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Antioxidant, Antibacterial and Cytotoxic Potential of Selected Macroalgae from the Red Sea, Sudan Coast

El Nur E.E.[1], Ali L.I.[1], Fadul E.[2] and Mohamed l.E.[1*]
[1]Department of Botany, Faculty of Science, University of Khartoum, PC, 11115, P. O. Box 321 Khartoum, Sudan
[2]Department of Medicinal Biochemistry, Medicinal, Aromatic and Traditional Research Institute (M.A.P.R.I.), National Center for Research, P. O. Box 2404 Khartoum, Sudan
ietidalem11@gmail.com

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Abstract

Marine biodiversity is a source of unique chemical hits with its potential to develop into drug leads. Many studies have reported that macroalgae from marine is considered as an essential origin of antibacterial, antioxidant and antitumor agents. In this study, three marine macroalgae, Jania rubens, Halimeda tuna and Turbinaria decurrens were collected from the Red Sea, Sudan coast near to Port Sudan city. The collected algae were successively extracted using four solvents with increasing polarities. Extracts of the marine macroalgae were evaluated for secondary phytochemicals as well as antioxidant and antibacterial activities using Iron chelating assay and cup plate agar diffusion methods respectively. Dichloromethane crude extracts of J. rubens and T. decurrens were tested for cytotoxic activity.The most bioactive extracts were subjected to column chromatography. Then the fractions evaluated for antioxidant and antibacterial activities. The results revealed that, the algal extracts contained major groups of secondary metabolites; flavonoids, triterpenes and alkaloids. The antioxidant results showed that the chloroform and ethanol extracts of J. rubens and H. tuna were more active than those of the ethyl acetate and the petroleum ether ones (highest activity 86%). The highest positive antibacterial activity were recorded for ethanol extracts against S. aureus and E. coli; while the chloroform extract exhibited positive results against E. coli in all investigated extracts. The fractions indicated that some fractions of the three algae have moderate to low activity against E. coli. J. rubens revealed unprecedented cytotoxic activity with IC_{50} less than 3µg/ml and selectivity against two of six tumor cell line. A major compound was isolated in pure form using preparative thin layer chromatography. The isolated pure compound was identified as β- sitosterol glucoside using authentic samples. To draw final conclusion, further research is needed, high performance liquid chromatography together with a detailed spectroscopic analysis, particularly the red algae specimens from which the potent antimicrobial and antioxidant activity is being attribute as well as cytotoxicity.

Keywords: Sudan, Red Sea, macroalgae, antioxidant, antibacterial, antitumor potential.

Introduction

Marine macroalgae or seaweed is a unique source of remarkable natural agents of biological importance that are not present in the land wild plant species[1]. From previous reports, algae from marine water consider to be extraordinary origin of natural products chemicals including different carbohydrates, fatty acids, phenolic compounds, peptides, terpenoids, vitamins and minerals[2-4]. Such compounds might be responsible for many biological activities, for examples antibacterial, antioxidant, antitumor and antifungal[5-7]. Compounds derived from natural sources such as marine algae can be used as safe antimicrobial, antitumor agents[8,9]. Seaweeds are rich with abundant quantity of natural polyphenolic compounds therefore, exhibited strong antioxidants capability[6]. The Red Sea is considered as a rich environment with algal biodiversity; its pharmaceutical importance remains undiscovered and more researches in the chemical entities they contain, in the medicinal value of this entities in order to develop novel drugs, is needed. The aim of our present investigation is to carry out the antibacterial, antioxidant and cytotoxic activity of three macroalgae collected from the Sudan coast of the Red Sea.

Materials and methods

Collection of the algal materials: The algal samples were collected from the coast of the Red Sea at Port Sudan city Eastern Sudan. In order to remove foreign particles, the specimens were washed with sea water. To remove the salt from the surface of the samples, they were then washed using tap water, the samples were dried using tissue papers. They were identified using authentic samples in the Faculty of Marine Sciences and Fisheries, University of Red Sea, Sudan as; *Jania rubens*, (Linnaeus) J. V. Lamouroux (*J. rubens*, red alga), *Turbinaria decurrens* Bory de Saint-Vincent (*T. decurrens*, brown algae), *Halimeda tuna*, J. Ellis and Solander (*H. tuna*, green algae).

Preparation of Crude Extracts: The dried materials of the three collected marine macroalgae were coarsely powdered. For

preliminary antibacterial, antioxidant activities and qualitative phytochemical analysis, two hundred grams of the three powdered material were extracted using successive solvents of increasing polarities from less polar petroleum ether to the most polar solvent, ethanol (95%), the ethanolic crude extracts resulted were further fractionated into chloroform and ethyl acetate extracts. For cytotoxic activity, new dried material about 50 grams of *J. rubens* and *T. decurrens* were defatted with petroleum ether then extracted with dichloromethane. All the materials were soaked overnight in the solvents then were filtered. The solvent were evaporated using a rotatory evaporator and the dried resultant extracts were weighed and kept in a refrigerator at 4°C till used.

Qualitative Test of Secondary Metabolites: The three ethanol crude extracts were subjected to initial screening for secondary metabolites such as flavonoids, alkaloids, triterpenes, sterols, tannins, saponins and coumarins using the techniques described by Harborne[10].

Evaluation of Antioxidant Activity: The antioxidant activity throughout this study for crude extracts and fractions as well were evaluated according to the iron chelating method established by Kexue *et al*[11] with some modifications. Measurements and analyses were carried out three times. Ion chelating activity (percentage) was calculated as follows:

Radical scavenging activity (RSA) (%) = $[(A_{control} - A_{sample})/ A_{sample}] \times 100$

Antibacterial Activity: To assess the antibacterial activity of the algal extracts and fractions, the cup plate agar diffusion method was applied with some inconsiderable alteration[12]. Three species of pathogenic bacteria were used; *Escherichia coli*, *Klebsiella pneumoniae* and *Staphyllococcus aureus*. Standardized bacterial suspensions were prepared according to well established method[12] and 2ml were mixed thoroughly with 250ml nutrient agar at 45°C. The inoculated agar (20-25ml) was poured into sterile Petri dishes. After solidification of agar 3 wells were made using a sterile cork borer (No 9), of 9mm in diameter. The test concentrations were made by dissolving weighted amount of extracts in dimethyl sulphoxide (DMSO) to prepare two concentrations, 100 and 200mg/ml for crudes and 50mg/ml for fractions. After 18-24 hours, the inhibition zone diameters of each treatment were measured. Three replicates were carried out for each extract/ fraction. Streptomycin 40µg/ml was used as antibiotics control.

Cytotoxicity: A monolayer cytotoxicity and proliferation assay of the CH_2Cl_2 crude extracts of *J. rubens* and *T. decurrens* was carried out according to the method established by Roth *et al*.[13] using human tumor cell lines as was reported previous[14].

Column Chromatography of Chloroform Extracts: A glass column of 2×40cm fill with silica gel of 70-230 mesh particle size was used to run column chromatography of the chloroform extracts of the three macroalgae. The extracts were applied to the column after being mixed with small amount of SiO_2. Elusion was made using stepwise gradient solvents. 12 fractions (100ml portion) were collected. The resulted fractions were dried in reduced pressure rotatory evaporator and were used for further antioxidant and antibacterial activities after were being dried, weighed and re-dissolved in DMSO solvent to prepare the tested concentrations.

Results and discussion

Qualitative Test of Secondary Metabolites: The algal samples ethanolic crude extracts were tested to check the presence of major secondary metabolites. The results illustrated the existence of major groups of natural products in the samples. The results indicated the presence of triterpenes/ steroids; in a large amount in *J. rubens* and *H. tuna*, and a moderate amount in *T. decurrens*, while alkaloids were found in mild or small amounts. Flavonoids were found in moderate to mild amounts in all samples. On the other hand, the test for tannins, coumarins and saponins revealed negative results in all of the studied samples (Table-1). In earlier similar study[15], saponins, cardiac glycosides and phenolic was recorded in high amount in methanol extracts of, *H. tuna*, and *J. rubens*, while alkaloids and terpenoids was found in a moderate level. Many secondary metabolites were detected in *T. decurrens* extract such as flavonoids, terpenoids/or steroids, alkaloids, whereas, saponins and tannins were not detected in the previous screening[16]. Methods adopted in extraction, seasonality, taxonomy of the species, the way of harvest and climatic or geographical conditions, all are factors affecting phytochemical contents of the algae extracts[16]. The difference in these results compare to literature findings may be related to one or all of these factors. The results showed that the three algae species examined may be suitable sources for flavonoids, triterpenes, steroids and alkaloids, which can be separated and further screened for specific biologically active compounds relative to their traditional and medical uses. It is widely recognized that certain phytochemicals isolated from species of algae can be used as therapeutics. Examples are sodium laminarin sulphate and fucoidan, which are used as blood coagulants. Also, carotenoids that have provitamin-A activity were reported to be produced by algae[17]. Smith reported that most of the xanthophyll compounds are present in the Phaeophyta and some are present in the Chlorophyta, whereas the Rhodophyta contains only lutein[18]. Quantitative analyses may be effective to lead the scientists on which bioactive group of phytochemical they concentrate on, in order to isolate target entities.

Antioxidant Activity of the Crude Extracts: The algae species crude extracts were tested for antioxidant activity *In vitro* test, the standard procedure of the ferrous ions chelating was used. From the results, it may be deduced that the most potent antioxidant crude extract was that of ethanol and chloroform extract of *J. rubens* with 86±0.00% and 81±0.02% antioxidant effects respectively compare to the EDTA as a positive control

with radical scavenging activity 98%. The ethyl acetate and petroleum ether extracts showed moderate to weak activities 72 ±0.00% and 53±0.03% respectively (Figure-1). As for the brown algae *T. decurrens*, ethyl acetate extract showed medium activity 62±0.03, compared to weak activity of the other extracts 51±0.02% for petroleum ether, 53±0.00% for ethanol and 58±0.01% for chloroform extract (Figure-1). For the green algae *H. tuna* extracts, the chloroform and ethanol extracts were observed generally have medium activities (75±0.02% and 61±0.00%) than the extracts of petroleum ether and ethyl acetate 42± 0.02% and 23±0.02% respectively (Figure-1). Antioxidant activities of algal extracts were estimated from their ability to decrease ferric ion in the assay system. Through measuring the conversion of Fe^{3+} to Fe^{2+}, any transformation ability of the extracts may consider as a remarkable indicator of the antioxidant potential of the extracts. Marine chemical constituents recognized with their efficient antioxidant power attributed to their content of carotenoids and polyphenolic compounds, for instance, phlorotannins, fucoxanthin, bromophenols and flavonoids[19]. The antioxidant activities of such compounds might be related to their high concentration of free radical scavenger[20,21]. The antioxidant capacity is correlated well with the phenolic contents in the marine algae and also it depends on algal species used and the solvent of the extraction used as well[22]. Previously, extraction of several macroalgae species with non polar solvents (hexane, petroleum ether) resulted in strong antioxidant activity in contrast to our study, this may be due to the high contents of soluble lipids, carotenoids, steroids and/or terpenoids and low polar phenolic compounds[23]. Similar antioxidant activity results were reported earlier for *J. rubens* and *H. tuna*[24,25]. Ismail *et al*[16], reported the highest DPPH Scavenging activity of 61.6% for acetone extract of *T. decurrens*.

Antibacterial Activity of the Crude Extracts: Preliminary antibacterial screening was carried out for the crude extracts of the three investigated marine macroalgae. Nine ethanol, ethyl acetate and chloroform crude extracts of the three algae species, were tested *in vitro* against three bacterial pathogens. The justification of choosing these bacterial strains depends mainly on the bacterial cell wall structure; the Gram-positive bacteria, Gram-negative bacteria and capsulated bacteria. Among the nine extracts tested all of them showed antibacterial activity against at least one bacterial strain, the results showed that extracts of 200mg/ml concentration inflicted the highest antibacterial activities. The most susceptible bacteria strains were *S. aureus* and *E. coli* with the highest inhibition zone values which ranged between 15-19mm at the higher concentration 200mg/ml, as was recorded in Table-2, Figure-2. Chloroform extract of *J. rubens* exhibited remarkable antibacterial potential towards the three bacteria at the two concentrations 200 and 100mg/ml; *K. pneumoniae* was resistant at the lower concentration (Figure-2). While the brown algae *T. decurrens* showed high activity against *E. coli*, 16mm inhibition zone (Table-2, Figure-2). The green algae *H. tuna* extract with the same organic solvent showed moderate activity against *E. coli and K. pneumoniae* with inhibition zone of 15 and 14mm respectively (Figure-2). The ethanol extracts of all algae species were considered active against *S. aureus* and *E. coli*, while only ethanol extract from *J. ruben* at higher concentration was active against *K. pneumoniae* (Table-2, Figure-2). On the other hand, ethyl acetate extract of *J. rubens* revealed moderate activity at a higher concentration against only *K. pneumonia*; whereas *T. decurrens* and *H. tuna* ethyl acetate extracts revealed mild activity against two bacteria *E. coli* and *S. aureus* at higher concentration as was recorded in Table-2 and represented in Figure-2. Such extracts lack activity may be due to that ethyl acetate extracts were more or less did not contain enough active metabolites or the compounds they contain do not possess antibacterial inhibitory potential. These results show that algae crude extracts revealed promising antibacterial action against Gram positive and Gram negative bacteria. A previous report indicated that the Gram +ve bacteria is more susceptible when treated with crude algal extracts compare with Gram -ve bacteria, this is related to a more complicated cell wall structure of the Gram -ve bacteria[26]. In the current study, the suitable solvents for extracting the antibacterial agents from the red algae *J. rubens* were ethanol and chloroform; meanwhile, they revealed the highest antibacterial affectivity against the studied pathogens. This is due to the solubility rate of different bioactive chemical compounds in these solvents. Our results agree with those of Bai[27], who suggested that the highest activity against tested pathogens resulted when ethanol was used as the solvent to extract marine algae. Another report considered ethanol as an ideal solvent for extracting antimicrobial principles than ethyl acetate[28], in other studies, chloroform was the best solvent in respect to ethanol[29]. The bioactive chemical compounds contributed directly in the analysis of bacterial cells, or lead to damage cell wall structure, through enzyme inhibition, different chemical reactions; peroxidation or production of degradable auto oxidation materials. Bacteriostatic and/or bactericidal inhibitory potential of active algae extracts might be attributed to Phlorotannins and polyphenols which have an affinity to react well with proteins[30]. In earlier published research[31], methanol and chloroform extracts of *J. rubens* indicated promising antimicrobial power with a large inhibitory potential which was similar to the present results. In parallel to our findings, many studies were found bacterial strains susceptible to methanol extracts of *T. decurrens* and *H. tuna* in previous[32,33].

Cytotoxicity: The cytotoxic activity of dichloromethane crude extracts of two macroalgae *J. rubens and* T. *decurrens* against tumor cancer cell lines was carried out (Table-3). The extract of *J. rubens* exhibited novel unprecedented cytotoxicity (IC_{50}= 0.184µg/ml) and selectivity towards two out of six cell lines. The CH_2Cl_2 extract of *T. decurrens* showed selective cytotoxic activity against one type of cell line with IC_{50}= 5.450µg/ml. The promising cytotoxicity of *J. rubens* may be attributed to brominated diterpenes which give remarkable activity against tumor cells[9]. Polysaccharides from *J. rubens* were reported as antitumor agents[34]. Cytotoxic activity in red and brown seaweed

might be due to steroid, polysaccharide and bromophenol[35]. These results may suggest the interference of cellular mitosis and the other cell processes with some natural chemical metabolites present in the studied marine algae species to induce cell apoptosis[36].

Table-1: Phytochemical tests for the major groups of secondary metabolites in the algal extracts.

Algae	Tannins	Triterpens/Steroids	Coumarins	Alkaloids	Flavonoids	Saponins
Jania rubens	-	+++	-	+	++	-
Turbinaria decurrens	-	++	-	+	+	-
Halimeda tuna	-	+++	-	+	++	-

+ ≡ low amount; ++ ≡moderate amount; +++ ≡ high amount; - ≡ not detected.

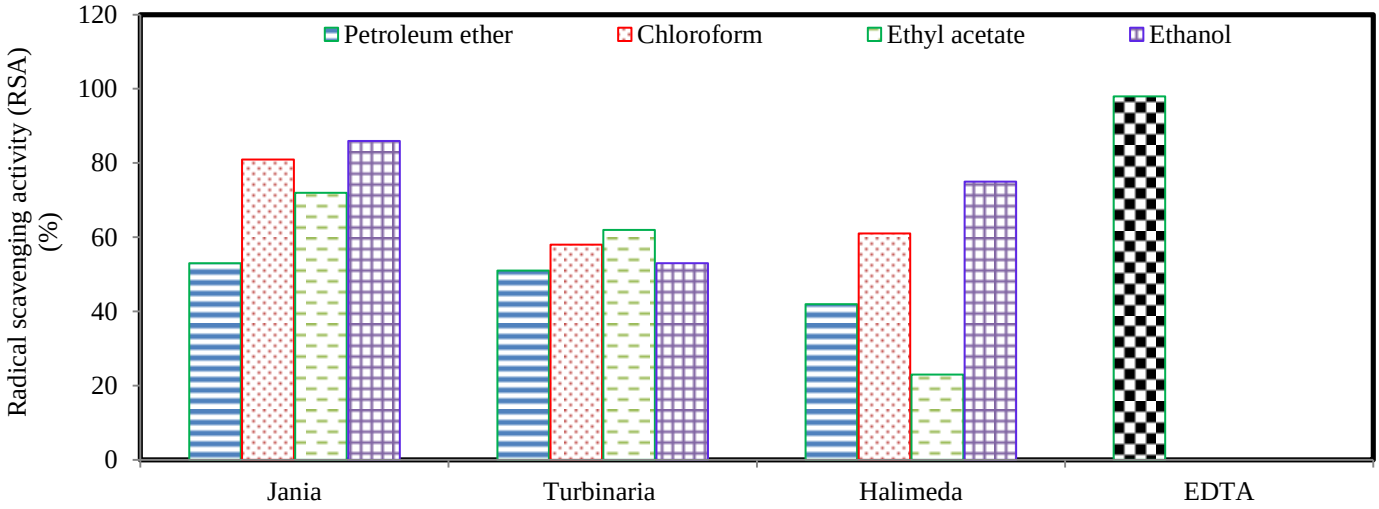

Figure-1: Antioxidant activity of macroalgae crude extracts.

Table-2: Antibacterial activity results of marine algae crude extracts.

Bacterial strains / Algal species	Bacterial strains I. Z. D. (M ± SD)					
	E. c.		*S. a.*		*K. p.*	
	Crude extracts Concentrations (mg/ml)					
	100	200	100	200	100	200
Jania rubens (C)	13 ±0.3	16± 0.5	17± 0.1	19± .05	0	13± 0.1
Jania rubens (EOH)	13±0.2	16±0.5	15±0.3	18±0.1	0	15±0.1
Jania rubens (E A)	0	0	0	0	0	14 ±0.1
Turbinaria decurrens (C)	0	16± 1.0	0	0	0	0
Turbinaria decurrens (EOH)	13±0.3	14 ±0.2	13 ±0.2	15 ±0.3	0	0
Turbinaria decurrens (E A)	0	14 ±0.2	0	13 ±0.2	0	0
Halimeda tuna (C)	0	15± 0.1	0	0	0	14± .03
Halimeda tuna (EOH)	15±0.1	17±0.3	12±0.3	16±0.2	0	0
Halimeda tuna (E A)	0	14 ±0.3	11± 0.1	14± 0.0	0	0
Streptomycin 40 µg/ml (control)	37 ±0.3		35 ±0.0		32 ±0.1	

I. Z. D. = The Inhibition Zones Diameter in mm, C= chloroform extract, EOH= ethanol extracts, EA= Ethyl Acetate extracts.

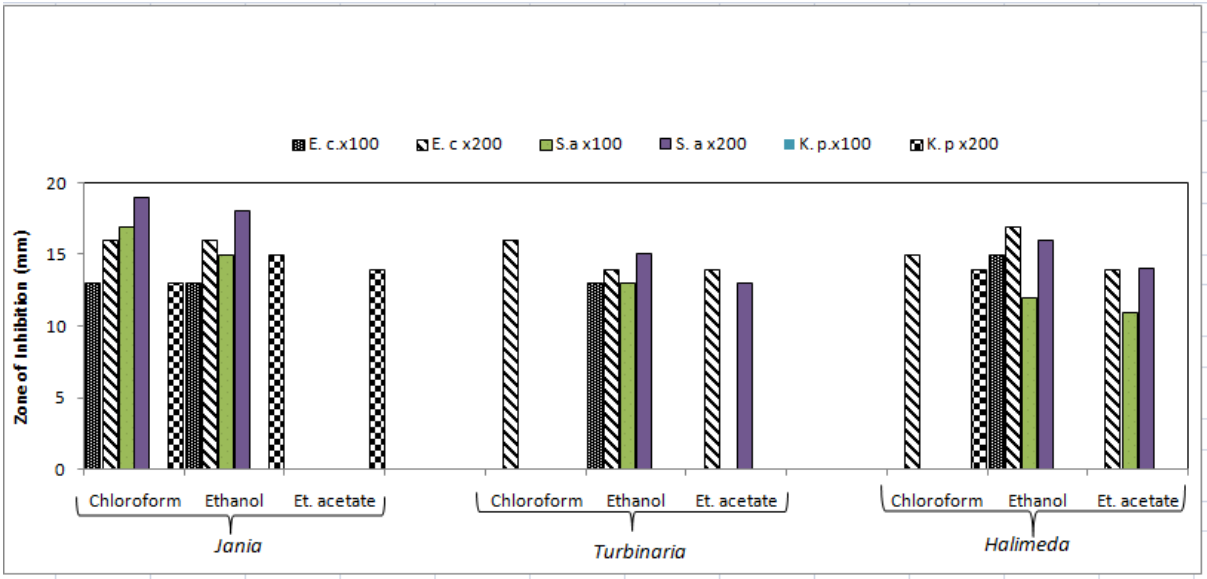

Figure-2: Antibacterial activity of crude extracts against three pathogenic bacteria.

Table-3: Cytotoxic potential of dichloromethane crude extracts of *Jania rubens* and *Turbinaria decurrens.*

Sample code	IC_{50}	IC_{70}	Active/total _ % (at)				Tumor selectivity		
	[µg/ml]	[µg/ml]	3 µg/ml		30 µg/ml		Selectivity	% Select	Rating[a)]
CH_2Cl_2 *Jania*	0.184	1.047	4/6	67%	6/6	100%	2/6	33%	+++
CH_2Cl_2 *Turbinaria*	5.450	9.974	1/6	17%	6/6	100%	1/6	17%	++

[a)]- =< 4% ≡ +; >= 10% ≡ ++ >= 20% ≡ +++

Fractionation of the Crude Chloroform Extracts: The chloroform crude extracts of the three specimens were further fractionated. Although the biological activities revealed that the polar fractions of ethanol crude extracts, for most of the samples showed activity; yet they were not further fractionated, due to that, the ethanol extracts of marine algae are mainly rich in polysaccharides in form of phlorotannins, sulphated polysaccharides and agar as well as a large amount of inorganic salts. From silica column chromatography, 12 fractions were obtained from each of the three chloroform extracts of marine algae and these fractions were then subjected to antioxidant and antibacterial activities.

Antioxidant Screening of the Fractions: The ion chelating activity of *J. rubens* fractions was presented in Figure-3, reflected that all fractions gave weak or negative results (less than zero), while only F7 eluted with 100% ethyl acetate, exhibited moderate antioxidant activity 61%. None of the fractions was able to cause activity similar to that observed for the crude extract 81%. Thus, it might be concluded that, in this case, the activity of crude extracts could be assigned for synergistic effect rather than one single compound. The antioxidant potential of fractions from *T. decurrens* revealed that F1 and F12 eluted with hexane 100% and methanol 100%, showed moderate activity (73 and 67%) in comparison to the crude extract which showed weak chelating activity 58%. The remaining fractions showed null activity or negative results (Figure-3). Regarding *H. tuna* fractions, F1 and F 11 gave high antioxidant when compared with the crude extract (75%), and F2, F4, F6, F10 and F12 had shown moderate antioxidant activity while F3, F5, F7, F8 and F9, exhibited weak or non effect (negative) as presented in Figure-3. It seems that some fractions were more potential than the crude extract, this might be due to the type of compounds they contain which could interact effectively and chelate Fe^{+2} followed by a decrease in the maximum absorbance of the iron and form ferrous-ferrozine complex. The antioxidant activity may be due to non polar lipids or polyphenol and polysaccharide as polar agents or semi polar compounds. This justifies our findings, since the elution of fractions varied from non polar to the most polar solvent. A higher antioxidant capacity of the fractions was expected, as these fractions were rich in polyphenols in the form of flavonoids and several antioxidant agents. Interpretation of null activity or negative results is that, these fractions might contain much fat that never dissolved well in the protocol solvent making the mixture turbid which makes the spectrophotometer recording faulty absorbance readings[37].

Antibacterial Screening of the Fractions: Chloroform fractions of *J. rubens* resulted from column chromatography were tested against *K. pneumoniae, E. coli* and *S. aureus* for their antibacterial activity. The results indicated that fractions F3, F4, F6 and F7 have moderate antibacterial activity against *E. coli;* while F3 and F6 showed low inhibitory effect against *S. aureus,* and F6, F7 showed moderate antibacterial activity against *K. pneumoniae.* The results of antibacterial inhibition of *Jania* fractions suggested more than one active compound or the quantity of compounds responsible for their activity were in mild amount. The antibacterial activity of red seaweed *J. ruben* may be resulted from the existence of saturated or unsaturated fatty acids in higher amounts as was reported previously for *Dictyophora echinovolvata*[38]. In the case of *T. decurrens* fractions, weak antibacterial activity was found in the polar fractions, F9, F10 and F12 eluted with ethyl acetate: methanol ratios and methanol 100% (Figure-4). In a previous study it had been reported that cyclohexane extract of *Turbinaria conoides* has remarkable antibacterial and antifungal activity[39], which in contrast to the effect of the polar fractions in the present study. The fractions of *H. tuna* were examined against bacterial strains, the results indicated that all fractions were found inactive against *S. aureu*, F6, F7 and F8 represented degrees of antibacterial activity against *E. coli* bacteria (Figure 4). *Halimeda* species synthesized different types of diterpenoids which could stimulate competent biological activities as was reported by Paul and Fenical[40]. The antibacterial of *Halimeda sp.* may be regarded to non polar fatty acids or polyphenol as polar ingredients as was remarked by Wan Razarinah[41]. None activity of fractions may refer to possible degradation or modification of the active compound during the fractionation process of the crude extracts. We may conclude that fractions were revealed antibacterial activity against *E. coli* as Gram negative bacteria, whereas *K. pneumoniae* although it is another Gram negative bacteria, it showed resistance to most of the fractions; it may be due to the presence of a capsular polysaccharide. The Gram negative bacteria were found more susceptible to algae chemicals in spite of the complicated structure of cell wall than Gram positive bacteria due to lipopolysaccharide layer. Earlier researchers speculated that natural polyphenol may affect the cell wall layer of Lipopolysaccharide this makes active substances to traverse the cell wall barrier[42]. The remarkable antibacterial activity of marine fractions against Gram negative bacteria *E. coli* necessitate future researches to isolate and to develop new drugs from natural sources to treat such pathogenic bacteria.

Isolation of Pure Compound from the Active Fractions: From antibacterial and antioxidant results of fractions, fraction seven eluted with chloroform: ethyl acetate (9:1) of *J. rubens* chloroform extract proved to be bioactive and at the same time seems to be semi pure. Further investigations of this fraction to isolate the major compound were carried out. The selection of this fraction was based on its sufficient amount. The compound was purified on preparative-TLC and compound 1 was obtained as one major spot and appeared as purple colour with an R_f value of 0.47. This compound might be related to triterpenoids and/ or steroids. Comparing compound 1 with authentic samples of triterpenes and steroids using TLC and IR spectra, compound 1 was identified to be β- sitosterol glucoside (Figure-5).

Aknin *et al.*[43] reported that red algae contain variable sterols with different quantities between the species, the most dominant sterol in all member of the family Rhodophyceae is the cholesterol. Non toxic sterols, unsaponifiable, that possessed essential medicinal values were more common in marine algae extracts[44]. However, β-sitosterol glucoside might be the responsible compound for antimicrobial and antioxidant activities of this fraction. Interestingly plant extracts, reported to contain sitosterol 3-D glucopyranoside[45,46], were exhibited significant antimicrobial activity but no report concerning its antioxidant effect.

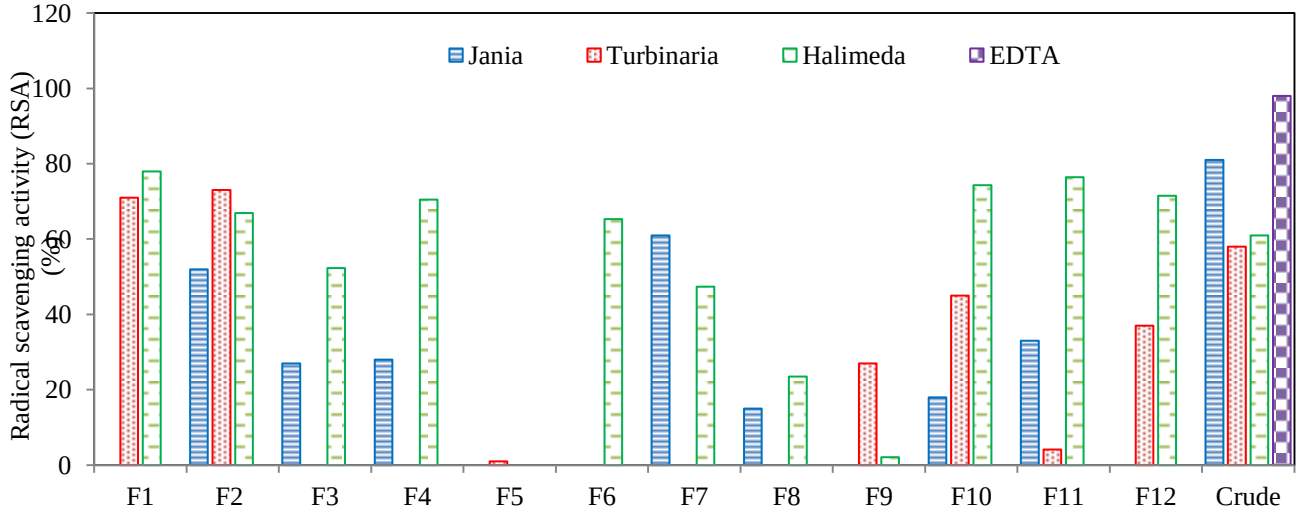

Figure-3: Antioxidant activity for the fractions of chloroform extracts.

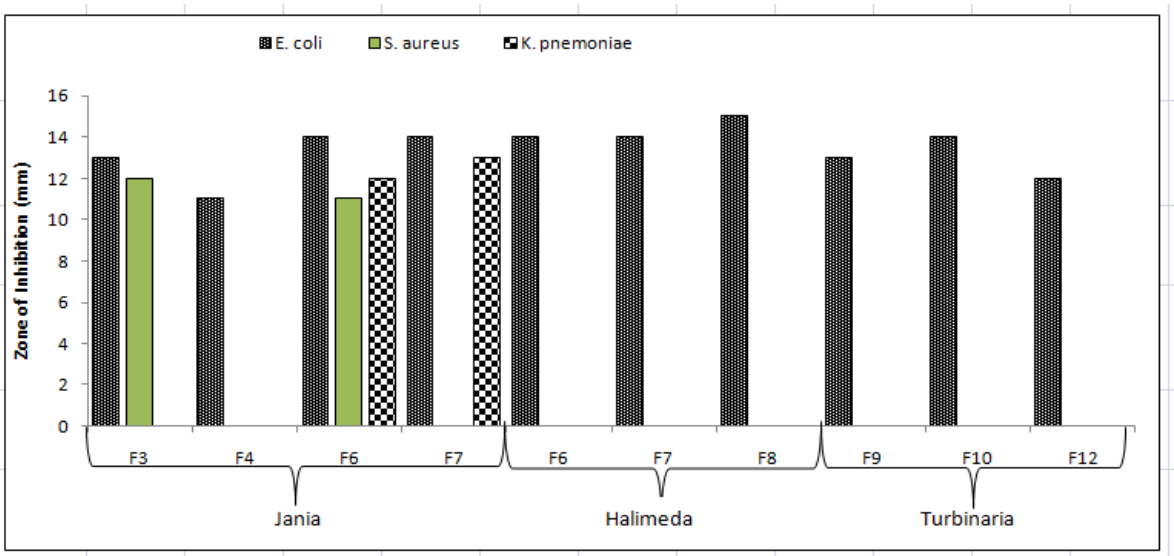

Figure-4: Antibacterial activity of the fractions of chloroform extracts against three pathogenic bacteria.

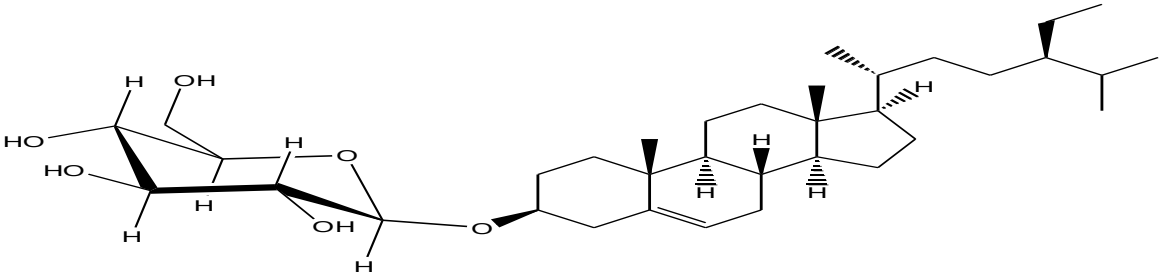

Figure-5: Compound 1 (β-sitosterol glucoside).

Conclusion

It may be concluded that marine macroalgae of the Red Sea at the Sudan coast might be a promising research target for unusual antibiotics, antioxidants and anticancer agents. The assay methods for antimicrobial and antioxidant activity used in our study were well-established ones. However, the investigation suggested that natural metabolites extracted from the marine algae may be regarded as responsible agents for promising bioactivities. The cytotoxicity of the crude extracts displayed extraordinary unprecedented results which were revealed promising selectivity against cancer cell lines. Thus bioassay-guided fractionation for the isolation of pure compounds from these marine algae species will be of great importance, followed by *in vivo* tests in order to develop candidates compounds for cancer chemotherapy.

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