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Original Research Article

Phytochemical Screening, Antioxidant Activity and Cytotoxicity of Methanolic Extracts of Selected Red Sea Macroalgae Exhibited Antimicrobial Activities

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Abstract

Objective: This study was carried out to detect presence of some secondary metabolites that may have antimicrobial activity and to evaluate the antioxidant activity and cytotoxicity of methanol extracts of six marine macroalgae belonging to green algae (Chlorophyceae), brown algae (Phaeophyceae) and the red algae (Rhodophyceae) collected from the intertidal area of the Sudanese Red Sea coast near Port Sudan. ***Methods:*** Preliminary phytochemical analysis, the DPPH radical scavenging and cytotoxicity screening were carried out in methanol extracts of the six macroalgae as per standard methods with few modifications. ***Results:*** Analysis revealed that secondary metabolites with higher medicinal activities such as saponin, cumarin, flavonoids, alkaloids, tannins triterpens and steroids were present. Anthraquinones, and cyanogenic glycosides were absent in all algal extracts. Most of samples were inactive in DPPH radical scavenging and has no cytotoxic effects. ***Conclusion:*** The study demonstrated clearly that macroalgae of the Red Sea can be significant sources of important compounds which may be used in formulation of drugs by the pharmaceutical industries.
Keywords: Macroalgae, Phytochemical Screening, Antioxidant activity, Cytotoxicity, Methanol extracts, Red Sea, Sudan.

INTRODUCTION

Algae are a group of marine organisms that live in aquatic environment [1]. They are considered as ecologically and biologically important components of the marine ecosystems. Marine macroalgae are the renewable living resources which are also used as food, feed, and fertilizer in many parts of the world [2]. Seaweeds are commonly used as sushi wrappings, seasonings, condiments and vegetables and can thus constitute between 10% and 25% of food intake by most Japanese [3, 4]. Seaweeds have been reported to contain secondary metabolites which include alkaloids, cyanogenic glycosides, flavonoids, saponins, tannins, steroids, and related active metabolites, most of which are phenolic compounds, which have medicinal potentials and have been extensively used in drugs and pharmaceutical industry [5]. Recently, researchers have proved that compounds originating from marine algae exhibit various biological activities [6]. Compounds with antibacterial, antiviral, antifungal and antioxidant activities have been detected in green, brown and red algae [7]. Reactive oxygen species such as hydroxyl, superoxide and peroxyl radicals are formed in cells resulted in extensive oxidative damage that in turn leads to geriatric degenerative conditions, cancer and a wide range of other human diseases [8]. Therefore, consumption of antioxidant and/or addition of antioxidant in food materials protect the body as well as foods against these events [9].Therefore, there is a new trend to isolate novel bioactive compounds and constituents from edible seaweeds [10].

Phytochemical screening, antioxidant activity and cytotoxicity screening of seaweeds can help the manufacturers in the identification and selection of raw materials for drug production. Therefore, in the present study, methanol extracts of six marine macroalgae belonging to *Chlorophyceae* (*Enteromorpha compressa* and *Caulerpa racemosa*), *Phaeophyceae* (*Cystoseira myrica* and *Sargassum* sp) and *Rhodophyceae* (*Gracilaria* sp and *Laurencia papillosa*) collected from the intertidal area of the Sudanese Red Sea coast near Port Sudan and exhibited antimicrobial activities [11, 12], were screened for their chemical compounds of medicinal importance and some other biological activities.

MATERIALS AND METHODS

Collection of Macroalgae

Marine macroalgae were collected by hand picking from the shallow intertidal waters of the Sudanese Red Sea coast near Port Sudan harbor during the period from June 2013 to June 2014. In the field, samples were washed thoroughly with seawater then transported to the laboratory as soon as possible and kept away from direct sunlight during transportation. In the laboratory epiphytic and extraneous matters were removed by washing samples with fresh water. Samples were authenticated and herbarium specimens were deposited at the Red Sea University then the samples were shade dried, cut into small pieces and powdered in a mixer grinder.

Preparation of Methanolic Algal Extracts

Fifty grams of the finely ground samples were weighed and mixed with 500 ml of 80% methanol (1:10, w/v). The mixtures were kept for two weeks at room temperature and mixed at regular intervals. After two weeks the mixtures were filtered with Whatman filter paper No. 1. The filtrate (Crude extracts) was freed from solvent by evaporation at room temperature.

Phytochemical Screening

Phytochemical screening was carried out on each methanolic extract of the algal samples using the methods described by [13-17] with few modifications. Phytochemical screening tests were done as follow:

Test for Saponins

Three hundred milligrams of each extract were placed in a clean test tube. 10 ml of distilled water were added, the tube was stoppered and vigorously shaken for about 30 seconds. The tube was then allowed to stand and observed for the formation of foam, which when persisted for at least an hour, was taken as evidence for presence of saponins.

Test for Cumarins

Two hundred milligrams of each extract were dissolved in 10 ml distilled water in test tube. A filter paper with a spot of 0.5N KOH put on it was attached to the test tube to be saturated with the vapor. The filter paper was then inspected under UV light; the presence of cumrins was indicated if the spot adsorbed the UV light.

Test for Alkaloids

Five hundred milligrams of each extract were heated with 5 ml of 2N HCl in water bath and stirred for about 10 minutes, cooled filtered and divided into two test tubes. To one test tube few drops of Mayer's reagent were added while to the other tube few drops of Valser's reagent were added. A slight turbidity or heavy precipitate in either of the two test tubes was taken as a presumptive evidence for the presence of alkaloids.

Tests for Flavonoids

Five hundred milligrams of each extract were washed with hexane then dissolved in 30 ml of 80% ethanol. The filtrate was used for the following tests:

A/ to 3 ml of the filtrate in a test tube, 1ml of 1% aluminum chloride solution in methanol was added. Formation of a yellow color indicated the presence of flavonoids; flavones and/or chalcone.

B/ to 2 ml of the filtrate 0.5ml of magnesium turnings were added. Production of defiant color to pink or red was taken as a presumptive evidence that flavonenes were present in the algal sample.

Identification of Tannins

Five hundred milligrams of teach extract were dissolved in 10 ml hot saline solution and divided in two tests tubes; to one tube 2-3 drops of ferric chloride were added and to the other one, 2-3 drops of gelatin salts reagent were added. The occurrence of a blackish blue color in the first test tube and a turbidity in the second denoted the presence of tannins.

Test of Sterols and Triterpenes

Five hundred milligrams of each extract were washed with hexane and dissolved in 10 ml of chloroform. To 5 ml of the solution, 0.5 ml acetic anhydride was added and then 3 drops of concentrated sulphuric acid at the bottom of the test tube. At the contact zone of the two liquids a gradual appearance of green, blue pink to purple color was taken as an evidence of the presence of sterols (green to blue) and or triterpenes (pink to purple) in the sample.

Test for Anthraquinone glycoside

Two hundred milligrams of each extract was boiled with 10 ml of 0.5N KOH containing 1ml of 3% hydrogen peroxide solution. The mixture was extracted by shaking with 10 ml of benzene. 5ml of the benzene solution was shaken with 3ml of 10% ammonium hydroxide solution and the two layers were allowed to separate. The presence of anthraquinones was indicated if the alkaline layer was found to have pink or red color.

Test for Cyanogenic Glycoside

Five hundred milligrams of each extract was placed in an Erlenmeyer flask and sufficient water was added to moisten the sample, followed by 1ml of chloroform (to enhance activity). A piece of freshly prepared sodium picrate paper was carefully inserted between a split cork which was used to stopper the flask. A change in color of the sodium picrate paper from yellow to various shades of red was taken as an indication of the presence of cyanogenic glycoside.

Antioxidant Activity

The DPPH radical scavenging was determined according to the method of [18] with some modifications. In 96-well plate, the test samples were

allowed to react with 2.2Di (4-tert-octylphenyl)-1-picryl-hydrazyl stable free radical (DPPH) for half an hour at 37°C. The concentration of DPPH was kept as (300µM). The test samples were dissolved in DMSO while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517nm using multiplate reader spectrophotometer. Percentage radical scavenging activity by samples was determined in comparison with a DMSO treated control group. All tests and analysis were run in triplicate.

Cytotoxicity Screening

Micro-culture-tetrazolium MTT-assay was utilized to evaluate the cytotoxicity of algal extracts. This colorimetric assay is based on the capacity of mitochondria-succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) into an insoluble, blue colored-formazan, a product measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells [19].

Cell Line and Culture Medium

L20B (Normal cell line) cells were cultured in a culturing flask containing a complete medium consisting of 10% fetal bovine serum and 90% minimal essential medium (MEM) and then incubated at 37^{o}C. The cells were sub cultured twice a week.

Cell Counting

Cells were counted using the improved Neubauer chamber. The cover slip and chamber were cleaned with detergent, rinsed thoroughly with distilled water and swapped with 70% ethanol, then dried. An aliquot of cell suspension was mixed with equal volume of 0.4% trypan blue in a small tube. The chamber was charged with cell suspension. After cells had settled, the chamber was placed under light microscope. Using 40 X objective, cells in the 4 large corner squares (each containing 16 small squares) were counted. The following formula was used for calculating the cells:

$$(\text{Cells/ml})\ N = \frac{\text{Number of cells counted X Dilution factor X } 10^4}{4}$$

MTT assay

Serial dilutions of extract were prepared in a 96 well flat bottomed plate (Nalge Nunc, Inter.). The outer wells of the plate were filled with 250 µl of in-complete culture medium except the last row 6 middle wells (B - G), which were used for the negative control receiving 50 µl of culture medium and 2µl of sterile 0.5% Triton X. 50 µl/wells complete culture medium (CCM) were added and 30 µl more were added to second column wells (B – G) that were used as first extract dilution wells. To the first dilution wells in the row, 500 µg of c suspension extract were added to the 80 µl. extract were then serially diluted by two-fold dilution from well B3 till B11 by transferring 250 µl to the next well after proper mixing. From the last dilution wells (B-11), 50 µl were discarded. Each compound was tested in triplicate. Cell suspension in a complete culture medium containing 2.5 X 10^5 /ml was properly mixed, and 150 µl of it were transferred into each well of the plate. The plate was covered and placed in 5% CO_2 incubator at 37 °C for three-five days (72-120 hours). On the third/fifth day, the supernatant was removed from each well without detaching cells. MTT ((3- (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) stock (5 mg/ml) was prepared earlier in 100 ml PBS (phosphate buffer saline). MTT suspension was vortexed and kept on magnetic stirrer until all MTT dissolved. The clear suspension was filter sterilized with 0.2 µ Millipore filter and stored at 4 or – 20°C until use. MTT was diluted (1:3:5) in a culture medium and brought to room temperature. To each well of the 96 well plates, 50 µl of diluted MTT were added. The plate was incubated further at 37° C for 2 to 3 hours in CO_2 incubator. MTT was removed carefully without detaching cells, and 200 µl of DMSO were added to each well. The plate was agitated at room temperature for 15 minutes then read at 540 nm using micro plate reader. The percentage growth inhibition was calculated using the formula below:

$$\% \text{ cell inhibition} = 100-\{(Ac-At)/Ac\} \times 100$$

Where,
At = Absorbance value of test compound
Ac =Absorbance value of control.

Statistical Analysis

All data were presented as means ± S.D. Statistical analysis for all the assays results were done using Microsoft Excel program (2016).

RESULTS

Phytochemical Screening

In the present study, qualitative and quantitative phytochemical screening was carried out with methanolic extracts of the selected marine macroalgal species. The results were shown in Table-1 and reveal that secondary metabolites with higher medicinal activities such as saponin, flavonoids and triterpens were present in high quantities while cumarin, alkaloids, tannins and steroids were either present in low quantities or absent. Anthraquinones, and cyanogenic glycosides were absent in all algal extracts

Table-1: Phytochemical Screening of Methanolic Extracts of Selected Red Sea Macroalgae exhibited Antimicrobial Activities

No.	Algal species	Phytochemical compounds								
		saponin	cumarin	alkaloids	flavonoids	Tannins	steroids	Triterpens	Anthraquinone	Cyanogenic glycosides
1	*Enteromorpha compressa*	+++	+	+	+++	+	+	+++	–	–
2	*Caulerpa racemosa*	+	+	–	+++	–	–	+++	–	–
3	*Cystoseira myrica*	++	+	–	+++	–	+	++	–	–
4	*Sargassum* sp	++	+	–	++++	–	–	+++	–	–
5	*Gracilaria* sp	+	+	–	+++	+	–	+++	–	–
6	*Laurencia papillosa*	+	+	–	+++	–	++	+++	–	–

Key: +++ High ++ Moderate + Trace – Negative

Antioxidant Activity

Free radical scavenging ability of methanol extracts of the selected macroalgae were expressed in percentage (%) and shown in Table-2. Maximum activity was found in *Laurencia papillosa* (6.7±0.37) and less activity was (0.2±0.15) in *Gracillaria* sp while the rest of samples were inactive.

Table-2: Radical scavenging activity (%) of methanolic extracts of selected Red Sea macroalgae with antimicrobial activities

NO.	Algal extracts samples and control	%RSA±SD(DPPH)
1	*Enteromorpha compressa*	Inactive
2	*Caulerpa racemosa*	Inactive
3	*Cystoseira myrica*	Inactive
4	*Sargassum* sp	Inactive
5	*Gracilaria* sp	0.2±0.15
6	*Laurencia papillosa*	6.7±0.37
7	Propyl Gallate*	90.2±0.01

*standard

Cytotoxicity screening

The effect of methanol extracts of the selected macroalgae on the viability of the normal cells was measured and expressed as percentage growth inhibition of the cells and shown in Table-3. All samples had no cytotoxic effects on normal Vero cells.

Table-3: Cytotoxicity screening of algal extracts on normal cell lines (Vero cell line) as measured by the MTT assay:

NO.	Algal species	Concentration (µg/ml) Inhibition (%) ± SD			IC_{50} (µg/ml)	IC_{50}
		500	250	125		
1	*Enteromorpha compressa*	72.28 ± 0.05	67.71 ± 0.07	46.86 ± 0.01	138.69	> 100
2	*Caulerpa racemosa*	51.14 ± 0.03	46 ± 0.03	36 ± 0.07	411.09	> 100
3	*Cystoseira myrica*	61.14 ± 0.07	53.71 ± 0.04	46.57 ± 0.05	174.00	> 100
4	*Sargassum* sp	70.28 ± 0.05	37.14 ± 0.07	31.43 ± 0.08	280.09	> 100
5	*Gracilaria* sp	67.71 ± 0.01	64 ± 0.05	44.57 ± 0.09	158.62	> 100
6	*Laurencia papillosa*	65.43 ± 0.03	54 ± 0.03	51.71± 0.02	134.42	> 100
7	*Control	96.28± 0.01				< 30

Key: IC_{50}< 30 µg/ml: high toxic, > 100 µg/ml: no toxic

*Control = Triton-x100 was used as the control positive at 0.2 µg/mL.

DISCUSSION

The phytochemical screening of the methanol extracts of the six selected marine macroalgal species showed the presence of the following secondary metabolites: saponin, cumarin, flavonoids, alkaloids, tannins triterpens and steroids which have been reported to be of great medicinal values [20]. These secondary metabolites have been extensively used in the preparation of drugs and in medicinal industry. They are also important for the survival of algae in their environment as they regulate the algal growth, inhibit or kill many bacterial strains, inhibit major viral enzymes and destroy some pathogenic protozoans [21].

Many studies demonstrated the presence of these compounds in algal extracts. Kavitha and Palani [22] in a qualitative phytochemical analysis of methanol, ethyl acetate and hexane extracts of *Chlorococcum humicola* revealed that methanol extract had better bioactivity. Flavonoids and carbohydrates were present in all the three extracts. Alkaloids and saponins were present only in ethanol extract. Terpenoids, anthraquinones, phenols and tannins were absent in all three extracts of *C. humicola*. Steroids were present only in the ethanol and ethyl acetate extracts while oils and resins were present only in the ethyl acetate and hexane extracts. From the study, it was observed that the alga *C. humicola* possesses medicinally important phytochemicals such as flavonoids, alkaloids and steroids.

Shankhadarwar [23] after a preliminary phytochemical screening found flavonoids, alkaloids, steroids, sapnonins and phenols in *Ulva lacuta and Enteromorpha intestinalis*; steroids and saponin were present in minimal amounts while flavonoids and alkaloids were present in good amounts.

Another phytochemical screening tests revealed that the ethanol extract of *Glidiella acerosa* showed many secondary metabolites than acetone extract [24]. Phytochemicals like alkaloids, carbohydrates, saponins, protein and amino acids, phytosterols, phenolic compounds flavonoids, and tannins were identified, and showed positive results in ethanol extracts, whereas terpinoids and glycosides were absent. In acetone extracts glycosides, proteins, phenolic compounds and terpinoids were absent whereas other metabolites were present**.**

Priyadharshini *et al*., [25] reported that seaweeds were an excellent source of components such as polysaccharides, tannins, flavonoids, phenolic acids, bromophenols and carotenoids that exhibited different biological activities.

Natural antioxidants are not limited to terrestrial sources and reports have revealed seaweeds are also rich in natural antioxidant compounds [26]. The presence of phytoconistituents such as phenols, flavanoids and tannins in seaweeds and seagrasses indicated a possible role that its extracts may have antioxidant activity. This activity was believed to help in preventing a number of diseases through free-radical scavenging activity [27].

The results of the antioxidant assays of this study indicated that the test seaweeds species are not good sources of the antioxidants compounds although they are rich in secondary metabolites of medicinal importance such as saponin, flavonoids and triterpens. This findings disagreed with many studies which indicated that seaweeds have good antioxidant activity e.g [28] who appeared that methanol extracts of *C.hornemannii* and *S. fusiformis* are the best source of antioxidant compounds. In another study, the DPPH assays were used to determine the antioxidant properties of ten types of seaweeds and sea grasses namely *Cymodeace rotundata, Acanthopora spicifera, Ulva lactuca, Ulva reticulate, Turbinaria conoides, Gracillaria edulis, Kappaphycus alvarezii, Gracillaria crassa, Gracillaria foliifera and Cymodeace serrulata*. The extracts were prepared with methanol and petroleum ether and appeared high antioxidant activity. In methanol extracts *C. rotundata, G. crassa* and *C. serrulata* showed the highest total antioxidant activity compared with other samples. *U. lactuca* exhibited the highest antioxidant and free radical scavenging activities in petroleum ether extracts [29].

The disagreement of this study with the previous studies may be due to the prolonged storage of the algal extracts which has been reported that it will considerably decrease the levels of these labile antioxidants such as L-ascorbate and BHT [28].

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