Antitumour and Antioxidant Activity of Some Red Sea Seaweeds in Ehrlich Ascites Carcinoma *in vivo*

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The antitumour activities of extracts from the Red Sea seaweeds *Jania rubens*, *Sargassum subrepandum*, and *Ulva lactuca* were investigated in an *in vivo* mice model based on intramuscular injection of Ehrlich ascites tumour cells. In parallel, antioxidant activities were measured. Tumour marker levels, liver biochemical parameters, and hepatic oxidant/antioxidant status were measured to prove the anticancer and antioxidant nature of the algal extracts. Significant decreases in carcinoembryonic antigen (CEA) and α-fetoprotein (AFP) levels, activities of liver enzymes, levels of nitric oxide (NO) and malondialdehyde (MDA), and an increase in total antioxidant capacity (TAC) were recorded in groups treated with the algal extracts. *Jania rubens* was selected for phytochemical screening of its phytoconstituents. In addition, carotenoids, halides, minerals, lipoidal matters, proteins, and carbohydrates were studied. Furthermore, 7-oxo-cholest-5(6)-en-3-ol (**1**) and cholesterol (**2**) were isolated from the dichloromethane fraction.

Key words: Seaweeds, Antitumour, Phytoconstituents

Introduction

Cancer is one of the most formidable afflictions in the world and it remains the second leading cause of death after heart disorders in developing as well as advanced countries (Kamenarska *et al*., 2009). The development of life threatening diseases like cancer is linked to the deficiency of antioxidant agents. Marine algae have been reported to exert multiple biological effects, including antioxidant and free radical scavenging abilities (Ganesan *et al*., 2008). Marine organisms are potential sources of highly bioactive secondary metabolites that might represent useful leads in the development of pharmaceutical agents (El-Gamal, 2010). For example in recent years, more and more researches have realized that marine organisms hold immense potential as a source of anticancer agents. It is even mentioned in the Ebers Papyrus that the ancient Egyptians used seaweeds to treat breast cancer (Teas, 1981). Lower cancer rates in Japan as compared to western countries have been related to dietary habits including seaweeds like *Ulva lactuca* in food. Over the past two decades, a global collaborative effort has been made to discover useful antitumour agents in marine organisms, especially algae (Cardozo *et al.*, 2007; Kamenarska *et al*., 2009).

The antitumourigenic activity of *Jania rubens* from the coastal area of Abou-Kir, Alexandria, Egypt has been reported (Ibrahim *et al*., 2005). The polysaccharide ulvan from *Ulva lactuca* was reported to have cytotoxic activity against tumour cell lines and can inhibit the tumour cell proliferation/differentiation program *via* induction of low cell reactivity (Zhang, 2006).

The main substances with potential economic impact in food science, pharmaceutical industry, and public health, biosynthesized by algae, are polyunsaturated fatty acids, sterols, minerals, polysaccharides, terpenoids, proteins, and halogenated compounds (Cardozo *et al.*, 2007). The aim of the present study was to evaluate three marine algae, *Jania rubens*, *Sargassum subrepandum*, and *Ulva lactuca*, belonging to the three known macroalgal divisions Rhodophyta (red algae), Phaeophyta (brown algae), and Chlorophyta (green algae), respectively, for antitumour activity and

antioxidant capacity in Ehrlich ascites carcinoma (EAC)-bearing mice. The study was extended to investigate the phytoconstitutents of the bioactive alga *J. rubens*.

Material and Methods

Algal samples

Samples of the red alga *Jania rubens* (Linnaeus) Lamouroux, the brown alga *Sargassum subrepandum* (Forsskal) C. Agardh, and the green alga *Ulva lactuca* Linnaeus were collected from the Red Sea coast at Sharm El-Sheikh, South Sinai, Egypt, in August 2008.

Preparation of algal extracts

The fresh algal samples were exhaustively extracted with methanol (90%, v/v) using a Soxhlet apparatus, and the solvent was evaporated in *vacuo* at 50 °C. The yields were 7.4%, 4.9%, and 11.5% (on fresh weight basis) for *J. rubens*, *S. subrepandum*, and *U. lactuca*, respectively.

Analytical procedures

An HPLC System (Thermo Separation Products, San Jose, CA, USA) consisting of: mobile phase, methanol/acetonitrile (20:80, v/v); injector volume, 100 μL; flow rate, 1 mL/min; UV detection wavelength, 450 nm, was used for the separation and detection of carotenoids. A Pye Unicam SP 1900 atomic absorption spectrometer (Cambridge, UK) was used for element analysis. Conditions for analysis of unsaponifiable material were as follows: column, OV-17 (methyl phenyl silicon); column dimensions: 1.5 x 4 mm; column temperature, 70–270 °C with a rate of 10 °C/min. Fatty acid methyl esters (FAMEs) were identified in a Shimadzu GC-14A gas chromatograph equipped with a flame ionization detector and a C-R4AX chromatopac integrator (Kyoto, Japan). The operating conditions for FAME analysis were as follows: column, SP-2300; column dimensions, 1.5 x 4 mm; column temperature, 70–190 °C with a rate of 8 °C/min. Generally, the injection temperature was 250 °C (N_2 gas) and the detector temperature was 300 °C (H_2 gas). An automatic Kjeldahl-Foss apparatus, model 16210 (Foss America Inc., Fishkill, NY, USA), and a Markham distillation apparatus (Markham, Ontario, Canada) were used for determination of total protein content. HPLC analysis of the amino acid composition was performed on a Pico-Tag stainless steel amino acid analyzer (Millipore Corporation, Bedford, MA, USA); column dimensions, 150 x 3.9 mm; rate, 1 mL/min; detection at 254 nm with a Spectra Physics P 2000 variable wavelength detector (San Jose, CA, USA). The solvents used were sodium acetate trihydrate (0.1 M), pH 4.6, and 60% acetonitrile. The ^{1}H NMR spectra were recorded on a Varian Mercury 300 NMR spectrometer (International Equipment Trading Ltd., Vernon Hills, IL, USA), and δ values are reported in ppm relative to TMS in the appropriate solvent.

Biological experiments

Female Swiss albino mice (160; 2–3 months old; 20–25 g body weight) obtained from the Animal House Colony of the National Research Centre, Cairo, Egypt, were used. Animals received human care in compliance with the guidelines of the Ethical Committee of Medical Research of the National Institute of Cancer, Cairo, Egypt. The animals were classified into eight groups. Group 1 served as untreated control group; groups 2, 3, and 4 were, separately, orally treated with *J. rubens*, *S. subrepandum*, and *U. lactuca* extract, respectively, in doses of 500 (Ratnasooriya *et al*., 1994), 300 (Wong *et al*., 2000), and 150 mg/(kg body weight d) (Devaki *et al*., 2009), respectively, for two months. Group 5 was injected intramuscularly (i.m.) in the right thigh with 0.2 mL of EAC containing $2.5 \cdot 10^6$ cells for tumour induction; the animals were left without any treatment for two months. Animals of groups 6, 7, and 8 were injected i.m. each with 0.2 mL of EAC; when their tumours had grown to about 10 mm in diameter, after 10 d then they were orally treated with either *J. rubens*, *S. subrepandum*, or *U. lactuca* extract in doses of 500, 300, and 150 mg/(kg body weight d), respectively, for two months.

At the end of the experimental period, blood samples were collected, and clear sera were obtained and stored at –20 °C until further analysis. The liver of each animal was dissected and immediately homogenized in phosphate buffer (2.45 mM, pH 7.4) to give 20% (w/v) homogenate. The homogenate was centrifuged at 1700 x *g* and 4 °C for 10 min, and the supernatant was stored at –80 °C until analysis.

Carcinoembryonic antigen (CEA) in serum was determined by enzyme-linked immunosorbent assay (ELISA) (Gold and Freedman, 1965) using

the International Immuno-Diagnostics kit (Foster City, USA). The concentration of α-fetoprotein (AFP) in serum was determined by an immunoenzymatic assay procedure (Uotilla *et al.*, 1981) using a commercial kit (Dia Metra Company, Faligno, Italy). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in serum were measured by the kinetic method (Bablok *et al.*, 1988) using a commercial kit (Stanbio Laboratory, Boeme, USA). Alkaline phosphatase (ALP) activity in serum was measured by a colorimetric method (Bessey *et al.*, 1946) using a kit from Quimica Clinica Aplicada (Amosta/Tarragona, Spain). Hepatic malondialdehyde (MDA) was measured using a kit from Biodiagnostic (Giza, Egypt) according to the method described by Berkels *et al.* (2004). Total antioxidant capacity (TAC) and nitric oxide (NO) radical scavenging were measured by a colorimetric method (Koracevic *et al.*, 2001) using a kit of Biodiagnostic. Statistical analysis of the data was performed using Student's t-test (Snedecor and Cochran, 1989).

Investigation of phytoconstituents of J. rubens

Phytochemical screening

Analysis of volatile constituents, nitrogenous bases (and/or alkaloids), flavonoids, tannins, anthraquinones, coumarins, cardiac glycosides, and sterols (and/or terpenoids) in the extracts from *J. rubens* followed the procedure of standard methods (Farnsworth, 1966; Harborne, 1984).

Carotenoids

The fresh sample (0.6 g) was extracted carefully with *n*-hexane (5 mL) containing 3 mL of 0.01% butylated hydroxy toluene (BHT) in ethanol (w/v) (Taungbodhitham *et al.*, 1998). During the extraction, light was limited and procedures were performed on ice to prevent degradation and isomerization of carotenoids. The mixture was homogenized using a vortex mixer, centrifuged at 200 x *g* for 5 min at –5 °C. The solvent was evaporated under nitrogen. The residue was taken up in the mobile phase, and the carotenoids were analysed by HPLC.

Halides

The dry algal tissue (2 g) was mixed with sodium carbonate (2 g) and moistened with ethanol to form a homogeneous paste. The paste fused completely in a muffle furnace for 3 h at 500 °C. After cooling, 25 mL of distilled water were added to the fused material and filtered. The neutralized filtrate (dilute nitric acid) was gently boiled and completed to 250 mL. Alkaline fusion and ashing of the dry algal tissue afforded an aqueous solution of the total halides which were subsequently determined by argentimetric titration using an adsorption indicator (Rao and Singbal, 1995). The percentage of chloride, bromide, and iodide, respectively, was determined according to Rao and Singbal (1995).

Elemental analysis

A mixture of acids (concentrated nitric, perchloric, and sulfuric acid, respectively, 8:1:1, v/v/v) was added to the sample [(1.00 ± 0.005) g] and digested with heating (Dean and Rains, 1975). Elements were determined in the digested solution by flame emission spectrometry (Na, Ca, Mg, P, and K) and atomic absorption spectrometry (Hg, Pb, Cd, Cu, Fe, Mn, Zn, Li, Se, and Co) (Dean and Rains, 1975).

Lipoidal matters

The fresh algal sample was exhaustively extracted with 90% methanol at room temperature. The methanol extract was extracted with petroleum ether (60–80 °C). The residue was saponified using 0.5 M alcoholic KOH to afford the fraction of unsaponifiable material (USM) (El-Said and Amer, 1965) and the fatty acids. The isolated fatty acids were subjected to methylation (MeOH, 4%–5% dry H_2SO_4) (Iverson and Sheppard, 1975). Identification of the components of the USM fraction and fatty acids was determined by gas-liquid chromatography (GLC) analysis.

Proteins

Total proteins were determined by the Kjeldahl method (AOAC, 1985) using a Markham distillation apparatus and a factor of 6.25 to convert the N content into the protein content.

Analysis of total amino acids

The amino acid composition of the protein sample was determined by the Pico-Tag method according to Cohen *et al.* (1989).

Polysaccharides

Total carbohydrates were determined after complete acid hydrolysis (Dubois *et al.*, 1956). The polysaccharide fraction was isolated (Harborne, 1984). The defatted algal sample was extracted with 90% methanol. The residue was dissolved in water and a 3-fold volume of ethanol was added

Table I. Effect of algal extracts on tumour markers in EAC-bearing mice.

Group	CEA [ng/mL]	Change (%)	AFP [ng/mL]	Change (%)
Control	0.71 ± 0.04	-	0.80 ± 0.02	-
J. rubens	0.65 ± 0.06	–5.2	0.75 ± 0.01	–6.3
S. subrepandum	0.77 ± 0.01	8.5	0.76 ± 0.01	–5.0
U. lactuca	0.78 ± 0.01	9.9	0.77 ± 0.01	–3.8
EAC	5.12 ± 0.19[a]	621.1	5.59 ± 0.17[a]	598.8
EAC + *J. rubens*	1.66 ± 0.08[b]	–67.6	1.82 ± 0.03[b]	–67.4
EAC + *S. subrepandum*	2.02 ± 0.18[b]	–60.5	2.46 ± 0.11[b]	–56.0
EAC + *U. lactuca*	1.75 ± 0.07[b]	–65.8	1.88 ± 0.06[b]	–66.3

Data are presented as mean ± SE of 7 mice/group.
[a] Significant change at $P > 0.05$ in comparison with control group.
[b] Significant change at $P > 0.05$ in comparison with EAC group.

to precipitate the polysaccharides overnight at 4 °C. The polysaccharides were tested for their identity by different analytical tests (Harborne, 1984). Total carbohydrates as well as soluble sugars were determined as glucose equivalents by the phenol-sulfuric acid method (Cohen *et al.*, 1989). The free sugars and the acid hydrolysates were examined by paper chromatography (PC). Ascending development with two different solvent systems, S_1 [*n*-BuOH/Me_2CO/H_2O (4:5:1, v/v/v)] and S_2 [*n*-propanol/EtOAc/H_2O (7:1:2, v/v/v)], was carried out. The spray reagent aniline hydrogen phthalate (0.9 g aniline and 1.6 g monopotassium phthalate in 100 mL *n*-butanol saturated with water) was used. Quantitative determination of the separated sugars was carried out according to Wilson (1959).

Investigation of the dichloromethane extract

The fresh algae (3.5 kg) were extracted with methanol in a mixer, the methanol extract was concentrated *in vacuo* and then partitioned between dichloromethane and water. The dichloromethane layer was dried and evaporated to dryness. The residue (0.7 g) was subjected to column chromatography on silica gel G (Merck, Darmstadt, Germany) with a step gradient of benzene/EtOAc (100:0–0:100, v/v). Fractions were monitored by thin layer chromatography (TLC) using the solvent systems cyclohexane/EtOAc (7:3, v/v) and CH_2Cl_2/CH_3OH (95:5, v/v) and spraying with anisaldehyde/sulfuric acid (0.5 mL *p*-anisaldehyde, 85 mL methanol, 10 mL glacial acetic acid, 5 mL concentrated sulfuric acid heated at 100 °C). Two compounds were isolated and subjected to physical, chemical, chromatographic, and spectral analyses as well as comparison with reference samples.

Results and Discussion

Antitumour effect of algal extracts

The effect of the algal extracts on the levels of the tumour markers CEA and AFP and their changes compared with the untreated control and untreated EAC are documented in Table I. Groups that received *S. subrepandum* or *U. lactuca* extract alone showed slight changes in serum CEA and AFP levels in comparison with the control group. The group that received *J. rubens* extract showed slight changes in both serum tumour marker levels in comparison with the control group (Table I). The post-treatment of the EAC-bearing mice with any of the tested algal extracts produced a significant decrease in the serum level of CEA and AFP in comparison with the untreated EAC-bearing mice. These data indicate that the methanol extract of each alga exhibits significant antitumour activity, which might be due to the antioxidant effects on EAC-bearing hosts (Dongre *et al.*, 2008).

Hepatoprotective activity of algal extracts

The effects of the algal extracts on liver enzyme activities (AST, ALT, and ALP) in serum under the aforementioned conditions are documented in Table II. Serum AST, ALT, and ALP were slightly affected by the oral administration of the selected algal extracts in comparison with the control group. The groups that received each of the tested algal extracts showed a significant decrease in serum ALP activity compared with

Table II. Effect of algal extracts on liver enzymes in EAC-bearing mice.

Group	AST [U/L]	Change (%)	ALT [U/L]	Change (%)	ALP [U/L]	Change (%)
Control	9.55 ± 0.67	-	9.07 ± 0.71	-	147.5 ± 1.55	-
J. rubens	9.08 ± 0.29	–4.9	7.75 ± 0.14[a]	–14.6	134.8 ± 0.98[a]	–8.6
S. subrepandum	9.26 ± 0.33	–3.3	9.78 ± 0.57	7.8	136.35 ± 0.88[a]	–7.6
U. lactuca	9.26 ± 0.31	–3.3	9.31 ± 0.37	2.6	135.48 ± 2.09[a]	–8.2
EAC	31.05 ± 1.16[a]	225.1	28.45 ± 2.04[a]	199.8	281.95 ± 3.68[a]	91.2
EAC + *J. rubens*	17.28 ± 0.68[b]	–44.3	13.36 ± 0.16[b]	–53.0	184.4 ± 0.67[b]	–34.6
EAC + *S. subrepandum*	19.46 ± 0.73[b]	–37.3	14.57 ± 0.12[b]	–48.8	186.4 ± 2.14[b]	–33.9
EAC + *U. lactuca*	16.3 ± 0.66[b]	–47.5	13.19 ± 0.63[b]	–53.6	178.4 ± 6.74[b]	36.7

Data are presented as mean ± SE of 7 mice/group.
[a] Significant change at $P > 0.05$ in comparison with control group.
[b] Significant change at $P > 0.05$ in comparison with EAC group.

Table III. Effect of algal extracts on oxidant/antioxidant status in EAC-bearing mice.

Group	Hepatic MDA [nmol/g]	Change (%)	Hepatic NO [μmol/g]	Change (%)	Hepatic TAC [μmol/g]	Change (%)
Control	37.4 ± 0.90	-	34.23 ± 0.49	-	33.5 ± 0.53	-
J. rubens	29.6 ± 0.81[a]	–20.9	26.53 ± 0.99[a]	–22.5	30.5 ± 1.16[a]	–9.0
S. subrepandum	35.3 ± 1.02	–5.6	29.9 ± 1.87[a]	–12.7	29.1 ± 0.95[a]	–13.1
U. lactuca	30.95 ± 1.86[a]	–17.2	26.9 ± 0.77[a]	–21.4	30.3 ± 0.99[a]	–9.5
EAC	69.8 ± 1.60[a]	86.6	94.6 ± 0.48[a]	176.6	14.6 ± 0.22[a]	–56.4
EAC + *J. rubens*	35.6 ± 1.21[b]	–49.0	65.4 ± 0.95[b]	–30.9	25.3 ± 0.44[b]	73.3
EAC + *S. subrepandum*	38.4 ± 0.42[b]	–45.0	67.2 ± 1.02[b]	–29.0	24.8 ± 0.66[b]	69.8
EAC + *U. lactuca*	37.3 ± 1.33[b]	–46.6	65.08 ± 1.25[b]	–31.2	24.3 ± 0.44[b]	66.4

Data are presented as mean ± SE of 7 mice/group.
[a] Significant change at $P > 0.05$ in comparison with control group.
[b] Significant change at $P > 0.05$ in comparison with EAC group.

the control group. AST, ALT, and ALP activities decreased significantly in the groups post-treated with each of the tested algal extracts in comparison with the untreated EAC-bearing mice. These findings indicate that the algal extracts possess strong hepatoprotective activity which could be attributed to their active antioxidant constituents (El-Gamal, 2010).

Antioxidant capacity of algal extracts

The effects of the algal extracts on the hepatic oxidant/antioxidant status represented by MDA, NO, and TAC and their percentage change from untreated and EAC-bearing mice are shown in Table III. Oral administration of each of the tested algal extracts produced a significant decrease in hepatic MDA, NO, and TAC levels compared to the untreated controls. Post-treatment with the algal extracts induced a significant decrease in hepatic MDA and NO levels with a concomitant significant increase in hepatic TAC when compared to the untreated EAC-bearing mice. Thus, the algal extracts have free radical scavenging activities and enhance the endogenous antioxidant defence system. A previous study suggested that an extract containing naturally occurring compounds was effective in exerting protective effects by modulating oxidative stress (Cardozo *et al.*, 2007).

These results of our current study complement those of another study revealing the potent cytotoxic effect of the dichloromethane extract of *J. rubens* towards tumour cell lines *in vitro* (Mayer and Gustafson, 2003). Also, compounds in the diethyl ether fraction, in particular terpenoids, were shown to suppress proliferation of many types of tumours (*in vitro*) (Awad, 2004).

Various bioactive phytoconstituents from algae, such as polysaccharides, proteins, and carotenoids, have been found to improve certain immune parameters in mice inoculated with mammary tumour cells (Cardozo *et al.*, 2007). The antitumour activity of the *J. rubens* extract against EAC in

the present study can be explained by antiproliferative action besides the enhancement of the immune response (El-Gamal, 2010). In the current study, *J. rubens* showed hepatoprotective effects against EAC as indicated by the significant reduction in the activities of liver enzymes in serum. The antioxidant activity of *J. rubens* was indicated by the significant decrease in hepatic MDA and NO levels with a concomitant increase in hepatic TAC in EAC-bearing mice. Bioactive phytoconstituents such as carotenoids and proteins of *Jania* species have been shown to possess powerful antioxidant properties against many diseases caused by oxidative stress (Cardozo *et al.*, 2007).

Various *Sargassum* species have been reported as sources of antitumour agents (Zhang, 2006). *S. horneri* and *S. fulvellum* were reported to cause significant inhibition of volume and growth of estrogen-independent human breast tumours (Kamenarska *et al.*, 2009). Appreciable inhibition of Ehrlich carcinoma by polysaccharides obtained from *S. fulvellum*, *S. thunbergii*, *S. confusum*, and *S. stenophyllum* (Cardozo *et al.*, 2007) was related to improved functions of the immune organs and enhanced antioxidative capacity in tumour-bearing mice. The current study showed that *S. subrepandum* significantly improved liver functions of EAC-bearing mice. This action of the extract could be attributed to its carotenoids and polysaccharides content. Polysaccharides from *S. wightii* were reported as hepatoprotective agents (Josephine *et al.*, 2008) exhibiting a potent mitigating effect on oxidative liver injury, through its antioxidant properties. Also, the methanol extract of *S. micracanthum* and *S. horneri* were reported to have hepatoprotective and potent antioxidant activities (Zhang, 2006), which were markedly correlated with the contents of phenolics and unsaturated fatty acids. The antioxidant activity of the *S. subrepandum* extract is documented in the present study by the significant reduction of hepatic MDA and NO levels and the elevation of hepatic TAC in EAC-bearing mice. This finding agrees well with the study of Kamenarska *et al.* (2009).

Ulva lactuca supplementation produced antitumour activity as it is evident from the significant reduction of CEA and AFP in EAC-bearing mice (Table I). In accordance with our finding, Lee *et al.* (2004) stated that the antitumour activity of *U. lactuca* could be attributed to the immunostimulating activity of polysaccharides in an aqueous alcoholic extract.

The present study revealed that oral administration of an *U. lactuca* extract produced significant improvement in liver functions in EAC-bearing mice. These results are in agreement with the previous study of Rao *et al.* (2004) who demonstrated that oral supplementation of a *U. reticulata* extract reduced the hepatotoxicity triggered by acetaminophen in rats. The antioxidant capacity of the *U. lactuca* extract is evident from its ability to inhibit hepatic MDA and NO levels with a concomitant rise in hepatic antioxidant capacity in EAC-bearing mice. In the present work, the antioxidant activity of *U. lactuca* extract may be attributed to its carotenoid composition (Rao *et al.*, 2004). Sathivel *et al.* (2008) demonstrated the antiperoxidative property of polysaccharides of *U. lactuca*.

Investigation of phytoconstituents of J. rubens

Red algae are being considered the most important source of many biologically active metabolites in comparison to other algal classes (El-Gamal, 2010). In the present study, the extract of *J. rubens* exhibited somewhat higher antitumour activity compared to the extracts of the other two seaweeds. The chemical composition of *J. rubens* from the Red Sea has not been thoroughly investigated. Thus, *J. rubens* was selected for phytochemical screening of its phytoconstituents.

Phytochemical screening

The phytochemical screening revealed that the algal species is free from flavonoids and cardiac glycosides. Other constituents such as nitrogenous bases (and/or alkaloids), anthraquinones, coumarins, and saponins are very scarce.

Volatile constituents, terpenoids and steroids are found in considerable amount. These detected compounds are valuable antitumour agents (Ibrahim *et al.*, 2005).

Total carotenoids

The total carotenoids content measured by HPLC was 1.30 μg/g fresh weight. The present study was in agreement with our previous study that demonstrated many carotenoid pigments upon separation in a single-step RP-HPLC procedure (Hegazi *et al.*, 1998). In accordance with our results, carotenoids may be prominently participating in antitumour, antihepatotoxic as well

as antioxidant properties of *J. rubens* (Cardozo *et al.*, 2007).

Halides

The percentage (data not shown) of chloride was 5.42%, while those iodide and/or bromide were 1.04% (the percentage is an average of three determinations). Halogenated secondary metabolites, while rare in terrestrial plants, are common in marine organisms due to the abundance of chloride and bromide ions in seawater. A significant number of algal halogenated metabolites exhibit an impressive array of biological properties. Certain halogenated metabolites from red seaweeds were reported to exhibit selective antitumour activity in the National Cancer Institute's human tumour- and disease-oriented *in vitro* screen (Cardozo *et al.*, 2007).

Micro- and macroelements

Macroelements, calculated with reference to air-dried algae (data not shown), were (in %): P (0.045), K (0.33), Mg (2.91), Ca (5.62), Na (3.50). Microelements were (in ppm): Fe (498), Mn (156), Zn (15), Cu (5.72), Li (0.29), Se (0.56), Co (0.31). Hg, Pb, and Cd were less than 0.1 ppm. Selenium (Se) is a rare antioxidant element and has an anticarcinogenic effect (Patrick, 2004); its content was not previously measured for *J. rubens*.

Minerals and trace elements play well-defined roles as components of metalloenzymes, and algae are good sources of them (Aslam *et al.*, 2009). It has been estimated that certain seaweeds are up to 30 times higher in minerals than in terrestrial food. Aslam *et al.* (2009) reported that the mineral-rich extract derived from certain red marine algae, which contains 12% Ca, 1% Mg, and detectable amounts of microelements, was effective in suppressing growth and inducing differentiation of colon carcinoma cells.

Sterols, hydrocarbons, and fatty acids

The yield for the lipoidal matter was 7.72% on dry weight basis. The percentage of total fatty acids amounted to 2.04%, on dry weight basis, and the USM amounted to 3.92%. GLC analysis of the USM content (Table IV) revealed cholesterol as the main compound and *n*-octacosane as the hydrocarbon with the highest content. In a previous report (Soliman *et al.*, 1994), *n*-dotriacontane (63.17%) was found as the major hydrocarbon, although it was a minor one in our study (2.09%). GLC analysis of fatty acid methyl esters (Table

Table IV. Gas-liquid chromatographic analysis of unsaponifiable matter of *J. rubens*.

R_t [min]	Compounds identified	Relative area (%)
12.09	*n*-Tetradecane	1.52
13.28	*n*-Pentadecane	2.20
16.91	*n*-Octadecane	3.11
18.63	*n*-Eicosane*	5.96
19.62	*n*-Docosane*	4.26
20.01	*n*-Tricosane*	4.25
20.99	*n*-Tetracosane*	2.37
21.63	*n*-Pentacosane*	4.70
21.95	*n*-Hexacosane	0.98
25.74	*n*-Octacosane	9.54
25.50	*n*-Triacontane	8.08
26.88	*n*-Dotriacontane*	2.09
33.92	Cholesterol	24.25
34.08	Campesterol	4.59
34.91	Stigmasterol*	0.98
36.22	*β*-Sitosterol*	7.02
Total hydrocarbons		49.06
Total sterols		36.84
Total identified compounds		85.90

R_t, retention time.
* Identified for the first time.

Table V. Gas-liquid chromatographic analysis of fatty acids of *J. rubens* as methyl esters.

RR_t [min]	Fatty acids identified	Relative area (%)
0.40	Caprylic (C8:0)	0.65
0.41	Pelargonic (C9:0)*	0.42
0.43	Capric (C10:0)	2.04
0.59	Lauric (C12:0)	1.01
0.72	Tridecylic (C13:0)*	0.35
0.78	Myristic (C14:0)	9.93
0.84	Myristoleic (C14:1)*	2.11
1.00	Palmitic (C16:0)	23.67
1.22	Palmitoleic (C16:1)*	6.48
1.53	Stearic (C18:0)	6.70
1.67	Oleic (C18:1)	13.25
1.68	Elaidic (C18:1)*	0.22
1.72	Petroselinic (C18:1)*	0.74
1.84	Linoleic (C18:2)*	1.34
1.87	Linolenic (C18:3)*	4.90
2.21	Arachidic (C20:0)*	1.02
2.29	Arachidonic (C20:4)	4.20
2.39	Lignoceric (C24:0)*	2.00
Total		81.11
Saturated acids		47.87
Unsaturated acids		33.24

RRt, relative retention time; Rt, retention time of palmitic acid methyl ester was 13.92 min.
* Identified for the first time.

V) revealed that the most abundant saturated fatty acid was palmitic acid, followed by myristic acid, while oleic acid was the main unsaturated fatty acid. Arachidonic acid was detected as minor constituent although it was reported to represent 41.52% in a previous study (Soliman *et al*., 1994). The data are in agreement with those previously reported; variation was observed with respect to constituents and major compounds. Also, the concentration of polyunsaturated fatty acids was unusually low for a marine alga.

Marine algae appear to be a potential source of unsaponifiable sterols. Different steroidal compounds were reported to have antitumour effects (Zhang, 2006). Also, it was reported in the literature that the cytotoxic activity of some red and brown algal species could be attributed to the presence of a mixture of organic acids such as: capric, lauric, linoleic, myristic, oleic, palmitic, and stearic acids, respectively (Kamenarska *et al.*, 2009).

Protein content

A considerable percentage of total protein (9.55% on dry weight basis) was detected. HPLC determination of the amino acid composition (Table VI) indicates that glutamic acid and alanine are the major amino acids. Methionine, threonine, and lysine are essential amino acids present in a remarkable amount. The total content of non-essential amino acids (43.26%) was higher than that of the essential ones (35.58%).

Table VI. Amino acid composition of *J. rubens* determined by HPLC.

Amino acid	Content (%)
Aspartic acid	5.16
Threonine*	7.62
Serine	3.02
Glutamic acid	13.90
Proline	0.33
Glycine	2.07
Alanine	2.241
Cysteine	1.98
Valine*	2.90
Methionine*	7.97
Isoleucine*	1.19
Leucine*	3.81
Tryosine	4.56
Phenylalanine*	4.24
Histidine*	0.56
Lysine*	6.29
Arginine	2.54

* Essential amino acid.

Recent studies have thrown light on the antitumour effect of different proteins isolated from marine sources (Cardozo *et al.*, 2007).

Polysaccharides

The conent of total hydrolysable carbohydrates was 23.29% on dry weight basis, while the content of soluble sugars was 14.08%. Qualitative PC analysis of the free sugars in the purified aqueous alcoholic extract of *J. rubens* revealed the presence of fucose, galactose, glucose, arabinose, and galacturonic acid. Tests for the identity of the isolated polysaccharides indicated their mucilaginous nature. The mucilaginous hydrolysate revealed that the monosaccharide composition ratio was (in %): xylose (7.5), mannose (2.4), glucose (3.5), rhamnose (1.9), galactose (4.5), glucuronic acid (28.2), fucose (23.7), and arabinose (10.8).

Marine algal polysaccharides, such as fucoid, have been reported as antitumour agents (Cardozo *et al.*, 2007). A certain type of carbohydrates was suggested to react with Ehrlich tumour cells, to stimulate macrophages and to inhibit tumour cell growth (Zhang, 2006). A glycoprotein containing 11.6% carbohydrates was reported to agglutinate Ehrlich ascites cells (Cardozo *et al.*, 2007). Polysaccharides isolated from certain algal extracts were reported to have good selective tumour cell growth inhibition effects on HL-60 and U-937 (El-Gamal, 2010).

Isolated compounds

In a previous study, the dichloromethane extract of *J. rubens* showed a cytotoxic effect (Mayer and Gustafson, 2003) *in vitro* which agrees with the present work. It was thus selected for isolation of its bioactive compounds.

Herein, we present an investigation on phytoconstituents from *J. rubens* collected from Red Sea coasts where the ecological aspects of secondary metabolites are different from those in the Mediterranean Sea (Soliman *et al*., 1994)

Both 7-oxo-cholest-5(6)-en-3-ol (**1**) and cholesterol (**2**) (Fig. 1) gave positive Liebermann-Burchard and Salkowski tests indicating their steroidal nature. The mass spectrum of **1** showed the molecular ion peak at *m/z* 400 (100%). The fragmentation pattern of **1** showed the loss of a water molecule and the side chain to give a fragment at 269 (14%). Another loss of the C_3H_6 group was represented by a fragment at *m/z* 224 (18%). Other fragment peaks were shown at *m/z* 173 (20%), 109 (19%), and 95 (43%). The molec-

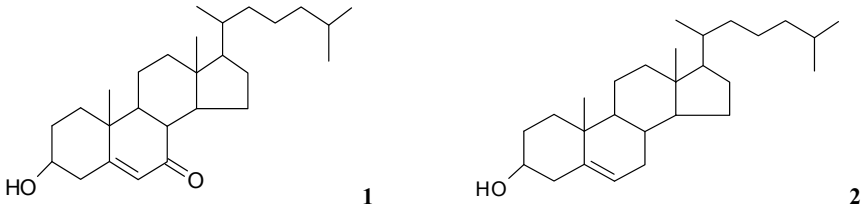

Fig. 1. Chemical structure of 7-oxo-cholest-5(6)-en-3-ol (**1**) and cholesterol (**2**).

ular formula of **1** was $C_{27}H_{44}O_2$ with the relative molecular weight 400.

The mass spectrum of **2** showed the molecular ion peak at *m/z* 386 (100%). The compound had lost a molecule of water giving a fragment at *m/z* 368 (42%), followed by further loss of the side chain giving a fragment at *m/z* 255 (10%). Other fragments appeared at *m/z* 57 (22%), 105 (16%), 301 (15%), and 371 (10%). The molecular formula of **2** was $C_{27}H_{46}O$ with the molecular weight 386. The ^{1}H NMR spectrum ($CHCl_3$, 270 MHz) of each compound exhibited typical steroidal methyl resonances. A signal at δ 0.68 ppm appeared in each compound's spectrum characteristic for the methyl group at position 18. Also, the presence of signals at δ 1.26 and 1.04 ppm characteristic for CH_3 at position 19 of **1** and **2**, respectively, were detected. Each spectrum showed the presence of signals in the range of δ 0.85–0.98 ppm representing protons of the three methyl groups at positions 27, 26, and 21. A characteristic hump of sterols in the range δ 1.4–2.3 ppm representing the cycloaliphatic region appeared as well as olefinic protons at $\delta \sim 5.4$ ppm characteristic for Δ^5 sterols. The compounds were identified as 7-oxo-cholest-5(6)-en-3-ol (**1**) and cholesterol (**2**) by comparison with the reported data of ^{1}H NMR spectroscopy (Anjaneyulu *et al.*, 1993). Oxysterols and cholesterol are compounds reported as biologically active antitumour compounds (Mayer and Gustafson, 2003). Recently, oxygenated derivatives of cholesterol have been shown to have antitumour effects in experimental models and in humans with no toxicity (Habib *et al.*, 2009).

Anjaneyulu V., Suresh B. J., and Rao K. N. (1993), 7-Oxo-cholesterol from *Jania rubens* of Lakshadweep Islands. Acta Cienc. Indica Chem. **19**, 80–82.

AOAC (Association of Official Analytical Chemists) (1985), Official Methods of Analysis, 12th ed. Washington, DC, USA, pp. 490–510.

Aslam M. N., Bhagavathula N., Paruchuri T., Hu X., Chakrabarty S., and Varani J. (2009), Growth-inhibitory effects of a mineralized extract from the red marine algae, *Lithothamnion calcareum*, on Ca^{2+}-sensitive and Ca^{2+}-resistant human colon carcinoma cells. Cancer Lett. **283**, 186–192.

Awad N. E. (2004), Bioactive brominated diterpenes from the marine red alga *Jania rubens* (L.) Lamx. Phytother. Res. **18**, 275–279.

Bablok W., Passing H., Bender R., and Schneider B. (1988), A general regression procedure for method transformation. J. Clin. Chem. Clin. Biochem. **26**, 783–790.

Berkels R., Purol-Schnabel S., and Roesen R. (2004), Measurement of nitric oxide by reconversion of nitrate/nitrite to NO. In: Methods in Molecular Biology, 279, Nitric Oxide Protocols. Humana Press, Inc., Totowa, NY, pp. 1–8.

Bessey O. A., Lowry O. H., and Brock M. J. (1946), Rapid colorimetric method for estimation of alkaline phosphatase in five cubic millimeters of serum. J. Biol. Chem. **164**, 321–329.

Cardozo K. H. M., Guaratini T., Barros M. P., Falcão V. R., Tonon A. P., Lopes N. P., Campos S., Torres M. A., Souza A. O., Colepicolo P., and Pinto E. (2007), Review: Metabolites from algae with economical impact. Comp. Biochem. Physiol. Part C Pharmacol. Toxicol. **146**, 60–78.

Cohen S. A., Mewyes M., and Trauin T. L. (1989), The Pico-Tag method. In: A Manual of Advanced Techniques for Amino Acid Analysis. Millipore Corporation, Bedford, USA.

Dean J. A. and Rains T. C. (1975), Flame Emission and Atomic Absorption Spectroscopy, Elements and Matrices. Marcel Dekker Inc, New York, pp. 101–150.

Devaki T., Sathivel A., and Raghavendran H. R. B. (2009), Stabilization of mitochondrial and microsomal function by polysaccharide of *Ulva lactuca* on D-galactosamine induced hepatitis in rats. Chem.-Biol. Interact. **2**, 83–88.

Dongre S. H., Badami S., and Godavarthi A. (2008), Antitumor activity of *Hypericum hookerianum* against DLA induced tumor in mice and its possible mechanism of action. Phytother. Res. **22**, 23–29.

Dubois M., Gilles K. A., Hamilton J. K., Rebers P. A., and Smith F. (1956), Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**, 350–356.

El-Gamal A. A. (2010), Biological importance of marine algae. Saudi Pharmaceut. J. **18**, 1–25.

El-Said M. E. and Amer M. M. (1965), Oils, Fats, Waxes, and Surfactants. Anglo-Egyptian Book Shop, Cairo, Egypt.

Farnsworth N. R. (1966), Biology and phytochemical screening of plants. Pharm. Sci. **55**, 225–276.

Ganesan P., Kumar C. S., and Bhaskar N. (2008), Antioxidant properties of methanol extract and its solvent fractions obtained from selected Indian red seaweeds. Bioresour. Technol. **9**, 2717–2723.

Gold P. and Freedman S. O. (1965), Demonstration of tumour specific antigen in human colonic carcinoma by immunologic tolerance and absorption techniques. J. Exp. Med. **127**, 349–462.

Habib N., Daaboul H., Hajj G., Jabbour A., and Kassem N. (2009), Antitumour activity of a new cholesterol derivative (24-ethyl-cholestane-3β,5α,6α-triol) in solid tumors. ASCO Annual Meeting Proceedings (Post-Meeting Edition). J. Clin. Oncol. **27**, 13541.

Harborne J. B. (1984), Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis, 2nd ed. Chapman and Hall, London, New York.

Hegazi M. M., Pérez-Ruzafa A., Almela L., and Candela M. E. (1998), Separation and identification of chlorophylls and carotenoids from *Caulerpa prolifera*, *Jania rubens* and *Padina pavonica* by reversed-phase high-performance liquid chromatography. J. Chromatogr. A **829**, 153–159.

Ibrahim A. M. M., Mostafa M. H., El-Masry M. H., and El-Naggar M. M. A. (2005), Active biological materials inhibiting tumour initiation extracted from marine algae. Egypt. J. Aquatic. Res. **31**, 146–155.

Iverson J. L. and Sheppard A. J. (1975), Programmed temperature gas chromatographic analysis of esters of fatty acids. J. Chromatogr. Sci. **13**, 505–508.

Josephine A., Nithya K., Amudha G., Veena C. K., Preetha S. P., and Varalakshmi P. (2008), Role of sulphated polysaccharides from *Sargassum wightii* in cyclosporine A-induced oxidative liver injury in rats. BMC Pharmacol. **8**, 4.

Kamenarska Z., Serkedjieva J., Najdenski H., Stefanov K., Tsvetkova I., Dimitrova-Konaklieva S., and Popov S. (2009), Antibacterial, antiviral, and cytotoxic activities of some red and brown seaweeds from the Black Sea. Bot. Mar. **52**, 80–86.

Koracevic D., Koracevic G., Djordjevic V., Andrejevic S., and Cosic V. (2001), Method for the measurement of antioxidant activity in human fluids. J. Clin. Pathol. **54**, 356–361.

Lee D. G., Hyun J. W., Kang K. A., Lee J. O., Hyun J. W., Kang K. A., and Lee E. Y. (2004), *Ulva lactuca*: a potential seaweed for tumour treatment and immune stimulation. Biotechnol. Bioproc. Eng. **9**, 236–238.

Mayer A. M. S. and Gustafson K. R. (2003), Marine pharmacology in 2000: antitumour and cytotoxic compounds. Int. J. Cancer **105**, 291–299.

Patrick L. (2004), Selenium biochemistry and cancer: A review of the literature. Altern. Med. Rev. **9**, 239–258.

Rao K. and Singbal S. Y. S. (1995), Seasonal variations in halides in marine brown algae from Porbandar and Okha coasts (NW coast of India). Indian J. Mar. Sci. **24**, 137–141.

Rao H. B. R., Sathivel A., and Devaki T. (2004), Antihepatotoxic nature of *Ulva reticulata* (Chlorophyceae) on acetaminophen-induced hepatoxicity in experimental rats. J. Med. Food **7**, 495–497.

Ratnasooriya W. D., Premakumara G. A., and Tillekerantne L. M. (1994), Post-coital contraceptive activity of crude extracts of Sri Lankan marine red algae. Contraception **50**, 291–299.

Sathivel H., Raghavendran H. R. B., Srinivasan B., and Devaki T. (2008), Anti-peroxidative and anti-hyperlipidemic nature of *Ulva lactuca* crude polysaccharide on D-galactosamine induced hepatitis in rats. Food Chem. Toxicol. **46**, 3262–3267.

Snedecor W. G. and Cochran G. W. (1989), Statistical Methods, 8th ed. Iowa State University Press, Ames, IA, USA.

Soliman F. M., El-Tohamy S. F., Fathy M. M., Ramdan A., Afify N. A., and Sanad O. A. (1994), Phytochemical and pharmacological investigation of the red alga *Jania rubens* (L.) Lamx. Carbohydrates, mannitol and pharmacological screening. J. Drug Res. Egypt **21**, 165–180.

Taungbodhitham A. K., Jones G. P., Wahlgvist M. L., and Briggs D. R. (1998), Evaluation of extraction method for the analysis of carotenoids in fruits and vegetables. Food Chem. **63**, 577–584.

Teas J. (1981), The consumption of seaweed as a protective factor in the etiology of breast cancer. Med. Hypotheses **7**, 601–613.

Uotilla M., Ruoslahti E., and Engvall E. (1981). Two-site sandwich enzyme immunoassay with mono clonal antibodies to human alpha fetoprotin. J. Immunol. Methods **42**, 11–15.

Wilson C. M. (1959), Quantitative determination of sugars on paper chromatograms. Anal. Chem. **31**, 1199–1201.

Wong C. K., Ooi V. E., and Ang P. O. (2000), Protective effects of seaweeds against liver injury caused by carbon tetrachloride in rats. Chemosphere **41**, 173–176.

Zhang Y. P. (2006), Progress on marine anticancer drugs in R & D. Prog. Pharmaceut. Sci. **30**, 433–442.