

Research Article**Free radical scavenging activity of methanolic extract of marine red algae *Actinotrichia fragilis*****Baskaran Krishnan*, Rathi M. A., Nirmaladevi N.**

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Received: 7 February 2019

Revised: 29 March 2019

Accepted: 10 April 2019

Abstract

Objective: Antioxidants in seaweeds have attracted increasing interest for its role in protecting human health. The present study was designed to assess the antioxidant and reactive oxygen species (ROS) scavenging activities of 70% methanol extract of the red algae *Actinotrichia fragilis*. **Material and Methods:** The evaluation of antioxidant properties was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), ferric reducing antioxidant power (FRAP), nitric oxide scavenging assay (NO), reducing power, hydroxyl, superoxide radical scavenging (SOD), hydrogen peroxide radical assay, the metal chelating activity as well as phosphomolybdenum assay. **Results:** Among the tested red algae, the maximum antioxidant activity was recorded in the methanol extract of *Actinotrichia fragilis*. Whereas methanol crude extract of red algae and diatom showed good antioxidant potential. **Conclusion:** This study suggests that methanol crude extracts contain different potential antioxidant compounds capable to scavenge different types of free radicals.

Keywords: *Actinotrichia fragilis*, Free radical scavenging, Antioxidant activity

Introduction

Antioxidants perform multiple functions which include defending against oxidative damage and cell signaling. One major function of antioxidants in biological system is to prevent the damage of cellular components by reactive oxygen species. Various free radicals with different biological, chemical and physical properties such as hydroxyl, alkoxy, peroxy, superoxide, nitric oxide, sulfur and nitrogen centered radical damages cells by chain reactions. As a result the oxidative stress generates and causes neural disorders, cardiovascular diseases, cancer, diabetes and arthritis (Devasagayam et al., 2004). Natural antioxidants with a multifunctional potential area of high interest as alternatives for synthetic antioxidants to prevent oxidation in complex food systems like muscle food. Aquatic plants are also gaining interest as a potential source of antioxidants. Algae are grouped into two main categories; the green algae, found in both benthic and littoral habitats and also

throughout the ocean waters as phytoplankton, and the green algae or seaweeds, which occupy the littoral zone, and can be classified as red (Rhodophyta), brown (Phaeophyta) or green (Chlorophyta), depending on their nutrient and chemical composition (Dawczynski et al., 2007; Gupta and Abu, 2011).

Recently, the potential antioxidant compounds were identified as some pigments (fucoxanthin, astaxanthin, carotenoid e.g.) and polyphenols (phenolic acid, flavonoid, tannins e.g.). Those compounds are widely distributed in plants or seaweeds and are known to exhibit higher antioxidant activities. Seaweeds are noted to contain not only labile antioxidants (i.e. ascorbate, glutathione) when fresh (Kakinuma et al., 2001), but also, more stable molecules such as carotenoids (Yan et al., 1999), mycosporine-like amino acids (Nakayama et al., 1999) and a variety of polyphenols (e.g. catechins, phlorotannins) (Yoshie et al., 2000). More reports that are recent revealed seaweeds to be a rich source of antioxidant compounds (Lim et al., 2002; Park et al., 2004; Kuda et al., 2005; Duan et al., 2006).

Many synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroxyquinone (TBHQ), ascorbic acid (AA) have been widely used in different food products. However,

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DOI: <https://doi.org/10.31024/ajpp.2019.5.5.3>2455-2674/Copyright © 2019, N.S. Memorial Scientific Research and Education Society. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

because of the potential health hazards, their use as food additives is under strict regulation in many countries. Marine algal extracts have also been demonstrated to have strong antioxidant properties (Yuan and Walsh, 2006). The Phaeophyta (brown seaweeds) shows comparatively higher antioxidant activity than green and red algae (Al-amoudi et al., 2009).

Actinotrichia fragilis is red or yellow with cylindrical branches about 1 mm in diameter that are covered with closely spaced rings of short, dark, stiff hairs, Figure.1. This lightly calcified seaweed is from 2 to 6 cm high and grows in tide pools, on reef flats, and in deeper subtidal habitats. *Actinotrichia fragilis* is a small (1±5–5 cm high) calcified, dichotomously divided multi-axial species, with an Indo-Pacific tropical distribution (Itono, 1979). Tetrasporophytes and dioecious gametophytes are isomorphic.

Seaweeds are considered as a source of bioactive compounds as they are able to produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities (Cox et al., 2010) with antiviral, antibacterial and antifungal activities (Delval et al., 2001) which acts as potential bioactive compounds of interest for pharmaceutical applications (Solomon et al., 2008). Most of these bioactive substances isolated from marine algae are chemically classified as brominated, aromatics, nitrogen-heterocyclic, nitrosulphuric-heterocyclic, sterols, dibutanoids, proteins, peptides and sulphated polysaccharides (Kolanjinathan et al., 2011). Therefore, the present investigation was attempted to study the antioxidant properties of methanolic crude extracts of marine diatom red algae *Actinotrichia fragilis*.

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).

Algal materials

Actinotrichia fragilis red algae were collected from the Rameswaram area on January 5th, 2018. The freshly collected seaweeds were washed with clean seawater to remove salt, epiphytes and sand attached to the surfaces of the samples and transported to the laboratory. The samples were carefully rinsed with tap water, wiped with paper towel. For *Actinotrichia fragilis* the stipes and hapteres were removed and the new and old parts of the blades were separated. The samples were lyophilized for 72 h, pulverized into powder and stored at 80°C prior to extraction.

Preparation of sample extract

Take 5 g of *Actinotrichia fragilis* powdered seaweed was



Figure 1. Marine red algae *Actinotrichia fragilis*

extracted overnight with 100 ml methanol at room temperature and centrifuged at 2800 rpm for 10 min. 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 freeze dried. These extracts were kept at 80 °C until analysis. The freeze dried extracts were redissolved in methanol and used for the analysis.

DPPH radical scavenging activity

Various concentrations of *Actinotrichia fragilis* of the sample (4.0 mL) were mixed with 1.0 mL of methanol solution containing DPPH radicals, resulting in the final concentration of DPPH being 0.2mM (Subhashini et al., 2011). The mixture was shaken vigorously and left to stand for 30 minutes, and the absorbance was measured at 517nm. AA was used as a control. The percentage of DPPH decolorization of the sample was calculated according to the equation:

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

IC₅₀ values (mg extract/mL) where the inhibitory concentration at which DPPH radicals were scavenged by 50%. AA was used for comparison.

ABTS⁺ scavenging activity

Samples were diluted to produce 200-1000 µg/mL. The reaction was initiated by the addition of 1.0 mL of diluted ABTS⁺ to 10 mL of different concentrations of *Actinotrichia fragilis* of the sample or 10 mL methanol as control (Huang et al., 2011). 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 A₀ is the absorbance of control reaction and A₁ was the absorbance of test compound.

Ferric-reducing antioxidant power assay (FRAP)

A stock solution of 10mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) in 40mM HCL, 20Mm FeCl₃.6H₂O and 0.3M acetate buffer (pH 3.6) was prepared (Xu et al., 2009). The FRAP reagent contained 2.5 mL TPTZ solution, 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 *Actinotrichia fragilis* of the sample and standard antioxidant solution. The reaction mixture was then 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 (Fe³⁺-TPTZ) complex was reduced to ferrous (Fe²⁺) form. The absorption at 540 nm was recorded.

Nitric oxide radical activity

Nitric oxide radical generated from sodium nitroprusside was measured (Saket et al., 2010). Briefly, the reaction mixture (5.0 mL) containing sodium nitroprusside (5mM) in phosphate-buffered saline (pH 7.3), with *Actinotrichia fragilis* 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 incubation mixture with an equal amount of Griess reagent. The absorbance of the chromophore (purple azo dye) formed during the diazotization of nitrite ions with sulfanilamide and subsequent coupling with naphthyl ethylene diamine dihydrochloride was measured at 546 nm.

Reducing Power Assay

The reducing power was determined as described (Yen and Chen, 1995). Briefly, 0.13 mL of *Actinotrichia fragilis* different concentration (200-1000 µg/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) were added to the mixture to terminate the reaction. Then, the solution was mixed with 1.5 mL ferric chloride (0.1%, w/v) and the absorbance was measured at 700 nm.

Hydroxy radical activity

The reaction mixture 3.0 mL contained 1.0 mL of 1.5mM FeSO₄, 0.7 mL of 6mM hydrogen peroxide, 0.3 mL of 20 mM sodium salicylate, and varying concentrations of *Actinotrichia fragilis* sample (Naskar et al., 2010). 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:

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

Where A₀ was the absorbance of the control (without extract), A₁ was the absorbance in the presence of the extract, and A₂ was the absorbance without sodium salicylate.

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 methosulfate (PMS) under aerobic condition (Chou et al., 2009). 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.

Hydrogen peroxide radical

Actinotrichia fragilis against H₂O₂ was measured according to the method (Delpour et al., 2009). A solution of 40 Mm H₂O₂ was prepared in phosphate buffer (pH-7.4). Next, 1.4 mL of different concentrations (200-1000 µg/mL) of the *Actinotrichia fragilis* was added to 0.6 mL of the H₂O₂ solution. The assay mixture was allowed to stand for 10 minutes at 25°C and the absorbance measured against a blank solution at λ max 230 nm. The *Actinotrichia fragilis* on H₂O₂ scavenging capacity index was calculated as follows:

$$\text{Scavenging capacity index} = \frac{A_{\text{Blank}} - A_{\text{Test}}}{A_{\text{Blank}}} \times 100$$

Actinotrichia fragilis was expressed as IC₅₀, which is defined as the concentration (mg/mL) of the *Actinotrichia fragilis* required to scavenge 50 % of H₂O₂. A is the absorbance.

Metal ion Chelating activity

The reaction mixture contained 1.0 mL of various concentrations of the *Actinotrichia fragilis* sample, 0.1 mL of 2mM FeCl₂, and 3.7 mL methanol (Smirnoff and Cumbes, 1989). The control contained all the reaction reagents except the sample. The reaction was initiated by the addition of 2.0 mL of 5mM 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 iron chelating ability. The capacity to chelate the ferrous ion was calculated by

$$\% \text{ chelation} = [1 - (\text{ABS}_{\text{sample}} / \text{ABS}_{\text{control}})] \times 100.$$

Phosphomolybdenum assay:

The phosphomolybdenum assay used for determining the antioxidant capacity is based on the reduction of M₀(VI) – M₀(V) by the antioxidants and subsequent formation of a green phosphate/M₀(V) complex at acid pH 0.3 mL of *Actinotrichia fragilis* 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. AA is utilized as a reference standard. The absorbance of the mixture is then measured at 695 nm with methanol blank. The antioxidant activity is expressed as the number of gram equivalents of AA (Sahaa et al., 2008).

Statistical analysis

All the assays were carried out in triplicate. Experimental results are expressed as mean \pm 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

DPPH have been used extensively as a free radical to evaluate reducing substances (Cotelle et al., 1996; Revathi et al., 2015). This purple color generally fades/disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals (i.e., by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them to a colorless/bleached product (i.e., 2, 2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance at 517nm. Hence, the more rapidly the absorbance decreases, the more potent the antioxidant activity of the extract. The DPPH radical assay is a suitable model for estimating radical scavenging activities of antioxidants. *Actinotrichia fragilis*

exhibited a significant dose dependent inhibition of DPPH activity. This had a lesser activity then the standard of Ascorbic acid (AA). The results are presented in figure.2 the IC₅₀ value of AA and *Actinotrichia fragilis* was 65.57 μ g/mL, 71.26 μ g/mL, respectively.

ABTS assay is a simple indirect method for determining the activity of natural antioxidants. In the absence of phenolics, ABTS radical is rather stable, but it reacts energetically with an H-atom donor such as phenolics, being converted into a non-colored form of ABTS (Roginsky and Lissi, 2005; Pandian et al., 2015). The ABTS radical cation-scavenging assay performed showed that the antioxidant activity increases with an increase in the concentration. The inhibition capacity of the radical ABTS⁺ and the AA% values, expressed in *Actinotrichia fragilis*, for the different concentration were presented in figure.3 the IC₅₀ value of AA and *Actinotrichia fragilis* was 65.57 μ g/mL, 69.51 μ g/mL, respectively.

Ferric-reducing power is an important indicator of the antioxidant potential of a compound or an extract (Vinayak et al., 2011).The ability to reduce ferric ions indicates that the antioxidant compounds are electron donors and could reduce the oxidized intermediate of lipid peroxidation processes, thus acting as primary and secondary antioxidants (Matanjum et al., 2008; Yen and Chen,1995).The antioxidant activity of the methanolic crude extract determined by FRAP assay varied as seen in

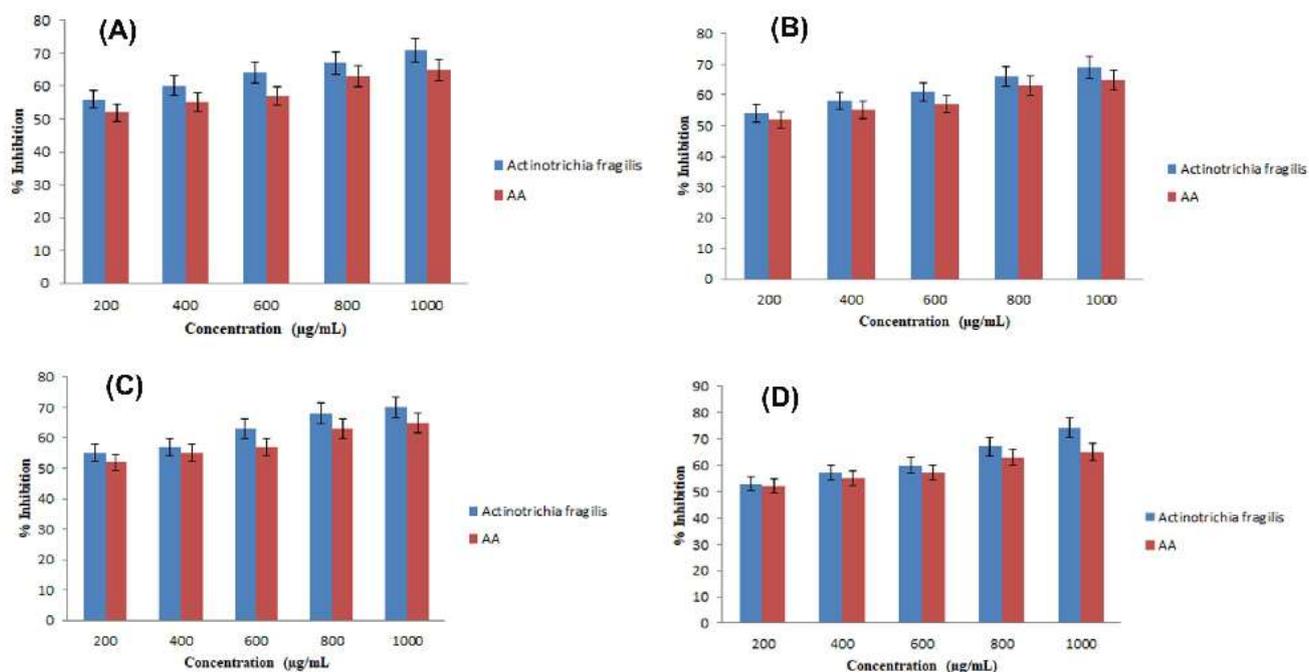


Figure 2. Figure shows radical scavenging activity of *A. fragilis* : (A) DPPH radical scavenging activity, (B) ABTS radical scavenging ability, (C) FRAP radical scavenging ability, (D) Nitric Oxide Radical scavenging. Each value is expressed as mean \pm standard deviation (n=3)

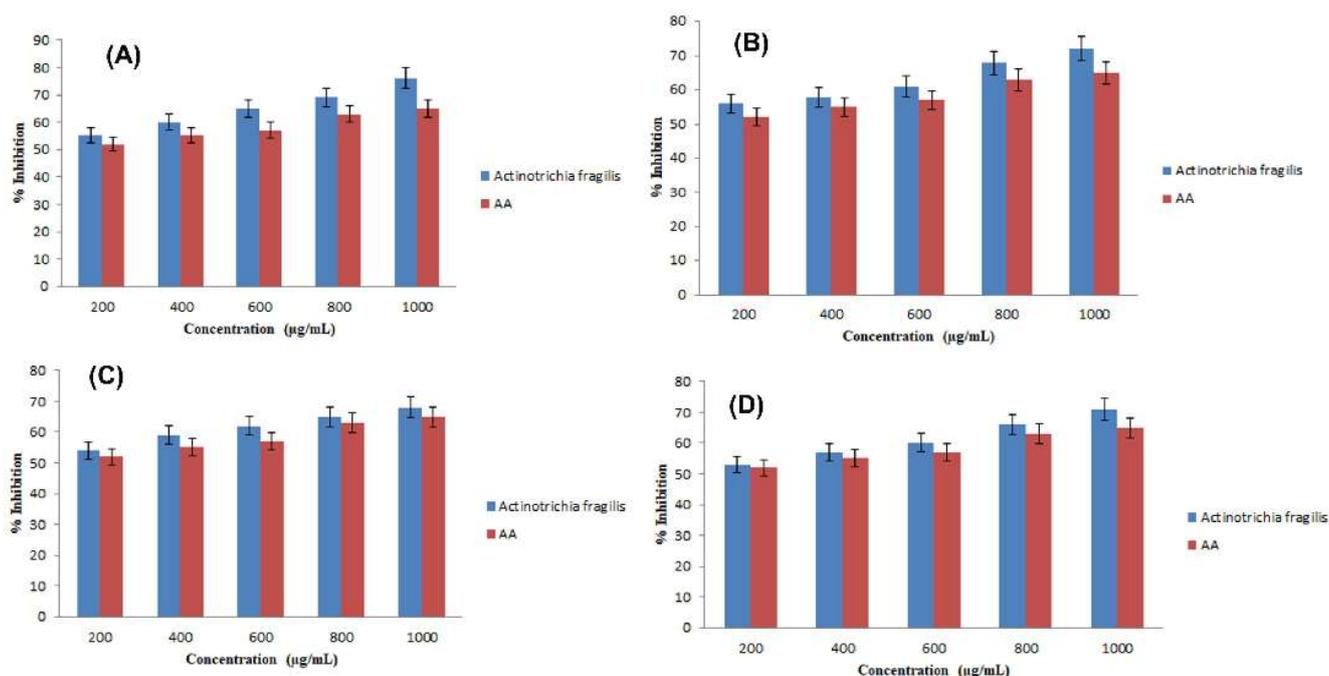


Figure 3. Figure shows radical scavenging activity of *A. fragilis* in different assays: (A) Reducing Power Radical scavenging ability, (B) Hydroxy Radical scavenging ability, (C) Superoxide Anion Radical scavenging ability, (D) Hydrogen Peroxide Radical scavenging ability Each value is expressed as mean \pm standard deviation (n=3).

figure 4. The reducing power were found to be higher in methanolic extract. At concentration of 70.43 $\mu\text{g/mL}$ of *Actinotrichia fragilis* 50% of FRAP generated by incubation was scavenged. The IC_{50} value of AA was 65.57 $\mu\text{g/mL}$.

Active oxygen species and free radicals are involved in a variety of pathological events nitric oxide radicals play an important role in inducing 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 (Moncada et al., 1991). Nitric oxide is generated when sodium nitroprusside reacts with oxygen to form nitrite. Seaweeds inhibit nitrite formation by competing with oxygen to react with nitric oxide directly. These compounds alter the structure and function of many cellular components. Any compound, natural or synthetic, with antioxidant properties might contribute towards the partial or total alleviation of this damage (Sanja et al., 2009). The nitric oxide radical scavenging assay was also performed with methanolic crude extract of the seaweed samples. The scavenging of nitric oxide by *Actinotrichia fragilis* was increased in a dose-dependent manner as illustrated in Figure.5. At concentration of 74.65 $\mu\text{g/mL}$ of *Actinotrichia fragilis* 50% of nitric oxide generated by incubation was scavenged. The IC_{50} value of AA was 65.57 $\mu\text{g/mL}$.

Reducing capacity is considered as a significant indicator of potential antioxidant activity of a compound or sample (Nakayama et al., 1999). The presence of reductants (i.e.

antioxidants) causes the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form. Therefore, by measuring the formation of Perl's Prussian blue at 655 nm, the amount of Fe^{2+} can be monitored. Higher absorbance indicated higher reducing power (Rashmi et al., 2010). Figure.6 elucidated the reductive capabilities of *Actinotrichia fragilis* compared to

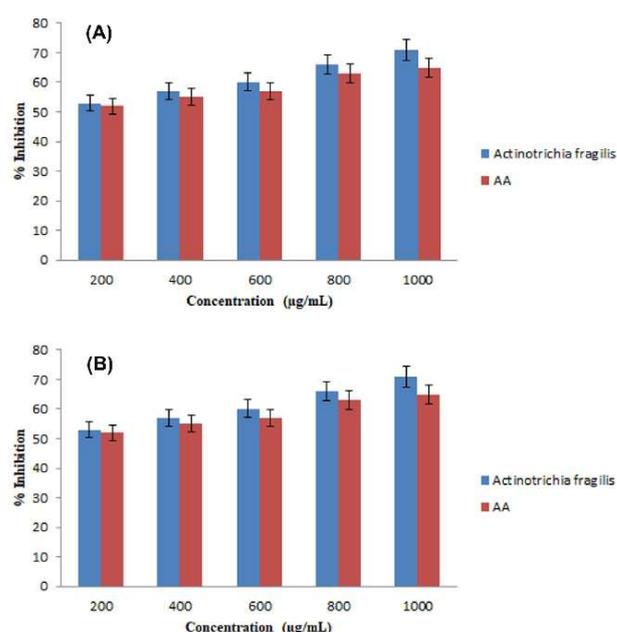


Figure 4. (A) Metal Chelating ability, (B) Phosphomolybdenum Radical scavenging ability of *A. fragilis*. Each value is expressed as mean \pm standard deviation (n=3).

AA. The reducing power of *Actinotrichia fragilis* was increased with quantity of sample *Actinotrichia fragilis* could reduce the most Fe^{3+} ions, which had a lesser reductive activity than the standard of AA. The IC_{50} value of *Actinotrichia fragilis* and AA was 76.08 $\mu\text{g/mL}$ and 65.57 $\mu\text{g/mL}$ respectively.

Hydroxyl radical are the major active oxygen causing lipid peroxidation in enormous biological damage. The highly reactive hydroxyl radical can cause oxidative damage to DNA, lipid and protein (Baskaran et al., 2014; Subashini and Prasanth, 2014). In this study, *Actinotrichia fragilis* was found to scavenge O_2 significantly and in dose dependent manner and may protect the DNA, protein and lipid from damage. The results for hydroxyl scavenging assay are shown in figure 4. The concentrations for 50% inhibition was found to be 72.46 and 65.57 $\mu\text{g/mL}$ for the *Actinotrichia fragilis* and AA respectively.

Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals that are generated after oxygen is taken into living cells. Superoxide anion changes to other harmful ROS and free radicals such as hydrogen peroxide and hydroxyl radical, which induce oxidative damage (Srikanth et al., 2010). The decrease the absorbance at 560 nm with the *Actinotrichia fragilis* thus indicates the consumption of superoxide anion in the reaction mixture. The antioxidant activity of the methanol crude extract determined by superoxide anion radical assay varied. The reducing power were found to be higher in methanol extract. At concentration of 68.65 $\mu\text{g/mL}$ of *Actinotrichia fragilis* 50% of Superoxide anion radical generated by incubation was scavenged. The IC_{50} value of AA was 65.57 $\mu\text{g/mL}$, respectively.

Many species of seaweed possess scavenging ability of hydrogen peroxide (Siriwardhana et al., 2010). It can cross membranes and may slowly oxidize a number of compounds. Hydrogen peroxide itself is not very reactive, but sometimes it can be toxic to cells because of rise in the hydroxyl radicals in the cells. The H_2O_2 radical scavenging assay was also performed with the methanolic crude extract of the seaweed samples. From the results, *Actinotrichia fragilis* showed concentration dependent activity and the H_2O_2 scavenging effect at a concentration was 71.01 $\mu\text{g/mL}$. This activity was comparable to the scavenging effect at the concentration of AA 65.57 $\mu\text{g/mL}$.

The metal iron chelating ability of seaweeds may be attributed to the presence of endogenous chelating agents, mainly phenolics because certain phenolic compounds have properly oriented functional groups, which can chelate metal ions (Wang et al., 1992). Ferrozine can quantitatively form complexes with Fe^{2+} . In the presence of chelating agents, the complex formation is disrupted with the result that red color of the complex is decreased. The metal chelating activity of *Actinotrichia fragilis* was 77.09 $\mu\text{g/mL}$. This was comparable to the scavenging effect

at the concentration of AA was 65.57 $\mu\text{g/mL}$.

Phosphomolybdenum assay, which is a quantitative method to evaluate water-soluble and fat-soluble antioxidant capacity (total antioxidant capacity), the extract demonstrated high electron-donating capacity showing its ability to act as chain terminators, transforming relative free radical species into more stable non-reactive products (Zheng et al., 1992; Baskaran et al., 2015). In the ranking of the antioxidant capacity obtained by this method. This revealed that increased phosphomolybdenum reduction of *Actinotrichia fragilis* to the quantity of the sample. The IC_{50} value of *Actinotrichia fragilis* was 75.82 $\mu\text{g/mL}$ and 65.57 $\mu\text{g/mL}$ as a standard AA.

Conclusions

In the present study the methanolic crude extracts of seaweeds at varying concentrations were shown as a potential DPPH, ABTS, FRAP, nitric oxide, reducing power, hydroxyl, superoxide radical scavenging, hydrogen peroxide radical assay, the metal chelating activity as well as phosphomolybdenum assay. Some extracts showed a higher antioxidant activity when compared to the commercial antioxidants. From the present study it can be concluded that the solvent extracts of marine macro algae exhibit good antioxidant activity. The methanolic extract showed a good result when compared to the AA. The results shown here indicate that the seaweed extracts can be a good source of natural antioxidant. Further investigation is needed to isolate and identify the specific class of compound that is responsible for the antioxidant activity.

Conflict of interest

The authors declare that they don't have any conflict of interest.

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