum absorbance at 700 nm [28,29]. Percentage scavenging activities of reducing power examined at different concentrations of *Hypnea valentiae* was revealed in fig. 5. *Hypnea valentiae* were exhibited a maximum reducing power scavenging activity of (42.63%) at 50 mg/ml whereas BHT was found to be (37.74%) at 50 mg/ml.

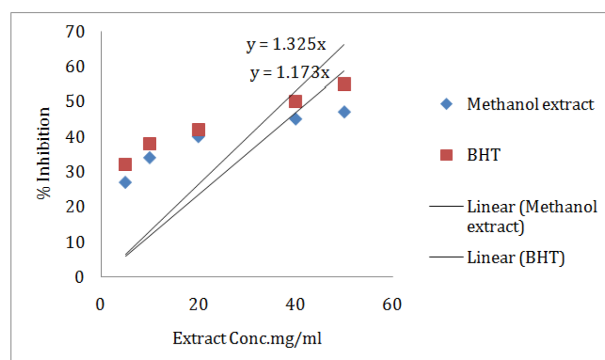

Fig. 5: Reducing power radical scavenging ability of *Hypnea Valentiae*. Each value is expressed as mean±standard deviation (n=3)

Superoxide anion radicals are formed from cellular oxidation actions in organisms, including in humans. Although it is a relatively weak oxidant, it decomposes to produce stronger oxidative species, such as hydrogen peroxide and hydroxyl radicals, through dismutation and other types of reactions. It is also the source of the free radicals formed *in vivo*. SOA radicals and its derivatives are cell damaging, causing damage to DNA and cell membranes. Therefore, it is a great important to scavenge SOA radicals [30, 31]. Superoxide is a highly reactive molecule that reacts with various substances produced through metabolic processes. The percentage scavenging activity of superoxide anion examined at a different concentration of *Hypnea valentiae* was revealed in Fig.6. *Hypnea valentiae* were exhibited a maximum Superoxide Anion scavenging activity of (40.95%) at 50 mg/ml, whereas BHT (standard) was found to be (37.74%) at 50 mg/ml.

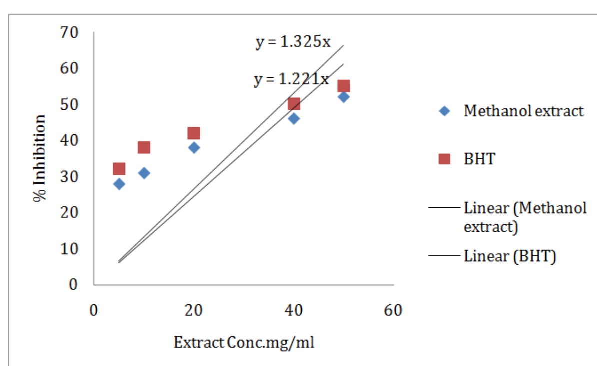

Fig. 6: Superoxide anion radical scavenging ability of *Hypnea Valentiae*. Each value is expressed as mean±standard deviation (n=3)

The hydroxyl radical is the most reactive free radical and can be formed from superoxide anions and hydrogen peroxides in the presence of metal ions, such as copper and iron. Hydroxyl radicals can cause damage to nearly all types of biomolecules, including proteins, DNA, polyunsaturated fatty acids, and nucleic acids [32]. The scavenging effect of OH was investigated using the Fenton reaction and the results shown as the 50% inhibition rate in fig. 7. *Hypnea valentiae* exhibited the inhibition of about (41.28%) but this is lower than the standard BHT (37.74 %).

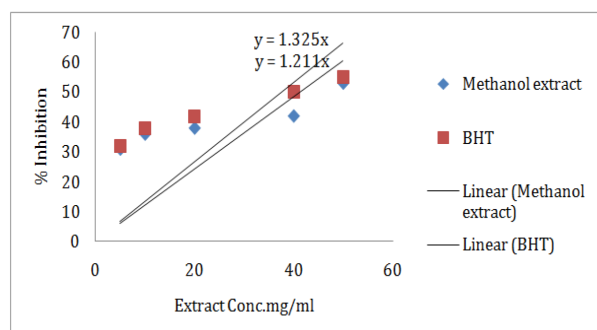

Fig. 7: Hydroxy radical scavenging ability of *Hypnea Valentiae*
Each value is expressed as mean±standard deviation (n=3)

H_2O_2 is a non radical compound, and is of potential biological significance because of its ability to penetrate biological membranes. H_2O_2 itself is not very reactive, but it can sometimes be toxic to the cell because it may give rise to hydroxyl radical in the cells (singlet oxygen and HO· Radicals) [33]. Thus, removal of H_2O_2 is very essential to protect the biological system in general, and food components, in particular. It was reported that extracts of some brown seaweeds registered more than (90%) H_2O_2 scavenging activity [34], thereby supporting the very fact that brown seaweeds are rich source of natural antioxidant compounds, which can scavenge H_2O_2 radical. Many other species of seaweeds were also reported in literature to possess potential H_2O_2 scavenging activity [35]. The percentage scavenging activity of H_2O_2 radical examined at different concentrations of *Hypnea valentiae* was revealed in Fig.8. *Hypnea valentiae* were exhibited a maximum H_2O_2 radical scavenging activity of (44.64%) at 50 mg/ml, whereas BHT (standard) was found to be (37.74%) at 50 mg/ml.

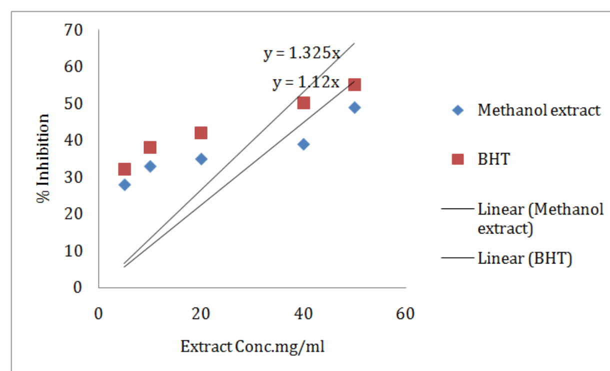

Fig. 8: Hydrogen peroxide radical scavenging ability of *Hypnea Valentiae*
Each value is expressed as mean±standard deviation (n=3)

Metal chelating ability in terms of ferrous ion chelating capacity is claimed as one of the important mechanisms of antioxidant activity. Ferrous ions are the most powerful prooxidants among various species of transition metals present in food systems. These ions react with hydrogen peroxide via the Fenton reaction and produce dangerous hydroxyl radicals [36]. However, dietary antioxidants (nutrients) having metal chelating ability may act as preventive or secondary antioxidants as they forms-bonds with metal ions and reduce the redox potential thereby stabilizing the oxidized form of the metal ions [47] fig. 9. The Metal chelating ability of the *Hypnea valentiae* was increased in a dose-dependent manner, using a concentration ranging from 5-50 mg/ml. The IC_{50} values were 39.84% and 37.74%, respectively, for *Hypnea valentiae* and BHT standards, indicating that the scavenging activity of BHT was significantly stronger than that of the *Hypnea valentiae.*

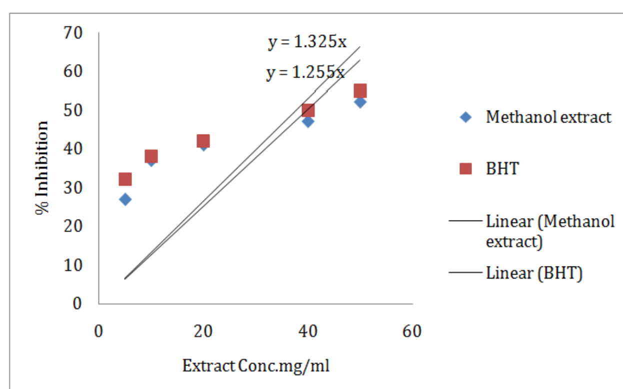

Fig. 9: Metal chelating ability of hypnea valentiae
Each value is expressed as mean±standard deviation (n=3)

This assay has been routinely used to evaluate the antioxidant capacity of extracts [38, 39]. Various extracts of *Hypnea valentiae* were also used to determine their antioxidant capacities by the

formation of green phosphomolybdenum complexes. The formation of the complex was measured by the intensity of absorbance in extracts at a concentration of 100 mg/ml at 95 C. The phosphomolybdenum method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and the formation of green phosphate/Mo (V) complex with the maximal absorption at 695 nm. Being simple and independent of other antioxidant measurements commonly employed, the assay was extended to plant polyphenols. In phosphomolybdenum assay, the concentration range from 5-50 mg/ml, The IC_{50} values were 45.00% and 37.74%, respectively, for *Hypnea valentiae* and BHT standards, indicating that the scavenging activity of BHT was significantly stronger than that of the *Hypnea valentiae.*

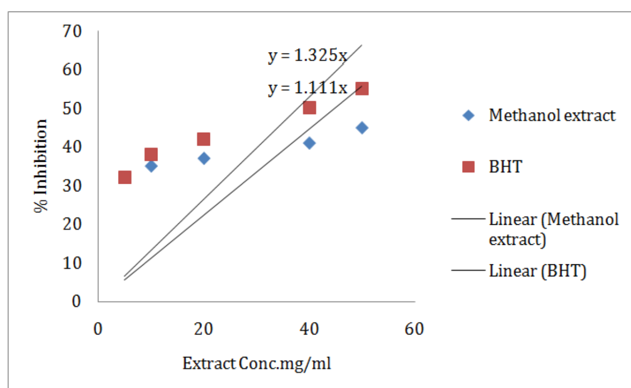

Fig. 10: Phosphomolybdenum radical scavenging ability of *Hypnea Valentiae*. Each value is expressed as mean±standard deviation (n=3)

CONCLUSION

The results of the present work indicated that the methanol extract of *Hypnea valentiae* was a fairly active scavenging assay system. The present findings seem promising to facilitate further experiments on the identification and characterization specific of compounds which are responsible for the relatively high antioxidant activities. Importantly, this research may contribute to a rational basis for the application of marine algal extract in possible therapy of diseases associated with oxidative stress and further supported that the antioxidant-rich extracts or fractions may be used as a dietary supplement, promoting good health.

CONFLICT OF INTERESTS

Declared None

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