International Food Research Journal 20(1): 291-297 (2013)
Journal homepage: *http://www.ifrj.upm.edu.my*

Free radical scavenging activity of *Sargassum siliquosum* J. G. Agardh

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Article history

Received: 11 May 2012
Received in revised form: 5 June 2012
Accepted:9 June 2012

Keywords

Free radical
hydroxyl radical
hydrogen peroxide
nitric oxide

Abstract

The ability of *Sargassum siliquosum* to prevent the initiation of free radicals to cause cellular damage was investigated *in vitro*. The plant was extracted exhaustively using methanol and partition using solvents of different polarities. Total phenolic contents (TPC) and flavonoid contents (TFC) were evaluated according to Folin-Ciocalteu and aluminium chloride colorimetric assays, respectively. TPC results showed significant difference in the mean gallic acid equivalent (GAE) ($p<0.001$) using the five fractions: water < (butanol-water = methanol-water) < hexane < dichloromethane (DCM), in which the mean GAE using DCM is significantly the highest with 69.03 mg/g GAE. TFC demonstrated a significant difference in the mean quercitin equivalent ($p<0.001$) in which the mean QE using dichloromethane is significantly the highest (65 mg/g) compared with other samples. Radical scavenging activities of fractions with the highest TPC and TFC were tested against OH, NO and H_2O_2. The strongest inhibition was demonstrated by DCM fraction against radical scavengers OH, NO and H_2O_2 in a dose-dependent manner with IC_{50} value of 0.28, 0.29, and 2.27 mg/mL, respectively. These results clearly indicate the beneficial effect of *S. siliquosum* as antioxidant and anticancer agent being a free radical scavenger.

Introduction

The *Sargassum* species, a brown warm water macroalgae with variously shaped plastids in each cell, is rich in carotenoids, fatty acids, phycobillins, vitamins, sterols, tocopherols and polysaccharides such as mannitol, cellulose, alginate, fucoidan, fucoxanthine, and laminaran (Yan *et al*., 1998; Dawczynski, 2007). Many *Sargassum* species have recognizable holdfast, stripes and blades that vary well in size and morphology (Haynes, 1975). Moreover, it is a good source of dietary fibers (Dawczynski, 2007). Its biological activity and chemical and nutritional composition may depend on many factors, including species, geographical origin or area of cultivation, seasonal, environmental and physiological variations, time of harvest, water temperature, and processing methods which can greatly influence its protein content, carbohydrates, lipids, fiber, and metabolites (El Gamal, 2010).

Several pre-clinical studies on *Sargassum* species revealed numerous physiological and biological activities; such as: antioxidant, anti-tumor, anti-angiogenic, anti-inflammatory, anti-coagulant, anti-viral and anti-vasculogenic (Gamal-Elden *et al*., 2009). The polysaccharides isolated from the *Sargassum* species possess medicinal value (Usov *et al*., 1998) and contain unique set of biomolecules that can provide health benefits for the human body (Biesalski, 2009). One of its noted sulfated polysaccharides fucoidan can enhance both innate and adaptive immune response by promoting tumoricidal activities by macrophages, natural killer cells, and T-helper cells (Li *et al*., 2008). Significantly, extracts of Sargassum latifolium inhibited about 60% of induced-DNA damage measured by Comet Assay, possessed anti-inflammatory activity, enhanced macrophage proliferation, inhibited nitric oxide (NO.) and COX-2, and showed selective toxicity against lympho- and blastic-anemia (Gamal-Elden *et al*., 2009). *In vitro* and *in vivo* pro-apoptotic activities of Sargassum sagaminarum on Keratinocytes cell line revealed that topical administration enhanced apoptosis including activation of caspase-3 (Hur *et al*., 2008). *In vitro* examination of the cytotoxic activity of *Sargassum thunbergii* on human promyelocytic leukemia cells

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(HL-60) induced shrinkage and formation of apoptotic bodies (Kim *et al*., 2008). Fractions of *Sargassum swartii* and *C. mystica* showed selective toxicity against proliferation of colorectal colon carcinoma cell (IC_{50}<100 µg/mL), and breast ductal carcinoma (IC_{50}<100 µg/mL) (Khavani *et al*., 2010).

Endemic to the Philippines, the S*argassum siliquosum* J.G. Agardh (Family *Sargassaceae*) is one of the major *Sargassum* species found in a number of places; namely: Batangas, Cagayan, Cavite, Ilocos, La Union, Masbate, Pangasinan, Quezon, Antique, Bohol, Cebu, Misamis Occidental and Zamboanga del Sur. It is locally called in Northern part of Luzon as aragan, bato-bato, or lusay-lusay while in Northern Visayas and Minadanao, it is known as samo (Montano *et al*., 2005). *Sargassum* species is considered as vegetables as a normal ingredient in salad and other local delicacies in the Ilocos region. Their common dish called Inabraw makes use of the boiled young part of brown seaweed plus fish and other vegetables (Martines-Gozz *et al*., 2001; Cordero, 2005; Trono *et al*., 1988). It is used as a health drink in Bohol and as fertilizer in both agricultural and horticultural fields by adding salt-free algae with any plant-potting media (Montano *et al*., 2005).

To date, no study has ever been published on the potential free radical scavenging activity of *S. siliquosum*. Marine organisms are seen as important natural and novel chemopreventive agents toward an inexpensive, non-cytotoxic, environment-friendly, and efficacious modality in cancer management and control. The study dealt on solubility of the marine plant on different organic solvents and the effect of the extracts on free radicals.

Materials and Methods

Fresh samples of *S. siliquosum* were collected from the seashore of Batangas, Philippines in August 2010. The collected specimens were submitted to the University of the Philippines, Diliman, Marine Science Institute for its authentication. Samples of *S. siliquosum* were washed several times with distilled water and were air-dried at 25-30^{O}C for about 3-5 days. The dried samples were ground using Wiley Mill and were sieved in 22 mm mesh size. The powder sample was kept in a clean, dried, well-sealed amber glass container to protect it from sunlight. The ground *S. siliquosum* was extracted using Kupchan's solvent partitioning scheme with some modifications (Sarker *et al*., 2006). Dry and ground sample was soaked in 100% methanol (MeOH) for 48 hours to extract the most polar compounds. The filtrate (crude methanolic fraction) was collected and reserved for further extraction. This procedure was repeated two more times. The residue were further extracted with 100% dichloromethane (CH_2Cl_2) using the same method to separate the non-polar components. Solvents from the residue were removed using rotary evaporator and the crude CH_2Cl_2 extract was partitioned using hexane and 90% MeOH/Water (H_2O) producing the hexane fraction and MeOH-H_2O fraction. The filtrate was partitioned between CH_2Cl_2 and H_2O to produce H_2O fraction and CH_2Cl_2 fraction. The resulting water-soluble compound from H_2O fraction was further separated using n-butanol producing H_2O-butanol fraction and H_2O fraction. A total of five fractions were subjected to different tests to identify the fraction with the highest free radical scavenging activity.

Determination of total phenolic content

Phenol in alkaline medium reacts with phosphomolybdic acid of Folin–Ciocalteau's reagent producing blue color complex (McDonald *et al*., 2001). Total phenolic content in the extract was determined using Folin-Ciocalteu (FC) reagent according to the method of Slinkard and Singleton (1977). Briefly, 1.0 mL of algae extract was diluted with 46 mL of distilled water in a 100-mL volumetric flask. Then, 1.0 mL of FC reagent (previously diluted with 1:10 v/v) was added and the content of the flask was mixed thoroughly. After 3 minutes, 3.0 mL of 2% Na_2CO_3 solution was added to the mixture and was allowed to stand for 2 hours with intermittent shaking. The absorbance was measured at 760 nm using spectophotometer. All tests were performed three times. Gallic acid was used as a standard phenolic compound. The amount of total phenolic compound in the extract was determined as micrograms of gallic acid equivalent (GAE) per milligram dry weight (Shukla, 2009).

Determination of total flavonoid content

The total flavonoid content was estimated using the method described by Koncic´*et al*. (2010) and Adedapo *et al*. (2008) with quercitin as standard. All tests were performed in triplicate. To 0.5 mL of extract solution, 0.5 mL of 2% $AlCl_3$ ethanol solution was added. Absorbance was measured at 420 nm after an hour of incubation at room temperature. Quercitin was used as the standard flavonoid compound prepared at different concentrations and treated in the same manner as the sample solution. The flavonoid content was calculated according to the equation that was obtained from the quercetin standard curve:

$$X = (A_o \times M_o)\ (A_1 \times M_1)$$

where, X is the flavonoid content, mg/mg extract in *Sargassum siliquosum*, A_1 is the absorbance of extract

solution, A_o is the absorption of standard quercitin solution, M_1 is the weight of extract, in mg and M_0 is the weight of quercitin in the solution, in mg.

Hydroxyl radical scavenging

The hydroxyl radical scavenging ability was determined using Fenton reaction. Fenton reaction mixture containing 3 mM deoxyribose, 0.1 mM ferric chloride, 0.1 mM EDTA, and 0.1 mM ascorbic acid and 2 mM H_2O_2 in 20 mM phosphate buffer pH 7.4 was added to various concentrations of extracts (63 µg/mL-1000 µg/mL in 95% ethanol). The reaction mixture was incubated for 30 minutes at 37°C and was added to 0.5 mL of 5% trichloroacetic acid (TCA) and 0.5 mL of 1% thiobarbituric acid (TBA) to yield a final volume 3 mL. The reaction mixture was kept in boiling water bath for 30 minutes and cooled. The absorbance was measured at 532 nm against an appropriate blank solution. All tests were performed three times. Ascorbic acid was used as a positive control. Percent inhibition in hydroxyl radical was calculated by the following expression: Percentage of inhibition = $[(A_o - A_1) / A_o] \times 100$, where A_o is the absorbance of the control and A1 is the absorbance of the sample.

Nitric oxide radical scavenging

The nitric oxide radical scavenging capacity of the fractions was measured by Griess reaction (Sangameswaran *et al*., 2009). Various concentrations of fractions (63 µg/mL-1000 µg/mL in 95% ethanol) were prepared. Sodium nitroprusside (1.5 mL, 10 mM) in phosphate buffer was added to 0.5 mL different concentrations of fractions. The reaction mixture was incubated at 25°C for 150 min. After incubation, 0.5 mL aliquot was removed and 0.5 mL of Griess reagent (1% (w/v) sulfanilamide, 2% (v/v) H_3PO_4 and 0.1% (w/v) naphthylethylene diamine hydrochloride) was added. The absorbance was measured at 546 nm. Ascorbic acid was used as reference standard and was treated the same way as that of fractions. Sodium nitroprusside in PBS (2 mL) was used as control. The nitric oxide radicals scavenging activity of fractions and ascorbic acid was calculated according to the following equation: Percentage of inhibition = $[(A_o - A_1) / A_o] \times 100$, where A_o is the absorbance of sodium nitroprusside in PBS (without fractions and ascorbic) and A_1 is the absorbance in the presence of the fractions and ascorbic acid.

Hydrogen peroxide scavenging

The hydrogen peroxide scavenging ability was determined according to the method described by Ebrahimzade (2010). Different concentrations of extracts and standard ascorbic acid in distilled water was added to 0.6 mL solution of 40 Mm hydrogen peroxide (H_2O_2) in phosphate buffer pH 7.4. After 10 min, absorbance of H_2O_2 was recorded at 230 nm against blank solution without H_2O_2. Percent inhibition in H_2O_2 was calculated by the following expression: Percentage of inhibition = $[(A_o - A_1) / A_o] \times 100$, where A_o is the absorbance of the control and A_1 is the absorbance of the sample.

Thin-Layer Chromatography (TLC)

TLC was performed on a silica gel plate (5 × 20 cm, Kieselgel 60F, 0.25 mm, Merck). An aliquot of DCM fraction was spotted on the silica gel plate with a solvent system of toluene : chloroform (9:11 v/v). The spots were visualized by spraying the plates with spray solution. Spray solution was a 1% solution of potassium ferricyanide in water and a 1% solution of ferric chloride in water. Blue color spots produced indicate that the fractions may be phenolic compounds.

Statistical analysis

Each data point was obtained by making at least 3 independent measurements. TPC and TFC level of significance was assessed using Analysis of variance (ANOVA), Duncan's Multiple Range Test (DMRT) was used for post hoc analysis. These statistical tests used 5% level of significance, with the aid of SPSS 17.0. Inhibitory concentration values were estimated using the four-parameter logistic (4PL) nonlinear regression model.

Results and Discussion

Extraction and yield of extract

Five different solvents were used in this study, since a wide range of extractant holds a better chance for the extraction and isolation of biologically active molecules for general screening of bioactivity (Kumar *et al*., 2008). The average percentage yield of each extracting solvent was based on triplicate analysis of 500-gram samples. Results showed that extraction with Hexane (3.30 ± 0.03) and DCM (3.07 ± 0.02) showed the highest yield. The hexane solvent yielded the highest amount of extract which means that the leaves of *S. siliquosum* contain mostly of lipophilic compounds such as waxes, cholorophyll, and fatty acids.

Total phenolic content (TPC) and total flavonoid content (TFC)

The TPC in *S. siliquosum* were in the range of 5.35±0.11-69.03±3.24 GAE mg/g. Water fraction presented the lowest amount of TPC (5.35±0.11

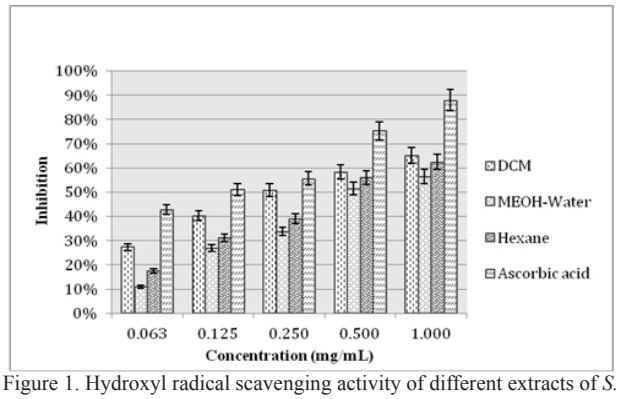

Figure 1. Hydroxyl radical scavenging activity of different extracts of *S. siliquosum* using Ascorbic acid as reference standard

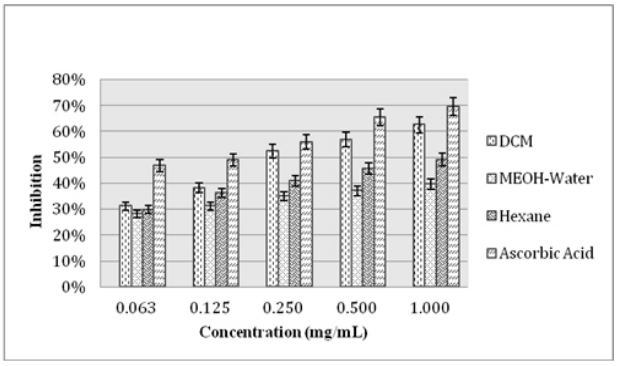

Figure 2. Nitric oxide scavenging activity of different extracts of *S. siliquosum* using Ascorbic acid as reference standard

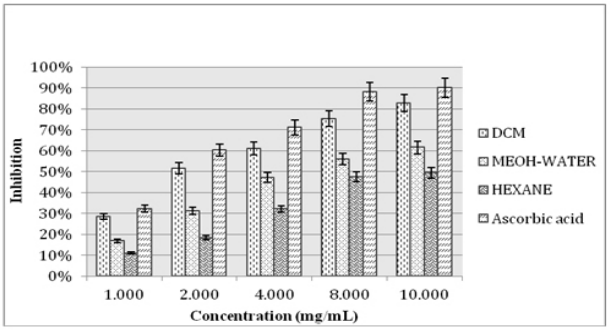

Figure 3. Hydrogen peroxide scavenging activity of different extracts of *S. siliquosum* using Ascorbic acid as reference standard

GAE mg/g) while the highest was observed in DCM fraction with a value of 69.03±3.24 GAE mg/g. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities (Nagavani *et al*., 2010). The high amount of phenols and flavonoids in extracts may explain their high antioxidative activities. TFC of the extracts were also determined using aluminum chloride assay, a widely adopted method in almost all published works to measure the total flavonoid content of plants. The principle of this colorimetric method is based on the acid-stable complex between aluminum chloride, and the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols (Chang *et al*., 2002). The content of total flavonoid (mg/g QE), TFC varied from 5.80±0.43 - 40.43±0.11. The DCM fraction has the highest amounts of TFC (40.43±0.11 mg/g QE), while the lowest amount (5.80±0.43 mg/g QE) was observed in water fraction. In this study, DCM fraction has the highest TPC and TFC, thus can be further tested for its ability to inhibit different free radicals.

Hydroxyl radical scavenging activity

Hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology (Li *et al*., 2008). This radical has a capacity to join nucleotides in DNA and cause strand breakage that contributes to carginogenesis, mutagenesis and cytotoxicity (Moskovitz *et al*., 2002 as cited by Manian *et al*., 2008 and Duan *et al*., 2007). Hydroxyl radical was measured by the deoxyribose method used by Samak (2009). Ferric-ascorbate–EDTA–H_2O_2 (Fenton reaction) generates hydroxyl radical, which react with deoxyribose to produce thiobarbituric acid reactive substances (TBARS) and upon heating with TBA will form pink chromogen. The hydroxyl quenchers reduce TBARS production and formation of pink chromogen by competing with deoxyribose for hydroxyl radicals. Hydroxyl radical scavenging activity of different fractions of *S. siliquosum* is shown in Figure 1. In this study, all fractions exhibited appreciable hydroxyl radical scavenging activity. DCM fraction has the strongest inhibition (65%) against hydroxyl radical at 1000 µg/mL concentration. Extrapolating from Figure 1, the DCM fraction was more effective than other extracts as evidenced by lower IC_{50} values. Hydroxyl radical scavenging activity of the extracts can be ordered as follows: DCM > hexane > methanol: water.

Nitric oxide scavenging activity

Nitric oxide, generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions, which is measured by Griess reaction (Sangameswaran *et al*., 2009). In this study, fractions were added to compete with oxygen leading to reduce production of nitric oxide that can be measured by Greiss reagent. The different fractions showed scavenging activity between 0.63 and 1 mg/mL. The % inhibition was increased with increasing concentration of the extract. The DCM fraction had shown better reducing power than other fractions (Figure 2). However, activity of ascorbic acid was higher than that of other fractions. In addition to reactive oxygen species, nitric oxide is

Table 1. R_f value and color of spots separated on TLC plate from the dichloromethane fraction of *S. siliquosum*

Rf	Visual	UV 254 nm	UV 366 nm	$K_3[Fe(CN)_6]$-$FeCl_3$ spray solution	Quercitin standard
0.52	Yellow	Dark spot	Yellow	Blue spot	
0.69	Light green			Blue spot	Blue spot
0.77	Dark green	Dark spot	Dark green		

also implicated in inflammation, cancer and other pathological conditions (Nabavi *et al*., 2008a,b).

Hydrogen peroxide scavenging activity

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly, and inside the cell, H_2O_2 probably reacts with Fe^{2+}, and possibly Cu^{2+} ions to form hydroxyl radical which may be the origin of many of its toxic effects (Nagavani *et al*., 2010). It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. The scavenging activity of the different fractions of *S. siliquosum* is shown in Figure 3. Ascorbic acid was used as the positive control. It was observed that the maximum scavenging activity (83%) was exhibited by DCM fraction at 10 mg/mL concentration, whereas ascorbic acid, at this concentration, displayed 90% scavenging activity. All fractions can inhibit H_2O_2 but lesser when compared to reference standards ascorbic acid.

Biochemical composition

Preliminary studies of DCM fraction using thin layer chromatography and quantitative analysis has revealed that *S. siliquosum* is enriched with alkaloids, flavonoids, saponins, tannins and phenolic compounds. Table 1 presents the Rf values of the TLC chromatograms of *S. siliquosum* (DCM fraction). A solvent system consisting of toluene: chloroform (9:11v/v) was used to separate the components of the DCM fraction. The migration time lasted for about 20 min. The fraction was shown to have 3 distinct spots with Rf values of 0.52, 0.69 and 0.77 (Table 1). One of the 3 spots produced blue color after spraying with $K_3[Fe(CN)_6]$-$FeCl_3$ spray solution, similar color that was observed with quercitin, the reference standard used. Therefore, on the basis of TLC analysis, one phenolic compound with reducing properties was identified to contain quercitin. The ability of *S. siliquosum* to inhibit free radicals (OH, NO, H_2O_2) may be attributed to the presence of quercitin compound in the extract, a flavonoid naturally present in food and beverages belonging to the large class of phytochemicals with potential antioxidant and anti-cancer properties.

An antioxidant is any substance that retards or prevents deterioration, damage or destruction by oxidation (Bhuiyan *et al*., 2009). These essential chemical substances have the ability to protect humans against detrimental oxidative effects caused by free radicals (Shukla *et al*., 2009) such as superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl (OH^-) and nitric oxide (NO^-) and collectively known as reactive oxygen species. These reactive oxygen species (ROS) are formed as a result of normal metabolic activity and endogenous resources. In pathologic conditions, the antioxidant mechanisms are often inadequate, as excessive quantities of ROS can generate (Halliwell *et al*., 2007). Generation of free radicals or reactive oxygen species (ROS) during metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative stress. Oxidative stress plays a role in heart diseases, neurodegenerative diseases, cancer, and in the aging process. Supplementation of natural antioxidants such as *S. siliquosum* can be used as a remedy to fight with oxidative stress.

Conclusion

The results obtained denote that *S. siliquosum* has a significant free radical scavenging activity against OH, NO and H_2O_2 radicals. Its ability to scavenge free radicals can predict its usefulness as an antioxidant and may constitute a good source of healthy compounds, therefore useful in the prevention of diseases such as cardiovascular, neurodegenerative diseases, diabetes and cancer, in which free radicals are implicated.

Acknowledgements

This research was funded by the Department of Science and Technology – Science Education Institute (DOST-SEI), the Office of Policy, Planning, Research and Information Research Division of the Commission on Higher Education (OPPRI-RD) and the Fund for Assistance to Private Education, Phil.

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