

Full Length Research Paper

## Exploration of a novel hydroxyl radical scavenger from *Sargassum myriocystum*

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*Sargassum myriocystum* was selected to evaluate for reactive oxygen radical scavenging capacities and antioxidant activities using commonly accepted assays. After shade drying, marine seaweeds were powdered and extracts were prepared by cold and Soxhlet methods using various organic solvents. Crude powder materials were used for determination of bio-chemical constituents like protein, carbohydrate and phenolic contents. It was found that phenolic contents constituted 22 mg gallic acid equivalents/ gm of dry plant materials. Methanol/chloroform extracts of Soxhlet showed highest reducing power, total antioxidant and hydroxyl scavenging activity. 80 to 120% inhibition of hydroxyl radical quenching activity was observed and demonstrated by all Soxhlet derived extracts, except petroleum ether. Hence, from the results we conclude presence of novel compound(s) in the crude extract of (methanol: chloroform) *S. myriocystum* is responsible for its hydroxyl radical scavenging activity.

**Key words:** Brown seaweeds, *Sargassum myriocystum*, antioxidant, hydroxyl radical.

### INTRODUCTION

Reactive oxygen species (ROS) produced from living tissues during metabolism lead to a wide range of human diseases and geriatric degenerative conditions (Ganesan et al., 2008). Oxidative rancidity and food decay are mainly due to secondary oxidants like heptanol and hexanal, which are the intermediates of lipid peroxidation

(polyunsaturated fatty acids) pathway induced by ROS (Devi et al., 2008). Defense against the ROS mediated complications are achieved using synthetic antioxidants that are mainly phenolic compounds like butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA). Experiments on rodents and kidneys reveals that BHT and related compounds leads to various metabolic abnormalities when they are supplemented in highest concentration (above 50 mg/kg/day) (Lee et al., 1996). As a result, efforts are being made to unearth the antioxidant compounds that occur naturally.

Search for naturally occurring antioxidants ended up in plants that are considered as a major source for diverse group of phenolic compounds, including simple phenolics, anthocyanins, hydroxycinnamic acid derivatives, and flavonoids. All the phenolic classes have the structural requirements of free radical scavengers and have potential as food antioxidants (Duan et al., 2006). Phenols are particularly effective antioxidants for

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**Abbreviations:** ROS, Reactive oxygen species; BHT, butylated hydroxy toluene; BHA, butylated hydroxy anisole; LOO, lipid peroxy radicals; DPPH, 2, 2-Diphenyl-1-picrylhydrazyl; DMSO, dimethyl sulphoxide; TCA, trichloro acetic acid; BSA, bovine serum albumin; HCl, hydrochloric acid; FeSO<sub>4</sub>, ferrous sulphate; EDTA, ethylene diamine tetra acetate; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; TBA, thiobarbituric acid; O<sub>2</sub><sup>•</sup>, singlet oxygen; OH<sup>•</sup>, hydroxyl radicals.

polyunsaturated fatty acids where they easily transfer a hydrogen atom to lipid peroxy radicals ( $\text{LOO}\cdot$ ), making it less toxic and quenches the radical process (Ruberto et al., 2001). Seaweeds are mainly targeted for their effective antioxidant compounds to defend oxidative stress mediated diseases in human body, and also food spoilage. This solely depends on diverse natural metabolites with unique structures in seaweeds as a result of extremely harsh marine environment. Especially, marine brown and red algae have gained great interest due to their potency to produce various bioactive derivatives (Li et al., 2009).

Traditionally, seaweeds have been used in diet by Asian population and on average in Japan a single person eats 1.4 kg of algae per year. This practice of seaweed diet has led to a large number of reports on the beneficial role of seaweed consumption in human health. These algae are rich in various macro and micro nutrients, polyphenols etc., which not only help in fighting disease but also help us to overcome age related disorders (Li et al., 2009; Burtin, 2003). Considering the nutritive value, seaweeds can be regarded as an under-exploited source of health benefit molecules for food processing and nutraceutical industry (Burtin, 2003).

*Sargassum*, one of the marine macroalgal genera that come under the class Phaeophyceae, is widely distributed in tropical and temperate oceans. Species like, *S. boveanum* with antioxidative and lipid peroxidation properties (Zahra et al., 2007), *S. hemiphyllum* with immune-stimulating activity (Hwang et al., 2010), *S. wightii* which shows a good amount of flavanoids in support of its antioxidant activity (Meenakshi et al., 2009), indicate that this genus is an ideal target for investigating presence of bio-molecules for various medical and industrial applications. *Sargassum myriocystum* is widely distributed on the southern coasts of Tamilnadu, India and many parts of Asia and it is reported to be used as animal feed, food ingredients and fertilizer. With no extensive study made on this particular species, the present work's focus was on determining biochemical composition and evaluating the radical quenching activity of *S. myriocystum*.

## MATERIALS AND METHODS

### Chemicals

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) was procured from Sigma Aldrich (Bangalore, India). All other chemicals, solvents and reagents used were purchased from Sisco research laboratories (Mumbai, India).

### Seaweed collection

Fresh brown seaweed, *Sargassum myriocystum* was collected (May, 2008 and 2009) from coast of Rameswaram, India and they were identified by Dr. Rangaswamy, CAS Botany, University of Madras, Tamilnadu, India. The collected plants were washed thoroughly with tap water and then with distilled water. Brown seaweeds were shade dried, powdered and used for further analysis.

### Solvent extraction

Cold (homogenization) and Soxhlet extractions were carried out as described below.

### Cold extraction (homogenization)

Ten grams of dried and powdered seaweeds was immersed in appropriate solvents. The solvent used were methanol, ethyl acetate, n-butanol, petroleum ether, chloroform and water. Extraction was carried out in a shaker for 24 h and this process was repeated twice. The liquid extracts of respective solvents were pooled in pre-weighed containers and allowed to dry under negative pressure. The weights of the extracts were calculated and % yield was determined.

### Soxhlet extraction and fractionation

The extraction by Soxhlet apparatus was followed by partitioning with different solvents that were used in the decreasing order of their polarity. A crude extract of methanol and chloroform (2:1) was soxhleted at 50 to 60 °C for 6 h. The crude extract was dried, weighed and then dissolved in 90% aqueous methanol for fractionation. Methanol fraction was further fractionated as described by Duan et al. (2006). Briefly, initial fractionation was carried out with 100 ml petroleum ether. Petroleum ether fraction was collected and aqueous methanol phase was evaporated under reduced pressure to give a semisolid. This portion was dissolved in equal volumes of distilled water and further fractionated with ethyl acetate, n-butanol and n-hexane. Resultant fractions including aqueous were evaporated to dryness. The dried fractions were dissolved in DMSO and stored at 4 °C for further assays.

### Biochemical composition

The dried plant materials were used for determining total protein and carbohydrate contents. Crude methanol-chloroform extract was used to determine the total phenolic contents in *S. myriocystum*.

### Estimation of protein in crude extract of *S. myriocystum*

One gram of sample was immersed in 4 ml of distilled water for 12 h and subsequently homogenized. The homogenized sample was mixed with 2.5 volumes of 25% TCA. (Barbarino and Lourenço, 2005). The contents were centrifuged under low speed and supernatant was recovered. Supernatant was used to determine the protein content as described by Lowry et al. (1956) using BSA as standard.

### Estimation of total carbohydrate

1g of sample was hydrolyzed with 5 ml of 2.5N HCl for 3 h. 1% sodium carbonate was added till the effervescence ceases, to neutralize the HCl. The content was made up to 100 ml and centrifuged at low speed. The supernatant was tested for total carbohydrate contents as described by Dubois et al. (1956) using glucose as standard.

### Determination of total phenol contents

The crude methanol-chloroform extract was used for determination of phenol contents as described by Taga et al. (1984). Gallic acid was used as positive standard and the result was expressed in gallic acid equivalents.

### Antioxidant assays

All the assays used to determine the antioxidant activity were carried out in 96 well microtitre plates (BD Falcon) and absorbance was read in

automated microplate reader (Bio-Tek Instruments Inc., USA). The concentration of the samples was maintained as 50 µg for all the assays.

#### DPPH radical scavenging activity

The scavenging effects of crude methanolic extract and fractions were determined as described by Yan and Chen (1995). Briefly, 50 µl of 0.16 mM 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) solution in methanol were added to 50 µl aliquot of sample. The mixture was vortexed for 1 min and kept at room temperature for 30 min in the dark. The absorbance of all the sample solutions was measured at 517 nm. The scavenging effect (%) was calculated by using the formulae given by Duan et al. (2006):

$$\% \text{ Inhibition} = ((\text{Blank-Test})/\text{Blank}) * 100$$

Blank and control samples were performed.

#### Deoxyribose radical scavenging activity

Deoxyribose non-site specific hydroxyl radical scavenging activity of extracts was determined as described by Chung et al. (1997). Briefly, 2.0 ml aliquots of sample was added to the test tube containing reaction mixture of 2.0 ml FeSO<sub>4</sub>·7 H<sub>2</sub>O (10 mM), 0.2 ml EDTA (10 mM) and 0.2 ml deoxyribose (10 mM). The volume was made up to 1.8 ml with phosphate buffer (0.1 M, pH 7.4) and to that 0.2 ml H<sub>2</sub>O<sub>2</sub> (10 mM) was added.

The mixture was incubated at 37°C under dark for 4 h. After incubation, 1 ml of TCA (2.8%) and TBA (1%) were added to the mixture, and then left to stand under boiling water bath for 10 min. 300µl of the content was transferred into a microtitre plate and absorbance was measured at 532 nm. If the mixture was turbid, the absorbance was measured after filtration (0.45 µm). Scavenging activity (%) was calculated using the formula:

$$\% \text{ Inhibition} = ((\text{Blank-Test})/\text{Blank}) * 100$$

Where, blank denotes the content without sample and test with the sample.

#### Superoxide anion radical scavenging

The superoxide radicals were generated by a pyrogallol autoxidation as described by Jing and Zhao (1995). Briefly, 9 mL of Tris-HCl buffer solution (50 mmol/L, pH=8.2) was added into a test tube, and contents was incubated in a water bath at 25°C for 20 min. A volume of 40 µL of pyrogallol solution (45 mM/L of pyrogallol in 10 mM/L of HCl), which was also pre-incubated at 25°C, was added to the aforementioned test tube. The mixture was incubated at 25°C for 3 min and then a drop of ascorbic acid was dripped into the mixture promptly to terminate the reaction. The absorbance at 420 nm was marked as A<sub>0</sub>. The same procedure was carried out with extracts instead of ascorbic acid and the absorbance was marked A<sub>1</sub>. A blank was run and noted as A<sub>2</sub> and the scavenging percentage was calculated using the following formula:

$$\% \text{ scavenging activity} = (A_0 - (A_1 - A_2)) / A_0 * 100$$

#### Estimation of reducing power

Reducing power of crude methanolic extract was determined as described by Oyaizu (1986). Briefly, 1.0 ml of methanol containing different concentration of sample was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (1%). Reaction mixture was incubated at 50°C for 20 min. After incubation, 2.5 ml of TCA (10%) was added and centrifuged (650 g) for 10 min. From the upper layer, 2.5 ml solution was mixed with 2.5 ml distilled water and 0.5 ml FeCl<sub>3</sub> (0.1%). 300 µl of the content was transferred into a microtitre plate and absorbance was measured at 695 nm. Increased absorbance indicates increased reducing power. The results were

expressed in Ascorbic acid equivalents.

#### Total antioxidant activity

Total antioxidant activities of crude methanolic extract and fractions were determined as described by Prieto et al. (1999). Briefly, 0.3 ml of sample was mixed with 3.0 ml reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95°C for 90 min under water bath. 300 µl of the content was transferred into a microtitre plate and absorbance was measured at 695 nm. Total antioxidant activity was expressed as the number of equivalents of ascorbic acid in milligram.

#### Statistical analyses

All the data were expressed as means ± standard deviation (SD) of ten replications, all statistic analyses were carried out using SPSS 11.01 for Windows and students *t*-test.

## RESULTS

### Biochemical composition

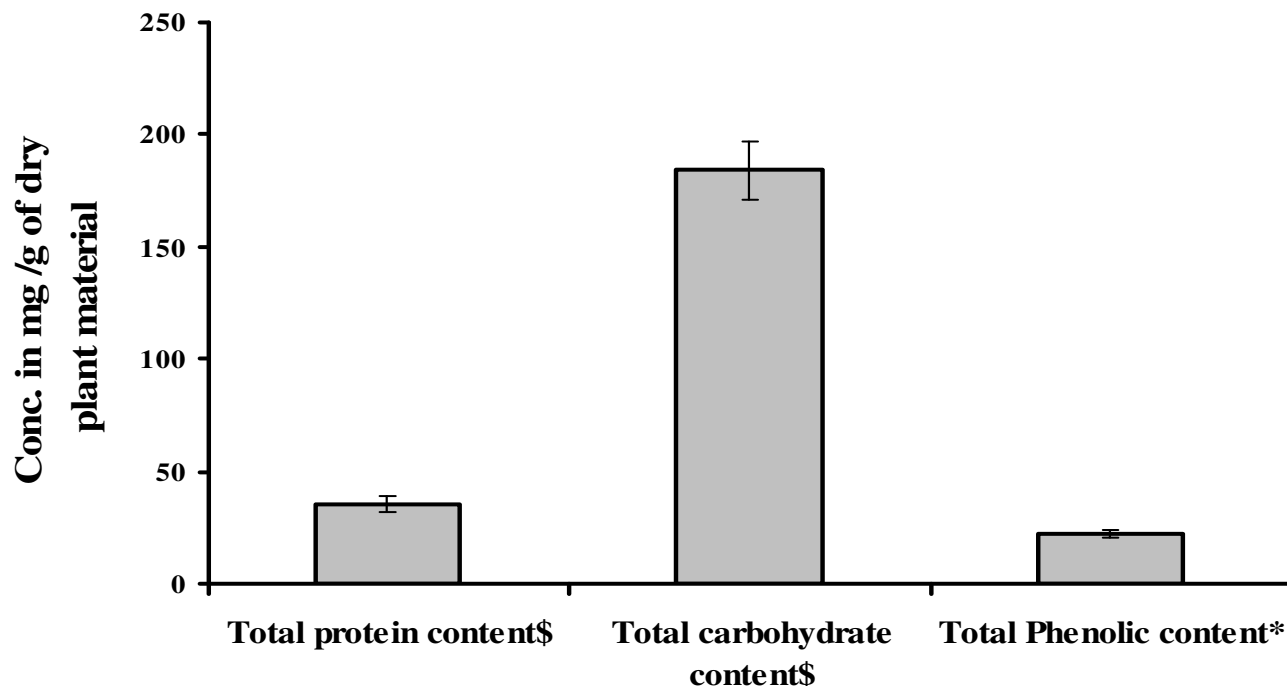
As shown in the Figure 1, the biochemical contents like protein and carbohydrate were quantified in crude powder. *S. myriocystum* has approximately 36 mg/gm of plant material (3.6%) and 184 mg/g of plant material (18.4%) of protein and carbohydrate respectively. It has been reported that protein and carbohydrate contents will vary according to seasonal and species level. The cell wall and intracellular polysaccharides contribute to the level of carbohydrates and pigments along with various enzymes for proteins. Our preliminary studies on phytochemical analysis of crude extract using thin layer chromatography and qualitative analysis has revealed that *S. myriocystum* is enriched with flavanoids (detected by ceric sulphate solution), tannins, alkaloids and terpenoids.

### Extraction and yield of extract

Six different solvents were used in this study, since a wide range of extractants hold a better chance for the isolation/extraction of biologically active molecules for general screening of bioactivity (Kumar et al., 2008). The yields of the extracts obtained by two extraction methods with six solvents are given in Tables 1 and 2. Cold extraction with chloroform (17.12%) and crude methanol: chloroform extracts (11.36%) showed the highest yield.

### Antioxidant activities

Figure 2(a) indicates reducing power of crude extract of *S. myriocystum*. Cold and Soxhlet extracts behaved similarly for reducing ferric ion to ferrous ion. Extraction using methanol: chloroform showed highest reducing



**Figure 1.** Biochemical constituents of *Sargassum myriocystum*. The results are analyzed by student T-test (n=6). \$ Total protein and carbohydrate are expressed in mg/g of plant material. \*Phenolic content is expressed in mg Gallic acid equivalents/g of dry plant.

**Table 1.** Final weight of the extract obtained by cold extraction and its % yield of each solvent.

Solvent	Weight of the extract	% yield
Water	0.558	5.58
Methanol	0.971	9.71
Ethyl acetate	0.615	6.15
n-Butanol	0.681	6.81
Petroleum ether	0.745	7.45
Chloroform	1.712	17.12

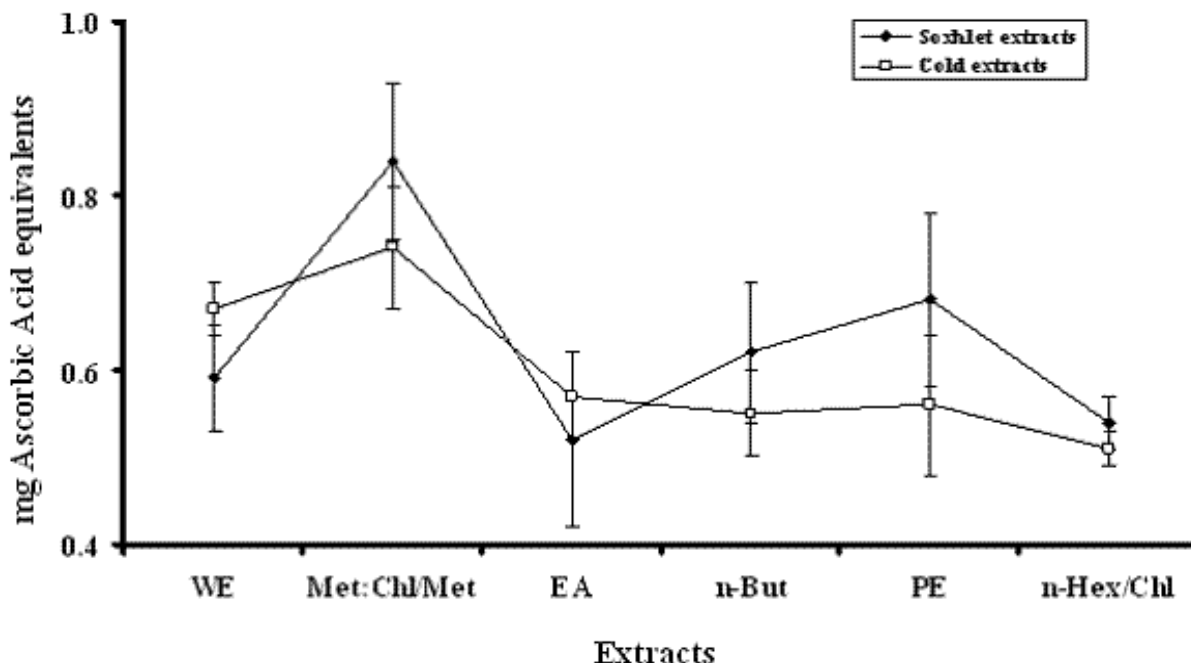
**Table 2.** Final weight of the extract obtained by Soxhlet extraction and its % yield of each solvent

Solvent	Weight of the extract	% yield
Water	0.131	1.31
Methanol/Chloroform	1.136	11.36
Ethyl acetate	0.166	1.66
n-Butanol	0.26	2.6
Petroleum ether	0.456	4.56
n-Hexane	0.011	0.11

power than methanol, n-butanol, petroleum ether, n-hexane and chloroform extracts in both extraction methods. At the same time both n-butanol and petroleum ether extracts from Soxhlet method showed higher reducing power than respective cold extracts.

Total antioxidant activity of *S. myriocystum* observed is

shown in the Figure 2(b). Polar solvent, water showed insignificant effect for cold and Soxhlet extractions as shown. Methanol: chloroform mixture of Soxhlet extracts showed highest antioxidant activity (0.22 mg ascorbic acid equivalent) than any other organic solvents as shown in the figure. n-Hexane extract obtained from



**Figure 2(a).** Reducing power of organic extracts of *Sargassum myriocystum* (cold and Soxhlet extracts) Met:Chl - Methanol:Chloroform, EA - Ethyl acetate, n-But - n-Butanol, PE - Petroleum ether, n-Hex - n-Hexane, WE - Water, Met - Methanol, Chl - Chloroform. (Met:Chl and n-Hex represents activity of Soxhlet extract and Met and Chl represents activity of cold extracts). The concentration of extract analyzed was 50µg/reaction volume. The results are analyzed by student T-test (n=6).

Soxhlet extraction demonstrated moderately increased antioxidant activity compared with cold extraction. Compared with Soxhlet extracts of ethyl acetate, n-butanol and petroleum ether, cold extraction using the same organic solvents showed better activity.

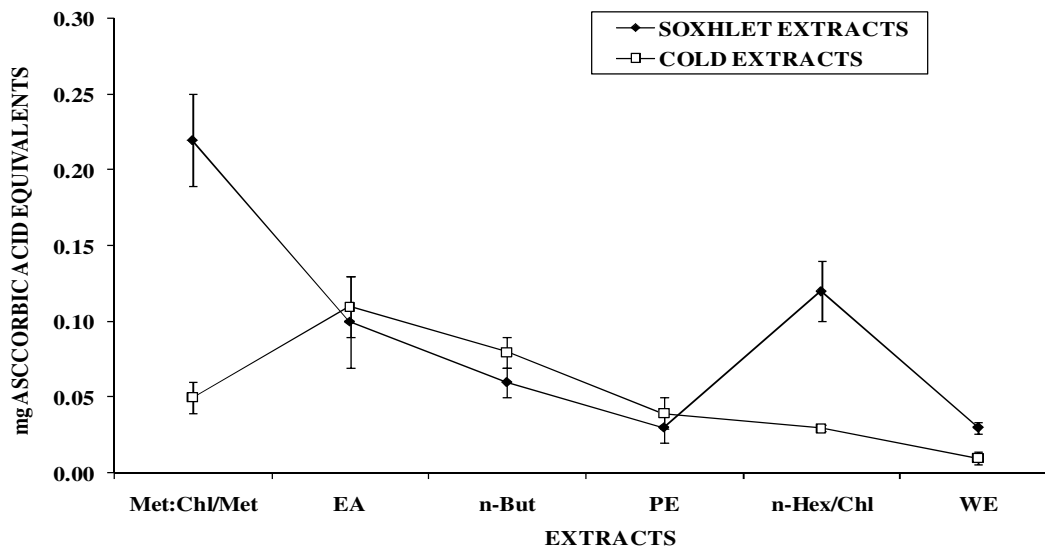
Radical scavenging effect of natural antioxidants could be evaluated using DPPH, a free radical donor under *in vitro* conditions (Jao and Ko, 2002; Matsukawa et al., 1997). Organic and water extracts of *S. myriocystum* were used for assessing its free radical quenching activity using DPPH. In Soxhlet extraction, water extract demonstrated lesser % inhibition than petroleum ether extracts as shown in the figure. n-Butanol extract of Soxhlet showed much less inhibition than rest of the extracts obtained from cold and Soxhlet methods. Water extract obtained from cold method demonstrated highest % inhibition than other extracts as seen in the Figure 2(c).

Pyrogallol auto-oxidation system was used to measure superoxide anion scavenging activity of algal extracts and the results were expressed in terms of inhibitory rate of the superoxide productivity. Super oxide anion radical scavenging assay was carried out in both forms of extracts as shown in the Figure 2(d). All the extracts showed similar radical scavenging activity of about 50% inhibition as shown in the Figure 2(d). Hydrogen peroxide is a relatively unstable metabolic product being responsible for the generation of hydroxyl radical and

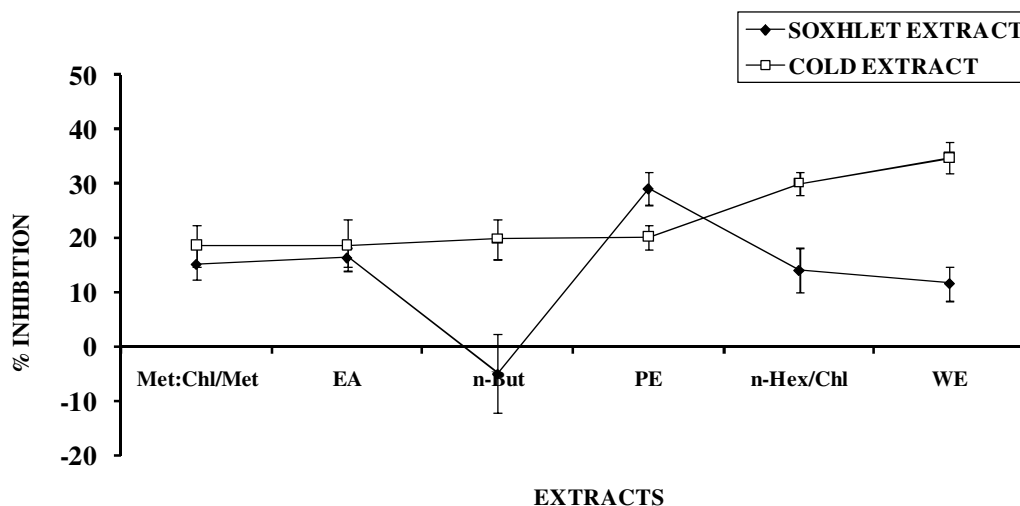
singlet oxygen, which are formed by fenton reaction. These formed hydroxyl radical initiate lipid peroxidation and subsequently damages the cells. Hydroxyl radical can be generated by the reaction of hydrogen peroxide with  $Fe^{2+}$  or  $Cu^{2+}$  and its scavenging activity was assayed in both forms of extracts as shown in the Figure 2(e). Cold extracts of methanol, chloroform mixture, n-butanol and petroleum ether extracts showed maximum % inhibition compared with ethyl acetate, n-hexane and water of the extracts obtained from cold extraction. Petroleum ether extract obtained from Soxhlet showed minimal inhibition (38%) compared with other extracts of the same, which showed ~ 85% inhibition. Cold extracts showed highest activity than Soxhlet extracts on hydroxyl radical scavenging as shown in the Figure 2(e) with statistical significance of  $p=0.001$ .

## DISCUSSION

Free radicals, showing deleterious effects are quenched by certain synthetic compounds having adverse side effects. This let various workers on exploration for natural sources of antioxidants with multifunctional potential as alternatives for toxic synthetic antioxidants, to prevent oxidation in any metabolic pathways. Nature has always served as a wealthy resource for medicinal plants for thousands of years and a remarkable number of drugs



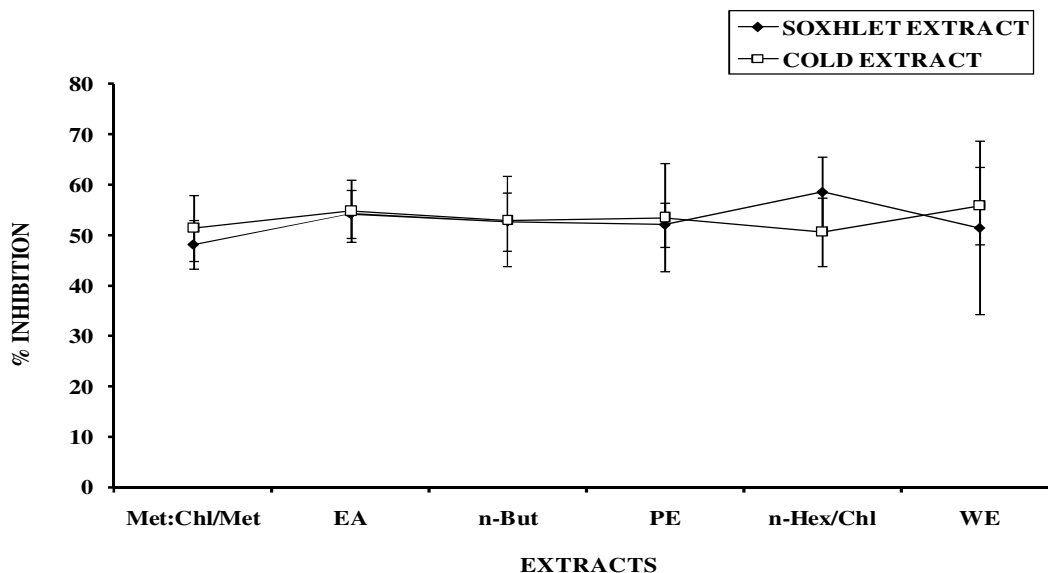
**Figure 2(b).** Total antioxidant activity of organic extracts of *Sargassum myriocystum* (cold and soxhlet extracts). Met:Chl, Methanol:chloroform; EA - ethyl acetate; n-But, n-Butanol; PE, petroleum ether; n-Hex, n-Hexane; WE, water; Met, methanol; Chl, chloroform. (Met:chl and n-Hex represents activity of soxhlet extract and Met and Chl represents activity of cold extracts). The concentration of extract analyzed was 50  $\mu\text{g}$ /reaction volume. The results are analyzed by student T-test (n=6).



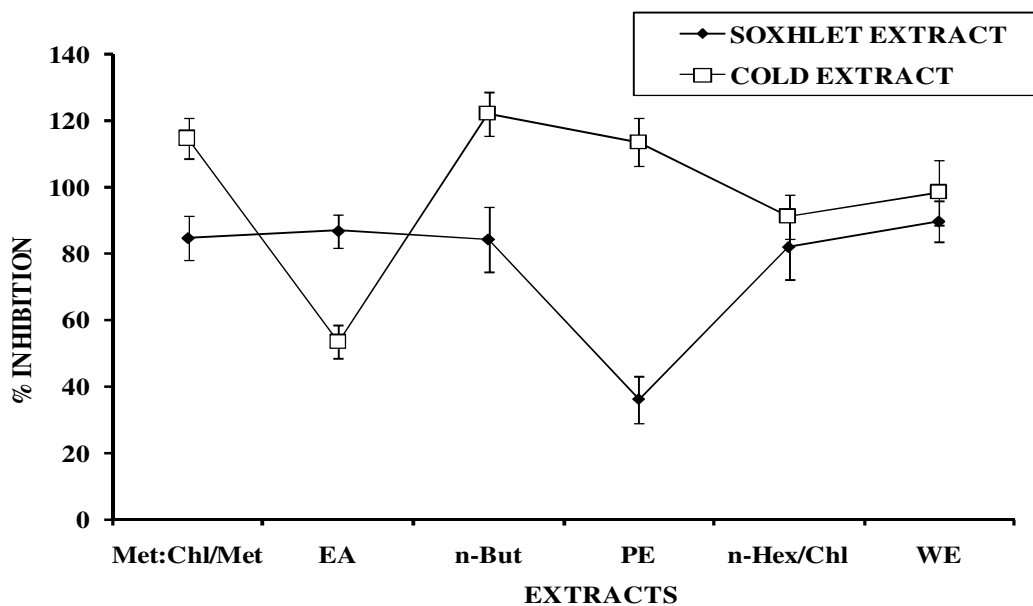
**Figure 2(c).** DPPH free radical scavenging activity of organic extracts of *Sargassum myriocystum* (cold and soxhlet extracts). Met:Chl, Methanol:chloroform; EA, ethyl acetate; n-But, n-Butanol; PE, petroleum ether; n-Hex - n-Hexane; WE, water; Met, methanol; Chl, chloroform. (Met:Chl and n-Hex represents activity of soxhlet extract and Met and Chl represents activity of cold extracts). The concentration of extract analyzed was 50  $\mu\text{g}$ /reaction volume. The results are analyzed by student T-test (n=6).

have been isolated and characterized, notably from plant origin (Cowan, 1999). For many years marine plants have served as good sources for antioxidant bio-molecules (Takamats et al., 2003). Marine algae are one of the abundant antioxidant sources that can fight against free radicals. For many years, *Sargassum* species have not only been used in human and animal nutrition but also for other industrial purposes.

Carbohydrate content was contributed not only by cell wall polysaccharides but also by some intracellular sulfated polysaccharide, which was considered to show various bioactivities such as anticoagulant, antitumor, antiviral and antioxidant (Hu et al., 2010). It was already found that protein content of marine algae depends on the seasonal period and climatic conditions (Fleurence, 1999).



**Figure 2(d).** Superoxide anion radical scavenging assay of organic extracts of *Sargassum myriocystum* (cold and soxhlet extracts). Met:Chl, Methanol:chloroform; EA, ethyl acetate; n-Bu, n-Butanol; PE, petroleum ether; n-Hex, n-Hexane; WE, water; Met, methanol; Chl, chloroform. (Met:Chl and n-Hex represents activity of soxhlet extract and Met and Chl represents activity of cold extracts). The concentration of extract analyzed was 50 µg/reaction volume. The results are analyzed by student T-test (n=6).



**Figure 2(e).** Hydroxyl radical scavenging assay of organic extracts of *Sargassum myriocystum* (cold and soxhlet extracts). Met:Chl, Methanol:chloroform; EA, Ethyl acetate; n-But, n-Butanol; PE, petroleum ether; n-Hex, n-Hexane; WE, water; Met, methanol; Chl – chloroform. (Met:Chl and n-Hex represents activity of soxhlet extract and Met and Chl represents activity of cold extracts). The concentration of extract analyzed was 50 µg/reaction volume. The results are analyzed by student T-test (n=6).

Reducing power activities were usually reported by the ability to develop reductones, which terminates free radical chain reactions by donating a hydrogen atom. Since most non-enzymatic antioxidative activity is

mediated by redox reactions, reports on antioxidant activity were concomitant with the reducing power (Costa et al., 2010).

Mixture of methanol and chloroform changed dielectric

constant and precipitates bio-molecules, which are responsible for reducing ferric ion. Total antioxidant activity can be used for structure-activity relationship of the antioxidant species (Devi et al., 2008). We could assume that active compound present in brown seaweeds dissolved extensively in Methanol/chloroform (2:1) mixture at 60°C, showed highest ability to reduce molybdenum VI (Mo6+) to form a green phosphate complex V (Mo5+) in terms of mg of ascorbic acid equivalents, compared with cold extraction.

Polar solvent, water showed higher radical scavenging activity, since algal extracts offered an electron and /or hydrogen for decolorization of DPPH (Molyneux, 2004). n-butanol showed negative % inhibition because of unavailability of free electron and/or hydrogen for decolorization of DPPH.

Singlet oxygen ( $O_2^-$ ) is formed in almost all the viable and living cells through several metabolic reactions (Fridovich, 1974) and its effect can be magnified because it produces other types of free radicals and oxidizing agent that can induce cell damage, which leads to apoptosis or necrosis (Lui and Ng, 1999). Antioxidants in algal extracts may inhibit both oxygen consumption and hydrogen peroxide formation. It has been shown that hydrogen peroxide together with reactive oxygen species (ROS) can damage several cellular components (Burdon, 1995). Hydroxyl radical has the highest 1-electron reduction potential (2310 mV) and can react with anything in living organisms at the second order rate constants of  $10^9$  to  $10^{10}$  mol /L /s (Korycka-Dahl and Richardson, 1978), among various free radical compounds. It can react with lipids, polypeptides, saccharides, nucleotides and organic acids, especially thiamine and guanosine and thereby cause cell damage (Ashok and Ali, 1999). From the results we could conclude that *S. myriocystum* showed highest inhibition against OH<sup>-</sup> radicals than other reactive oxygen species, as shown in the Figure 1, the algal crude extract has less phenolic contents than other marine seaweeds as reported.

## Conclusion

It is presumed from the phenolic contents, algal extracts have some other novel bio-molecules, which are responsible for OH<sup>-</sup> radical quenching activity. The authors had attempted to explore potential antimicrobial activity of *S. myriocystum* extracts (data not shown). However, antibacterial and antifungal studies using *in vitro* assays failed to demonstrate any appreciable activity. This could be a reflection of the biochemical makeup (such as fewer amounts of phenolics etc.) of this seaweed species (Abd El-Baky et al., 2008). Hence, further studies are warranted to demonstrate the active principle present in the *S. myriocystum*. We presume that the active principle responsible for highest OH<sup>-</sup> activity

may be a potential therapeutic compound to combat against OH<sup>-</sup> radical induced cellular damage.

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