Int. J. Drug Dev. & Res.
| October - December 2014 | Vol. 6 | Issue 4 | ISSN 0975-9344 |
www.ijddr.in

Screening of Antioxidant Potential of Green Alga *Codium adhaerens*

SUDHA K

MOHANA PRIYA K,

NANCY VEENA KUMARI,

PALANICHAMY V

School of Biosciences and Technology,VIT University, Vellore - 632014, Tamilnadu, India.

Corresponding Authors:
Email: vpalanichamy@vit.ac.in

Abstract:
Natural products from the marine source have drawn attention of researchers worldwide over the last few decades. Preliminary phytochemical screening and *in-vitro* antioxidant potential were analyzed for the red alga *Codium adhaerens* collected from the Pudumadam region, Rameswaram. Phytochemical analysis revealed the presence of major phytoconstituents phenolics, flavonoids, alkaloids, proteins, tannins and terpenoids. Ethyl acetate extract were tested for total phenolic and flavonoid content, total antioxidant activity, reducing power assay, scavenging activity on DPPH, Superoxide anion, nitric oxide and hydrogen peroxide radicals. The total phenolic and flavonoid content were found to be 53.11 ± 0.29 mg/g Gallic acid equivalents and 52.45 ± 0.37 mg/g Quercetin equivalents. The total antioxidant activity were found to be (74.61 ± 1.55)% at 1000µg. Ethyl acetate extracts exhibited maximum scavenging activity against free radical DPPH (72.92 ± 0.23)%, Superoxide anion (59.41 ± 1.58)%, Nitricoxide (52.41 ± 1.40)% and hydrogen peroxide radical (49.17 ± 0.83)% Hence, it can be concluded, red alga *Codium adhaerens* has bioactive compounds with remarkable antioxidant activity.

Keywords: *Codium adhaerens,* antioxidant, scavenging activity.

Introduction

Seaweeds are commonly called as marine macroalgae are classified into three divisions based on their colors such as Chlorophyta, Rhodophyta and Phaeophyta. Algae are renewable natural sources potentially rich in sulfated polysaccharides, with a predominance of one or another polysaccharide depending on the group. In red algae, there are mainly sulfated galactans, green algae have more heterogeneous sulfated polysaccharides that are rich in galactose, mannose, xylose, arabinose, glucose and brown seaweed are rich in fucans, laminarin and alginic acid (1). Seaweeds are growing in extreme environment, this factor implies that seaweed have some protective mechanisms and compounds (Matasukawa et al., 1997). They are considered as a major source of secondary metabolites with broad spectrum of biological activities. Seaweeds are rich in nutritional value mainly due to the presence of proteins, polysaccharides, minerals and vitamin content (2)

A free radical is a molecule with more unpaired electrons in the outer orbital. Most of these free radicals are in the form of high reactive oxygen and nitrogen species produce due to oxidative stress leads to serious damages in the tissue. In search of novel source of antioxidants in the last few decades, marine algae have been widely studied for their antioxidant activity. A recent report reveals that marine algae are rich sources of antioxidant compounds (3). Polyphenols are the major compounds for the antioxidant properties of seaweeds. Polyphenols antioxidant property is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (4). The objective of the current study was to evaluate the antioxidant potential of ethyl acetate extract of *Codium adhaerens*.

MATERIALS AND METHODS:

Sample collection

Green alga *Codium adhaerens* collected from the Pudumadam region, Mandapam, Southeast coast of India during the period of January 2014 by hand picking. After collection, they were washed thoroughly with seawater followed by distilled water thrice to remove salt and debris present. They were shade dried, grounded using mechanical grinder, packed in a sterile container and stored in the refrigerator until use.

Marine algal extract preparation

Soxhlet extraction of *C. adhaerens* was carried out with ethyl acetate solvent. Ten grams of algal powder was added with 200 ml of ethyl acetate was used for extraction and it was carried out in soxhlet apparatus for a period of 18 hours at room temperature and condensed using rotary vaccum evaporator and stored in the refrigerator at 4º C for further analysis (5).

Preliminary phytochemical screening

Phytochemicals present in the crude extract of *C. adhaerens* such as alkaloids, flavonoids, phenolics, proteins, saponins, steroids, tannins, terpenoids was determined by following the standard protocol described by Harborne, Trease and Evans (6-7)

Total phenolic content

The total phenolic content present in the crude extract of *C. adhaerens* were evaluated according to the method of Malik and Singh (8). 1 ml of extracts were taken and made up to 3 ml with distilled water. Then, 0.5 ml of 1:1 dilution of Folin's ciocalteau reagent and 2 ml of 20% Sodium carbonate were added to it. The mixture was boiled for a 1 min, cooled, incubated at 30 min at dark and absorbance was measured at 650 nm against the blank. A standard graph was plotted using known concentration of gallic acid. The concentration of phenols present in the graph were calculated from the calibration curve and expressed as mg/g of Gallic acid equivalents

Total flavonoid content

The total flavonoid content present in the extract of *C. adhaerens* was determined by following the method of Zishen *et al.* with little modifications (9). 1 ml of extract was mixed with 5 ml of distilled water and 300µl of 5% Sodium nitrite solution and incubated for 6 min. Then, 500 µl of 10% aluminium chloride was added, after 5 min, 2 ml of 1M sodium hydroxide was added to the mixture and then the volume was made up to 10 ml with distilled water and absorbance was measured at 510nm against the blank. Quercetin was used to calibrate the standard curve and the results were expressed as mg/g Quercetin equivalents.

Total antioxidant activity

The total antioxidant activity of the crude extract of *C. adhaerens* was determined by the phosphomolybdneum method (10). Various range of the extracts (200-1000) µg/ml was prepared. 1ml of each extract was mixed with 2 ml reagent (0.6M sulphuric acid, 28mM sodium phosphate, 4mM ammonium molybdate), incubated at 95ºC for 90 min and the absorbance was measured at 635nm using UV spectrophotometer. The percentage of antioxidant activity was calculated using the following formula.

$$\frac{Ac - As}{Ac} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Ac = Absorbance of control; As = Absorbance of sample.

Reducing power assay

Different concentration of the extracts (200-1000) µg/ml was prepared using distilled water. 1 ml of each extract were mixed with 2.0 ml of 0.2 M phosphate buffer and 2.5 ml of 1% potassium ferric

cyanide and incubated for 20 min at 50ºC. 2.5 ml of 10% Trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm for 10 min. 2.5ml distilled water and 0.5 ml of 1% Ferric chloride was mixed with 2.5 ml of supernatant and the absorbance was measured at 700nm.(11)

DPPH free radical scavenging assay

The scavenging activity of *C. adhaerens* extract against DPPH free radical was determined according to the procedure of Blios (12). Various range of concentration (200-1000) µg/ml was prepared using distilled water. 1 ml of each extract was mixed with 2ml of 0.16mM DPPH in methanol and incubated at dark for 20 min at room temperature. After incubation, the absorbance of the sample was measured at 520nm using UV spectrophotometer against the blank. The percentage of scavenging activity of *C. adhaerens* against the DPPH was calculated using the equation 1.

Superoxide free radical scavenging assay

The superoxide anion radical scavenging activity of *C. adhaerens* was evaluated by the method of Liu (13). Various range of concentration (200-1000) µg/ml was prepared using distilled water. 1ml of each extract was mixed with 0.5 ml of 2.52mM NBT, 0.5ml of 64µM NADH and 60µM phenanzine methosulphate and incubated at room temperature for 15 min. Absorbance of the sample was read at 560nm using UV spectrophotometer. The percentage of scavenging activity of *C. adhaerens* against superoxide anion was calculated using the equation1.

Hydrogen peroxide radical scavenging assay

The capability of *C. adhaerens* extract was determined by the method of Ruch (14). Various range of concentration (200-1000) µg/ml was prepared using distilled water. 40mM hydrogen peroxide was prepared using phosphate buffer (pH 7.4). 1ml of each extract was added with 600µl of 40mM hydrogen peroxide and incubated for 20 min at room temperature against the blank. The percentage of scavenging activity of *C. adhaerens* against hydrogen peroxide radical was calculated using the equation1.

Nitric oxide scavenging assay

Nitric oxide scavenging activity of *C. adhaerens* extract was measured by the Griess reaction (15). Various range of concentration (200-1000) µg/ml was prepared using distilled water. 500µl of 5mM sodium nitroprusside in phosphate buffer was added to 1 ml of each extract and incubated at 25ºC for 30 min.2 ml of Griess reagent (1% sulphanilamide, 0.1% napthylethylene diamine dichloride and 3% phosphoric acid) was added to the reaction mixture and absorbance was measured at 560nm using UV spectrophotometer against blank. The percentage of scavenging activity of *C. adhaerens* against nitric oxide free radical was calculated using the equation 1.

Results

In the present study, Green alga *Codium adhaerens* was collected and screened for the presence of major phytoconstituents. The ethyl acetate extract of *Codium adhaerens*reveals the presence of phytochemicals such as alkaloids, flavonoids, phenolics, glycosides, proteins, tannins, terpenoids and steroids as shown in the table 1 which could be the responsible for major bioactivity.

Covered in Scopus & Embase, Elsevier
Int. J. Drug Dev. & Res., October-December 2014, 6 (4): 103-111

Table 1: Phytochemical analysis of ethyl acetate extract of *C. adhaerens*

Phytoconstituents	Ethyl acetate extract of *C. adhaerens*
Alkaloids	Present
Flavonoids	Present
Proteins	Present
Tannins	Present
Terpenoids	Present
Glycosides	Present
Sterol	Present
Phenolics	Present

Phenolic compounds are commonly found in seaweeds have been reported to have a broad range of biological activities mainly antioxidant activity (16).The total phenolics present in the *C. adhaerens*ethyl acetate extract were determined by Folin's – ciocalteau reagent and the results were expressed as mg/g Gallic acid equivalents. The phenolic contents were found to be 53.11 ± 0.29 mg/g GAE equivalents.

Flavonoids are the most important natural polyphenolic due to their wide range of chemical and biological activities including antioxidant and free radical scavenging activities (17). The total flavonoids present in the ethyl acetate extract of *C. adhaerens* were evaluated by aluminium chloride method and the results were expressed as52.45 ± 0.37 mg/g Quercetin equivalents.

The total antioxidant activity of green alga *C. adhaerens* was evaluated by the phosphomolybdate method. *C. adhaerens* ethyl acetate extract exhibited the maximum antioxidant activity at 1000µg/ml as (74.61 ± 1.55) %.

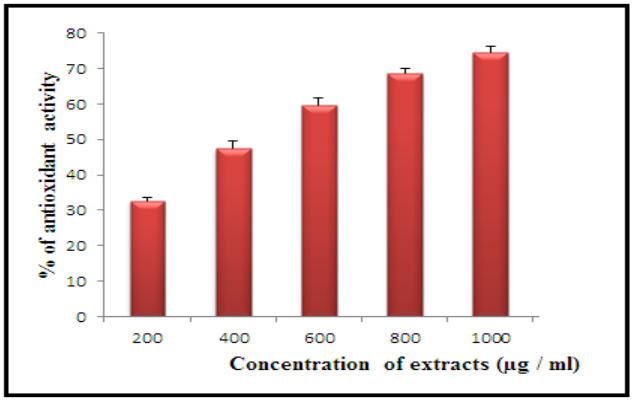

Fig.1: Total Antioxidant activity of *C. adhaerens* Experiments were done in triplicates. Values were expressed as mean ± SEM

Fe (III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action (18). The reductive potential of the crude ethyl acetate extract of *C. adhaerens* is the reduction of ferric to ferrous ion transformation. Increasing absorbance indicates the increasing reducing potential of extract. In this study, the reducing power increases with the increasing concentration of the sample as shown in the fig 2.

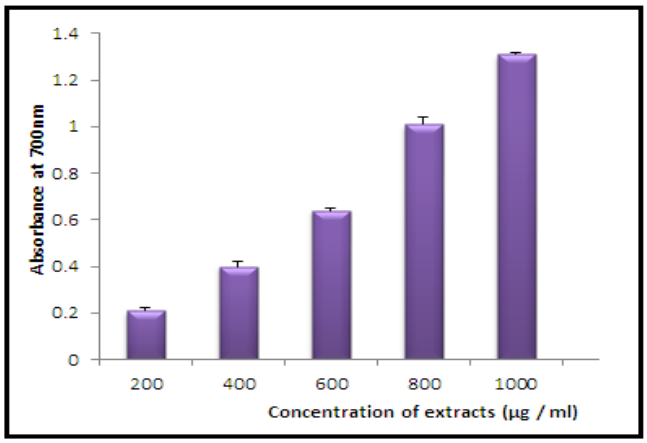

Fig 2: Reducing power of *C. adhaerens* Experiments were done in triplicates. Values were expressed as mean ± SEM

DPPH is a quick and extensively used method for evaluating the scavenging ability of the extracts. In this assay, the extracts have a capability to reduce the stable DPPH radical to the yellow color product, diphenyl picryl hydrazine. This assay is

based on the reduction of DPPH alcoholic solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction (19).It was found that the scavenging activity of the ethyl acetate extracts of *C. adhaerens* as increases with increasing concentration of sample as depicted in the fig 2. At 1000µg/ml, the maximum activity was found to be (72.92 ± 0.23)%,

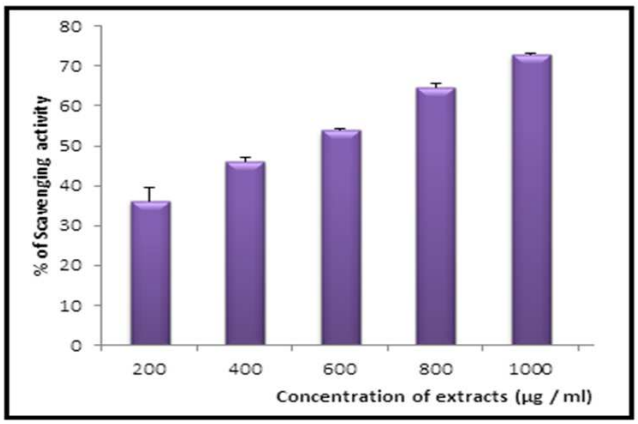

Fig 3: DPPH Radical scavenging activityof *C. adhaerens*
Experiments were done in triplicates. Values were expressed as mean ± SEM

The ethyl acetate extract of *C. adhaerens* was evaluated for superoxide scavenging assay by the nitroblue tetrazolium reduction method and the results are depicted in the figure 4. At 1000 µg / mL, maximum scavenging activity of (59.41 ± 1.58) %,was observed. This result indicates that the scavenging activity is directly proportional to the concentration of the compound.

Nitric oxide scavenging activity of the *C. adhaerens* extract was measured using Griess reaction. This assay is based on the principle at physiological pH, sodium nitroprusside in aqueous solution generates nitric oxide which interacts with oxygen to produce nitrite ions. Scavengers present in the extract compete with oxygen leads to the decreased production of nitrite ions (20). The extracts have shown the scavenging activity in a dose dependent manner as shown in the fig 5. At 1000µg/ml, the *C. adhaerens* has shown the scavenging activity as(52.41 ± 1.40)%

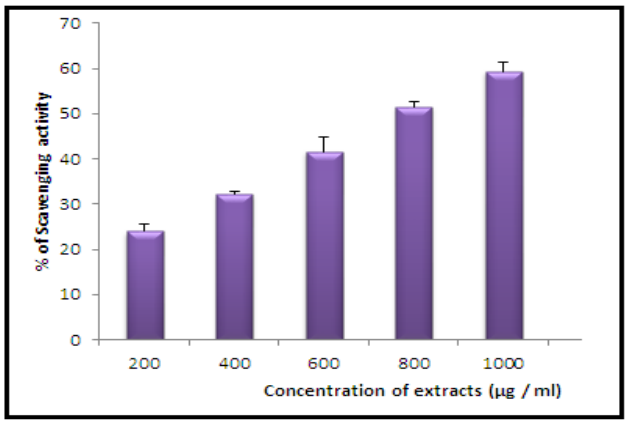

Fig 4: Superoxide anion Radical scavenging activity of *C. adhaerens*
Experiments were done in triplicates. Values were expressed as mean ± SEM

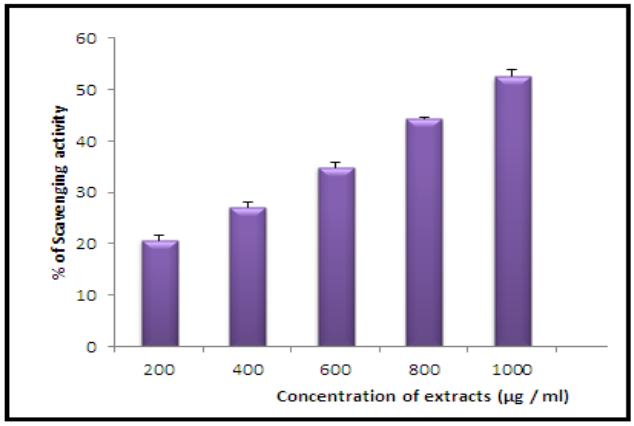

Fig 5: Nitric oxide scavenging activity of *C. adhaerens*
Experiments were done in triplicates. Values were expressed as mean ± SEM.

The capability of the *C. adhaerens* extract to effectively scavenge hydrogen peroxide was determined. Scavenging of H_2O_2 by extracts may be attributed to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water (21). The extracts were shown the scavenging activity in a concentration dependent manner. The scavenging activity increased with the increasing concentration of the sample as shown in the fig 6. At 1000µg/ml, it

has shown the maximum scavenging activity as (49.17 ± 0.83) %

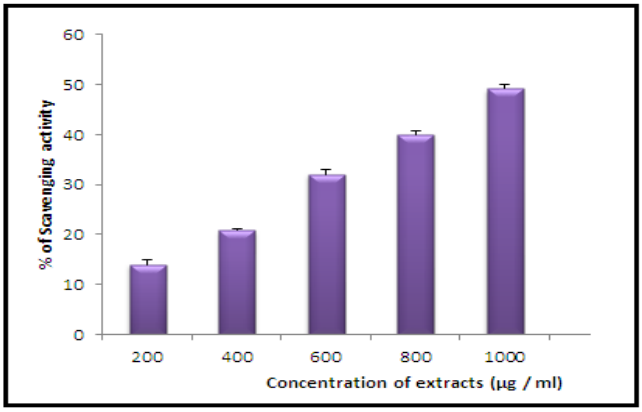

Fig 6: Hydrogen peroxide scavenging activity of *C. adhaerens*
Experiments were done in triplicates. Values were expressed as mean ± SEM.

Discussions

Seaweeds are considered as a major food ingredient in Asian countries includes Japan, Korea, Malaysia and china. In Asia, it has been used for centuries in the preparation of salads and soups (22). Naturally seaweeds are contains novel antioxidant compounds which control the free radical formation from metabolic reaction. Recent years, it has drawn attention of many researchers worldwide due to the rich source of antioxidant compounds (23). In preliminary phytochemical screening, major phytoconstituents were found in the ethyl acetate extract of *C. adhaerens.* Many types of seaweed were reported to contain active compounds that can cure diseases. Now a day, most of the people started to use remedies of natural origin for curing illness (24). Phytoconstituents such as phenolics, flavonoids, tannins have been found in the ethyl acetate extract of *C. adhaerens.* The presence of polyphenolic compounds is a good indicator of antioxidant activities in terms of their ability to scavenge free radicals (25). The uniqueness of their molecular skeleton and structures has contributed to the strong antioxidant activity. Polyphenols for instant uses its phenol rings as electron traps for free radicals (26).

Many methods are available to detect the antioxidant activity of the seaweeds. In this present study, the antioxidant activity was determined by the phosphomolybdate method, scavenging activity against free radicals DPPH, Superoxide, nitric oxide and hydrogen peroxide was done. In this present study, the green alga *C. adhaerens* has shown high phenolic content, antioxidant activity and scavenging activity. Likewise, previous reports have been demonstrated that green algae have high antioxidant potential and scavenging activity. Several studies have been demonstrated that the correlation between the phenolic content and antioxidant activity of certain seaweeds (27).

Antioxidant potential of the ethyl acetate extract of *C. adhaerens* was assayed by the phosphomolybdate method. It has shown the maximum antioxidant activity at the concentration of 1000 µg/ml. Reducing potential of the extract was measured by the potassium ferric cyanide method and it has shown high reducing capability. The reducing ability of the compound mainly depends on the presence of reductones, which exhibits the antioxidant potential by breaking the free radical chain by donating the hydrogen atom (16). It is a significant indicator of antioxidant potential. Similar studies have also been reported by several researchers in marine macro algae (28-29).

DPPH assay is a reliable, accurate method for assessing the scavenging activity of the compound. DPPH is a purple color compound generally fades into yellow color when an antioxidant is present in the medium. Thus

antioxidant molecules can quench the free radical DPPH by donating hydrogen atom or accepting electrons in hydrogen atom. Rapid decrease of absorbance indicates the more potent antioxidant activity of the extract (30). *C. adhaerens* has shown the maximum scavenging of (72.92 ± 0.23) %,Superoxide anion radicals are produced by various flavoenzymes like xanthine oxidase, which converts hypoxanthine to xanthine and subsequently touric acid in ischemia-reperfusion. In the PMS-NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT (31). The absorbance decrease at 560nm indicates the scavenging ability of extract in the reaction. *C. adhaerens* ethyl acetate extract has shown the scavenging activity against superoxide free radical as (59.41 ± 1.58) %,

Different species of seaweeds possess scavenging ability of hydrogen peroxide. It could cross membrane and might oxidize a compound. H_2O_2 alone is not a reactive but rarely it cause damage to the cells due to the production of hydroxyl radicals in the cells (32). Hydrogen peroxide scavenging activity of C, adhaerens ethyl acetate extract as found to be (49.17 ± 0.83) %

Antioxidant properties of phenolics might be due to their ability to act as reductones, hydrogen donors, free radical quenchers which prevents the formation of free radicals (33). *C. adhaerens* antioxidant activity might be due to the presence of higher polyphenolic content in it.

Conclusion

Based on the results observed, it can be concluded *C. adhaerens* has high antioxidant and free radical scavenging activity. The present study reveals *C. adhaerens* extract might be utilized as a natural source of antioxidant, supplement in food and pharmaceutical industry. Further study is must to isolate and characterize the specific bioactive compound present in it responsible for antioxidant activity.

Aknowledgement

We would like to thank the management of VIT University for providing the facilities and constant encouragement for the research.

References

1) Rocha HAO, Farias EHC, Bezerra LCLM, Albuquerque IRL, Medeiros VP, Queiroz KCS, Leite EL. Polissacar ídeossulfatados de algasmarin has com atividade anticoagulante. Infarma 2004; 16: 1-2.
2) Ruperez P, Calixto F S, Dietary and physicochemical properties of edible Spanish seaweeds. European Food Research and Technology2001; 212:349-354.
3) Shyamala Viswanathan, Ebciba C, Santhiya R Thangaraju Nallamuthu. Phytochemical Screening and In Vitro Antibacterial, Antioxidant and Anticancer Activity of *Amphiroa Fragilissima* (Linneaus) J V Lamoroux. International Journal of Innovative Research in Science, Engineering and Technology 2014; 3(5): 12933-47.
4) Rice-Evans CA. Measurement of total antioxidant activity as a marker of antioxidant status in vivo. Procedures and limitations, Free Radical Research 2000; 33:59-66.
5) Parekh J, Nair R, Chanda S. Preliminary screening of some folkloric plants from Western India for potential antimicrobial activity. Indian Journal of Pharmacology 2005; 37: 408-409.
6) Harborne JB, Williams CA. Advances in flavonoid research. Phytochemistry 2000; 55:481-485.
7) Trease GE, Evans WC. Pharmacognosy, 11th edn., Bailliere Tindall, London 1989; 45-50.

8) Malik EP, Singh MB. Plant Enzymology and Hittoenzymology (1st Edn). 1980; 292.

9) Zishen J, Mengcheng T, Jianming W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chemistry 1999; 64: 555-59.

10) Prieto P, Pineda M, Aguilar MM. Spectrophotometric quantification of antioxidant capacity through the formation of a Phosphomolybdenum complex; specific application to the determination of vitamin E. Analytical Biochemistry 1999; 269: 337-341.

11) Oyaizu M. Studies on product of browning reaction prepared from glucose amine. Japanese Journal of Nutrition 1986; 44: 307-15.

12) Blios MS. Antioxidant determinantions by the use of a stable free radical. Nature 1958; 26: 1199 - 1200.

13) Liu F, Ng TB. Antioxidative and free radical scavenging activities of selected medicinal herbs. Life Sciences 2000; 66: 725-735.

14) Ruch RJ, Cheng SJ, Klaunig JE. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis 1989; 10: 1003-1008.

15) Govindarajan, R.,Rastogi, S., Vijayakumar, M. Studies on antioxidant activities of *Desmodiumgangeticum.* Biological and Pharmacological Bulletin 2003; 26: 1424.

16) Tao Wang, Rosa Jonsdottir, Guorun Olafsdottir. Total phenolic compounds, radical scavenging and metal cheatation of extracts form Icelandic seaweeds. Food Chemistry 2009; 116: 240-248.

17) Kahkonen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS. Antioxidant activity of plant extracts containing phenolic compounds. Journal of Agricultural and Food Chemistry 1999; 47: 3954-3962.

18) Nabavi SM, Ebrahimzadeh MA, Nabavi SF, Fazelian M , Eslami B. In vitroantioxidant and free radical scavenging activity of *Diospyros lotus* and *Pyrusboissieriana* growing in Iran. PharmacognosyMagazine2009;4(18): 123-127.

19) Shon MY, Kim TH, Sung NJ. Antioxidants and free radical scavenging activity of *Phellinusbaumii* (Phellinus of Hymenochaetaceae) extracts. Food Chemistry 2003; 82: 593-597.

20) Ebrahimzadeh MA, Ehsanifar S and Eslami B .*Sambucusebuluselburensis* fruits: A good source for antioxidants. Pharmacognosy Magazine 2009*;* 4(19): 213-218.

21) Nabavi SM, Ebrahimzadeh MA, Nabavi SF and Jafari M. Free radical scavenging activity andantioxidant capacity of *Eryngium caucasicum Trautv* and *Froripiasubpinnata*. Pharmacology online 2008; 3: 19-25.

22) Jiménez-Escrig A, Sánchez-Muniz FJ. Dietary fibre from edible seaweeds: chemical structure, physicochemical properties and effects on cholesterol metabolism. Nutrition Research 2000; 20: 585-598.

23) Alekseyenko TV, Zhanayeva SY, Vendiktova AA, Zvyagintseva TN, Kuznetsova TA, Besednova N. Antitumor and antimetastatic activity of fucoidan, a sulfated polysaccharide isolated from the Okhotsk sea *Fucusevanescens* brown alga. Bulletin Experimental Biology and Medicine 2007; 147: 730-732.

24) Tyagi, N. and Bohra, A. Screening of Phytochemicals of fruit plant and antibacterial potential against *Pseudomonas aeruginosa*. Biochem. Cell Architecture 2002; 2: 21-24.

25) Chakraborty K, Praveen NK, Vijayan KK, Rao GS. Evaluation of phenolic contents and antioxidant activities of brown seaweeds belonging to *Turbinaria* spp. (Phaeophyta, Sargassaceae) collected from Gulf of Mannar. Asian Pacific Journal of Tropical Biomedicine 2013; 3(1): 8-14

26) Meenakshi, Manicka Gnanambigai D, Tamil Mozhi S, Arumugam M, Balasubramanian T. Total Flavonoid and *in vitro* Antioxidant Activity of Two Seaweeds of Rameshwaram Coast, Global Journal of Pharmacology 2009; 3(2): 59-62.

27) Siriwardhana N, Lee KW, Kim SH, Ha JW, Jeon YJ. Antioxidant activity of *Hizikia fusiformis* on reactive oxygen species scavenging and lipid peroxidation inhibition. Food science and technology international 2003; 9 (5): 339–346.

28) Kumaran A, Karunakaran RJ. In vitro antioxidant properties of methanol extracts of five *Phillanthus* species from India. Lebens Wissen and Technologie 2007; 40: 344-352.

29) Kumar Chandini S, Ganesan P, Bhaskar N. In vitro antioxidant activities of three selected brown seaweeds of India. Food Chemistry 2008; 107: 707-713.

30) Indu.H, Seenivasan.R. In Vitro Antioxidant Activity of Selected seaweeds From Southeast Coast of India. International Journal of Pharmacy and Pharmaceutical Sciences 2013; 5(2). 474 - 84.

31) Ihami Gulcin, HaciAhmetAlici, Mehmet Cesur. Determination of *in vitro* antioxidant and radical scavenging activities of propofol. Chemical and Pharmaceutical Bulletin2005; 53: 281 – 285.

32) Siriwardhana N, Lee KW, Kim SH, Ha JW, Jeon YJ. Antioxidant activity of *Hizikia fusiformis* on reactive oxygen species scavenging and lipid peroxidation inhibition. Food Science and Technology International 2003; 9 (5): 339–346.

Article History: ----------

Date of Submission: 10-11-2014

Date of Acceptance: 17-11-2014

Conflict of Interest: NIL

Source of Support: NONE