

Antioxidant Activity of Extract and Semi- Purified Fractions of Marine Red Macroalga, *Gracilaria Verrucosa*

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Abstract: The ethanol and its successive extracts of the marine red macroalga, *Gracilaria verrucosa*, were measured for antioxidant activity, using the α,α -diphenyl- β -picrylhydrazyl radical-scavenging assay system, and compared with those of the positive controls of butylated hydroxytoluene (BHT) and Butylated hydroxyanisole (BHA). The crude ethanolic extract was further fractionated to afford four fractions (PE, EA, BuOH and Water fractions), of which the pet.ether (PE) and ethyl acetate-soluble (EA) fraction exhibited the strongest antioxidant activity in the assay system. The EA fraction was further separated into eleven subfractions, designated as EA1–EA11, by silica gel column chromatography. In most cases, EA3 and EA4 were found to possess the strongest antioxidant activity. The total phenolic contents and reducing powers of the extract, fractions, and subfractions were also determined. Significant associations between the antioxidant potency and the total phenolic content, as well as between the antioxidant potency and the reducing power, were found for the tested fractions and subfractions.

Key words: Antioxidant activity; DPPH; *Gracilaria verrucosa*; Phenolic compounds; Reducing power, Seaweeds

INTRODUCTION

Fresh seaweeds, both wild and cultivated, have long been used in food diets, as well as traditional remedies in Asian countries, (China, Japan and Korea). It has been reported that seaweeds serve as an important source of bioactive natural substances (Smit, 2004). Many metabolites isolated from marine algae have been shown to possess bioactive effects (Faulkner, 2002). In fact, the discovery of metabolites with biological activities, from macroalgae, has increased significantly in the past three decades (Smit, 2004). On the other hand, seaweeds have recently received significant attention for their potential as natural antioxidants (Chandini *et al.*, 2008).

There has been interest in the contribution of free radicals reaction participating in reactive oxygen species to the overall metabolic perturbation that result in tissue injury and disease. Reactive oxygen species such as superoxide anion, hydrogen peroxide, and hydroxyl radical are generated in specific organelles of cells (Mitochondria and Microsomes) under normal physiological condition. These reactive oxygen species can damage DNA, so as to cause mutation and chromosomal damage, oxidize cellular thiols and abstract hydrogen atoms from unsaturated fatty acids to initiate the peroxidation of membrane lipids (Halliwell and Gutteridge, 1985; Ames *et al.*, 1993). Recently, various phytochemicals and their effect on health, especially the suppression of active oxygen species by natural antioxidant from tea, spices and herbs, have been intensively studied (Ho *et al.*, 1994; Elmastas *et al.*, 2006). Phenolic compounds play an important role in the oxidative properties of many plant-derived antioxidants (Canadanovic –Brunet *et al.*, 2006). Phenolic substances were also reported to possess a wide range of biological effects, including antioxidant, antimicrobial, anti-inflammatory and anticancer (Cole *et al.*, 2005; Fraga 2007; Fusco *et al.*, 2007; Stevenson and Hurst, 2007).

The most commonly used antioxidant at the present time is butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propylgallate (PG), and *tert*butylhydroxytoluene (TBHQ) (Sherwin, 1990). However, they are suspected of being responsible for liver damage and carcinogenesis in laboratory animals (Wichi, 1988; Grice, 1986). Therefore, the development and utilization of more effective antioxidant of natural origin are desired (Oktay *et al.*, 2003).

Egypt is endowed naturally with a very rich algae life such as the *Gracilaria verrucosa*. There are documentations about the use of these alga as a food source as indicated by Yan *et al.* (1999). They mentioned

that a single solvent extraction may not be enough to exhaustively extract certain compounds responsible for the activity. The objective of the present study was to investigate and evaluate antioxidant activities of the semipolar extract, fractions and subfractions of *Gracilaria verrucosa* and compared to two synthetic antioxidants, butylated hydroxyl toluene (BHT) and butylated hydroxyanisole (BHA). In addition, we also assessed the reducing power of fractions and subfractions of *Gracilaria verrucosa*. Furthermore, the correlation between antioxidant activity and phenolic content, as well as between the antioxidant activity and reducing power was also considered

MATERIALS AND METHODS

Chemicals:

α,α -diphenyl- β -picrylhydrazyl radical (DPPH), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and Folin-Ciocalteu reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Gallic acid (GA) was purchased from Shanghai Chemical Reagents Co. (Shanghai, China) and solvents, such as methanol, chloroform, ethyl acetate and n-butanol were purchased from common sources. All chemicals used were of the highest analytical grade.

Marine Alga Materials:

Fresh *G. verrucosa* (8-15 cm) was collected from Marsa Matrouh governorate sea area Egypt in spring season 2009. (The chemical and physical parameters of seawater were analyzed as reported by APHA (1998) and present in Table1). Authenticated by Dr. Fathy M. Soliman, Professor of Phycology, Pharmacognosy Department, Faculty of Pharmacy, Cairo University and Prof. Dr. Mahmoud H. M. Abdel-Rahman, Botany Department, Faculty of Science, El-Fayoum University. The fresh algae sample was rinsed with seawater to remove debris and epiphyte then washed in fresh water and dried far away from sunlight at room temperature, then finally milled and preserved in quick fit amber-colored glass bottles.

Preparation of Marine Algal Extract, Fractions, and Subfractions:

The dried *Gracilaria verrucosa* (115g) was ground into powder and extracted with 500 ml ethanol 70%. The extraction was repeated twice and the total extracts were filtered, and the obtained filtrates were concentrated under using rotary evaporator to dryness, the crude extract yields (6.5g). The crude extract was partitioned with 3x100 ml petroleum ether then successively partitioned with 3x100 ml of ethyl acetate, 3x100 ml of n- butanol, and 3x100 ml water respectively. The resulting four extracts were evaporated to dryness in vacuum, yielding petroleum ether (PE,1.1g) ethyl acetate (EA, 2.38g) n- butanol (0.8g) and water extract (0.85g), respectively. Since the EA fraction exhibited the highest activity in the DPPH assay, this fraction was further applied to a silica gel (200-300 mesh) column chromatographic separation, using step-gradient elution from chloroform/methanol 90: 10 to methanol 100% each of 300ml, to yield eleven fractions (EA1-EA11). Solvent in each subfraction was evaporated to dryness under vacuum and each subfraction stored at -20 °C until used.

Measurement of DPPH free-radical Scavenging Activity:

Quantitative measurement of radical scavenging properties was determined by the method of Blois, (1958). The reaction mixture contained 50 μ l of test samples (or 80% MeOH as a blank) and 5 ml of a 0.04% (w/v) solution of DPPH in methanol. Different standard antioxidants butylated hydroxytoluene (BHT,) and Butylated hydroxyanisole (BHA) were used for comparison or as a positive control. Discoloration was measured at 517 nm after incubation for 30 and 60 min. Measurements were performed at least in triplicate. The actual decrease in absorption induced by the test compounds was compared with the positive controls and IC50 value was calculated using the dose inhibition curve.

Determination of Reducing Power:

The reducing power of all samples was determined as described by Dorman *et al.*, (2003). 200ppm of fractions and sub-fractions were added to 1ml of distilled water and mixed with phosphate buffer (2.5 ml, 0.2 mol/ L, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50 °C for 20 min. A portion (2.5 ml) of trichloroacetic acid (TCA, 10%) was added to the mixture, which was then centrifuged at 3000 rpm (MSE Mistral 2000, UK) for 10 min. The reaction was initiated by the addition of 5 mmol/L ferrozine (0.2 ml) and the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The value is expressed as gallic acid equivalent (GAE ug/g seaweed dry weight).

Measurement of Total Phenolic Contents:

The amount of total phenolics of the fractions and subfractions were determined with the Folin-Ciocalteu reagent using the method of (Singleton *et al.*, 1999). To 50 ml of each sample (three replicates), 2.5 ml 1/10 dilution of Folin-Ciocalteu's reagent and 2 ml of Na₂CO₃ (7.5%, w/v) were added and incubated at 45 °C for 15 min. The absorbance of all samples was measured at 765 nm using a UV-vis spectrophotometer (GAT UV-9100). Results were expressed as micrograms of gallic acid.

Statistical Analysis:

The direction and magnitude of correlation between variables were done using analysis of variance (ANOVA) and quantified by the correlation. The P-values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION**Effect of Fractions and Subfractions on the DPPH Radical Scavenging Activity:**

In the present work, the DPPH radical scavenging assay was used for evaluation of the antioxidant activity of the crude extract, fractions and subfractions derived from *Gracilaria verrucosa*. This reaction has been widely used to investigate the ability of algal extracts and fractions and /or pure compounds of those, to act as free radical scavengers or hydrogen donors.

The values of percent DPPH scavenging activities of *Gracilaria verrucosa* crude extract and four fractions were summarized in Table (2) as comparable with known antioxidant such as BHT and BHA. In most cases, as shown in Table (2), the highest percent DPPH radical scavenging activity was observed in PE fraction and EA fraction, followed by crude extract, n-butanol and aqueous residue showed lower scavenging activity. This results show that compounds with the strongest radical scavenging activity in *Gracilaria verrucosa* are of medium polarity. At all concentrations tested, the all fractions exhibited dose and time-dependent to DPPH radical scavenging activity. Detailed analysis for the values listed in Table (2) show that at the tested concentration, the PE fraction has the lower antioxidant activity than the BHT and BHA. At higher concentration (200µg/g) the PE and EA fractions gave the highest antioxidant activity (89% and 85%, respectively). These results suggested that PE and EA fractions are good scavengers for DPPH radical. Fig. (1) shows the IC₅₀ value for the four fractions isolated from *Gracilaria verrucosa*. From this figure it was observed that the crude extract has a lowest IC₅₀ (85µg/ml) followed by PE and EA fractions (130 and 135µg/ml, respectively) but water extract and n-butanol gave the highest IC₅₀ (180 and 190 µg/ml, respectively).

Table (3) shows the DPPH radical scavenging activities of eleven subfractions (EA1-EA11) isolated from the EA fraction by silica column chromatography. As indicated, all of the eleven fractions (EA1-EA11) were a time-dependent DPPH - scavenging activity. EA3 and EA4 subfractions revealed similar and comparable activity to those BHT and BHA. In addition, the subfractions showed higher activity than the total EA fraction itself.

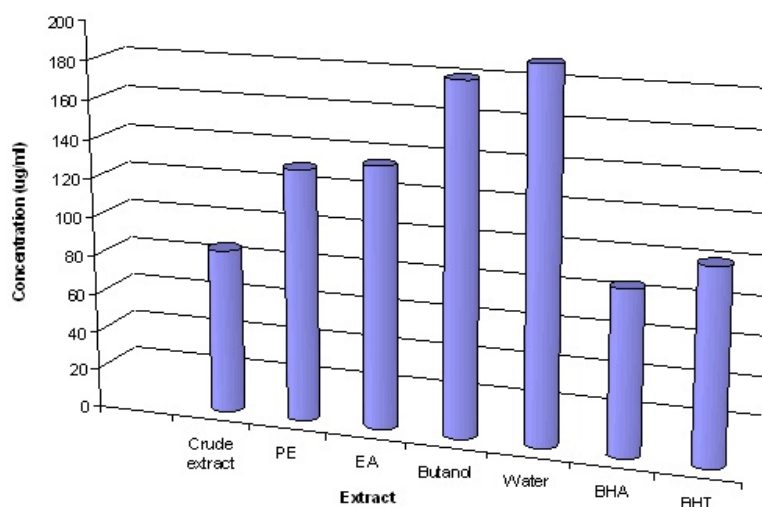


Fig. 1: IC₅₀ values of crude extract and its fractions of *G. verrucosa* against DPPH radical.

Table 1: Physico-chemical properties of sea water collected from Marsa Matrouh beach.

Parameters	Unit	El-Garam beach
pH		6.3
Turbidity	NTU	1.0
Electric conductivity	µmhose/cm	42300
Salinity	%	28
Total alkalinity (as CaCO ₃)	mg/l	146
Total hardness (as CaCO ₃)	mg/l	8000
Calcium hardness (as CaCO ₃)	mg/l	1360
Magnesium hardness (asCaCO ₃)	mg/l	6640
Chloride	mg/l	24000
Sulfate	mg/l	2812
Dissolved silica	mg/l	7.3
Nitrite	mg/l	0.0
Nitrate	mg/l	1.9
Total phosphorus	mg/l	1.4
Iron	mg/l	1.2
Manganese	mg/l	1.8
Calcium	mg/l	544
Magnesium	mg/l	1592
Sodium	mg/l	15549
Potassium	mg/l	5450

Table 2: DPPH radical-scavenging activities of *G. verrucosa* extract.

Fractions	Antioxidant scavenging activity (%)					
	100 µg/ml		150 µg/ml		200 µg/ml	
	30min	60min	30min	60min	30min	60min
Crude extract	53 ^c	63 ^d	66 ^{cd}	71 ^{bc}	75 ^{ab}	78 ^a
Petroleum ether	10 ^f	35 ^d	26 ^c	57 ^c	69 ^b	89 ^a
Ethyl acetate	12 ^f	38 ^d	15 ^c	53 ^c	64 ^b	85 ^a
n-butanol	3 ^c	17 ^d	5 ^c	32 ^c	53 ^b	64 ^a
Water	4 ^d	24 ^c	4 ^d	29 ^c	47 ^b	55 ^a
BHA	48 ^b	78 ^a	57 ^b	84 ^a	89 ^a	90 ^a
BHT	50 ^b	78 ^a	48 ^b	78 ^a	88 ^a	90 ^a

Each value is presented as mean of triplet treatments, means within each row with different letters (a-f) differ significantly at $P \leq 0.05$ according to Duncan's multiple range test, LSD =10

Reducing Power:

Several methods have been developed to measure the efficiency of antioxidants as pure compounds or in extract. These methods focus on different mechanisms of the oxidant defense system that is scavenging active oxygen species and hydroxyle radicals, reducing of lipid peroxy radicals, inhibiting of lipid peroxidation, or chelating of metal ions (Dorman *et al.*, 2003). The reducing power of different extracts, fractions and sub fractions of *Gracilaria* sp (to reduce ferric ions) was determined in this study. As observed in Table (4), amongst the four fractions, the highest amount of reducing power was observed in the PE fraction (212 µg/g GA) followed by EA fraction (163 µg/g GA). These results also indicated that compounds with strongest reducing power were concentrated in PE and EA fractions and were of medium polarity. Interestingly, the PE and EA fractions also exhibited the highest antioxidant activity in DPPH assay, suggesting the relationship between antioxidant activities and reducing power. Amongst the eleven subfractions (EA1-EA11), the highest reducing power was found in EA3 (815µg/g GA) and EA4 (419 µg/g GA), followed by EA5, EA2 and EA9 fractions. The EA1 and EA11 subfractions gave the lowest antioxidant activity, (Table 5). Interestingly, the EA3 and EA4 fractions also exhibited the highest antioxidant activity in DPPH assay. A relationship between antioxidant, reducing power and phenolic compounds was recently studied in extract and semi-purified fractions of the marine red alga, *Rhodomela confervoides* (Rhodomelaceae) (Wang *et al.*, 2009). This study agreed with that obtained in the present work.

Measurement of Total Phenolic Content:

The phenolic content of the *G. verrucosa* was determined using Folin-Ciocalteu reagent and was expressed as gallic acid equivalents (GAE). Typical phenolics that possess antioxidant activity have been characterized as phenolic acids and flavonoids (Kahkonen *et al.*, 1999). Phenolic acids have repeatedly been implicated as natural antioxidants in fruits, vegetables, and other plants. For example, caffeic acid, ferulic acid, and vanillic acid are widely distributed in the plant kingdom (Larson 1988, Canadanovic –Brunet *et al.*, 2006).

Table 3: DPPH radical-scavenging activities of *G. verrucosa* EA- subfractions.

Sub-Fractions	Antioxidant scavenging activity (%)	
	200 µg/ml	
	30min	60min
Crude EA fraction	64 ^b	85 ^a
EA1	16 ^f	19 ^f
EA2	16 ^f	27 ^c
EA3	89 ^a	90 ^a
EA4	90 ^a	91 ^a
EA5	27 ^d	35 ^d
EA6	11 ^s	55 ^b
EA7	17 ^f	27 ^c
EA8	22 ^c	32 ^d
EA9	34 ^c	43 ^c
EA10	34 ^c	43 ^c
EA11	34 ^c	43 ^c
BHA	89 ^a	90 ^a
BHT	88 ^a	90 ^a

Each value is presented as mean of triplet treatments, means within each column with different letters (a-g) differ significantly at $P \leq 0.05$ according to Duncan's multiple range test. LSD=10

Table 4: reducing powers of *G. verrucosa* fractions.

Fractions (200µg/ml)	(GAE µg/g)
PE	212 ^a
EA	163 ^b
n-butanol	31 ^d
Water	105 ^c

Reducing power is expressed as gallic acid equivalents (GAE µg/g seaweed dry weight)

Each value is presented as mean of triplet treatments, means with different letters (a-g) differ significantly at $P \leq 0.05$ according to Duncan's multiple range test. LSD = 13

Table 5: reducing powers of *G. verrucosa* EA- subfraction.

Subfractions (200 µg/ml)	(GAE µg/g)
EA1	145 ^c
EA2	218 ^c
EA3	815 ^a
EA4	419 ^b
EA5	225 ^c
EA6	188 ^d
EA7	171 ^d
EA8	195 ^d
EA9	214 ^c
EA10	142 ^c
EA11	164 ^c

Reducing power is expressed as gallic acid equivalents (GAE µg/g seaweed dry weight). Each Value is presented as mean of triplet treatments, means with different letters (a-e) differ significantly at $P \leq 0.05$ according to Duncan's multiple range test. LSD = 15

As determined (Table 6), the PE and EA fractions of *G. verrucosa* were rich in phenolic compounds, with 33.0 and 31.0 µg of gallic acid equivalents (GAE) per gram of dried seaweed. The lowest amount of phenolic compounds was found in water extract, which only presented as 9.5 µg/g GAE (Table 6).

From Table (7) it was observed that from the eleven subfractions (EA1-EA11), the highest phenolic compounds were found in EA3 fraction (58.3µg/g of GAE) and EA4 (37.5µg/g GAE), followed by EA5, EA2 and EA9, which were 16, 15.5 and 15.4µg/g GAE, respectively. An EA6 and EA8 fractions have the lowest values (Table 7). Interestingly, the EA3 and EA4 subfractions also exhibited the highest antioxidant activity in DPPH assay and highest reducing power, suggesting that there may be relationship between antioxidant activity, reducing power and phenolic compounds. These results are similar to that of Duan *et al.*, (2006) who found a significant correlation between the total phenolic content and the antioxidant activity in the EA fraction and its subfractions obtained from a red alga, *Polysiphonia urceolata*. Also the same results are obtained by Sreenivasan *et al.* (2007) who evaluated the antioxidant activity and phenolic content of *Gracilaria changii*. These results with that of the present study suggest that phenolic compounds may be responsible for the antioxidant activity of *G. verrucosa*.

Table 6: Total phenolic contents of *G. verrucosa* extract fractions.

Subfractions (200 µg/ml)	(GAE µg/g)
PE	33 ^a
EA	31 ^a
n-butanol	28 ^a
Water	9.5 ^b

Total phenolic content is expressed as gallic acid equivalents (GAE µg/g seaweed dry weight). Each value is presented as mean of triplet treatments, means with different letters (a-b) differ significantly at $P \leq 0.05$ according to Duncan's multiple range test. LSD = 5

Table 7: Total phenolic contents of *G. verrucosa* EA- subfractions.

Subfractions (200 µg/ml)	(GAE µg/g)
EA1	1.9 ^c
EA2	15.5 ^c
EA3	37.5 ^b
EA4	58.3 ^a
EA5	16.4 ^c
EA6	0.93 ^c
EA7	11.3 ^d
EA8	1.2 ^c
EA9	15.4 ^c
EA10	1.6 ^c
EA11	11.8 ^d

Total phenolic content is expressed as gallic acid equivalents (GAE µg/g seaweed dry weight). Each value is presented as mean of triplet treatments, means with different letters (a-b) differ significantly at $P \leq 0.05$ according to Duncan's multiple range test. LSD = 1.1

Conclusion:

The results obtained in the present study clearly demonstrate that the EA and PE fractions derived from *Gracilaria verrucosa*, are fairly active fractions for *in vitro* DPPH free radical scavenging activity. Also the subfractions EA3 –EA4 showed high scavenging activity and are concentration and time-dependent manner. The inhibitory characteristics of the extract, fractions and subfractions were determined in multiple ways, including the measurement of reducing power and phenolic content. In addition, the results suggest that phenolic compounds might be major contributors to the antioxidative activities of *Gracilaria verrucosa*. Our data may contribute to a rational basis for the use of marine algal extract in possible therapy of diseases related to oxidative stress, or have potential for developing phenolic –rich extracts and fractions. Our results also indicated that inclusion of antioxidant-rich extract or fractions of seaweeds as a dietary supplementary has beneficial effects for human health. The findings of the current work appear useful for further research aiming to isolate and identify the specific phenolic compounds responsible for the antioxidant activity of *Gracilaria verrucosa*.

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