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PHYTOCHEMICAL INVESTIGATION AND ANTIMICROBIAL ACTIVITY OF *ULVA LACTUCA* (L.)

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Abstract

Different growth stages of *Ulva lactuca* were harvested from different biotopes in the Suez Canal during February through April 2003 and subjected to bioassay to detect substances with biological activity. The total alcoholic extract of algal material was fractionated into light petroleum, chloroform, ethyl acetate, fatty acid methyl esters and unsaponifiable matter. The effect of extracts was more pronounced as antifungal than as antibacterial. Petroleum extract had the more prominent effect on *Aspergillus niger*. Fatty acid methyl ester had a greater effect on *Chrysosporium* sp, whereas, unsaponifable acetate extract affected *Fusarium* sp. *Bacillus subtilus* was found to be sensitive to all solvent extracts. Ethyl acetate extract affected *Staphylococcus aureus*. Unsaponifable acetate affected *Sarcina lutea*. The un-saponifable fraction was analyzed by GLC, GC/MS, and TLC. GLC analysis of fatty acid methyl esters and unsaponifiable fraction revealed the presence of twelve fatty acids, palmitic, oleic and margaric acids represented by 70.34, 20.9 and 2.26%, respectively, while the hydrocarbon C-28 was the major unsaponifiable one (63.76%).

Introduction

Natural products continue to play a major role in drug discovery due to the diversity of their active compounds and their role in treatment of diseases. Although much has been done in this regard, Harvey (2000) noted that only 10% of the world plants have been investigated for biological activity. In recent years, many publications have featured the discovery of bioactive compounds isolated from marine organisms such as tunicates, sponges, soft corals, sea hares, bryozoans, and marine microorganisms (Donia and Hamann, 2003; Haefner, 2003).

Marine waters contain more than 4500 described species of green, red and brown seaweeds or macroalgae (Dring, 1982). There are a number of seaweeds used as direct food (Dawes, 1998), others with economic potential (Critchley *et al.*, 1998), but have received comparatively less bioassay attention. However, some species were shown to have antimicrobial activity (Ahmed *et al*., 1992; Saleh *et al*., 1993; Awad *et al*., 1998; and Donia and Hamann, 2003). The green alga, *Ulva lactuca* was shown to posses an anti-inflammatory compound

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(Faulkner, 2002). *Ulva lactuca* contains active polysaccharide and heterosaccharide that exhibited antiviral activity against a number of human and ovian influenza, and stimulated macrophages, β-cells and T-cells in mice (Ivanova *et al*., 1994 a and b). The compounds already isolated from seaweeds are providing valuable ideas for the development of new drugs against cancer, microbial infection and inflammation (Premila *et al*., 1996; Kim *et al*., 1997; Okai *et al*., 1997; Elena *et al*., 2001; and Vitor *et al.*, 2002).

This work aimed to assess the potentialities of different organic extracts of *Ulva lactuca* from the Suez Canal against some pathogenic bacteria and fungi isolated from clinical samples.

Materials and methods

1- Harvest and manipulation of algal material

Ulva lactuca was harvested from the Suez Canal (Fig. 1) during the period from February through April 2003. Harvesting was done by snorkeling and carried out in a manner to represent different stages of *Ulva* and different biotopes. Algal material was packed in nylon bags, and kept in an icebox for subsequent manipulation. Careful sorting of algal material was carried, in the laboratory, to remove epiphytes. *Ulva* was quickly rinsed with tap water to remove salts and impurities, dripped, weighed, dried at room temperature and packaged in paper bags. A fresh algal sample was stored in 4% formalin-seawater for identification. The species was identified using Burrows (1991) and Aleem (1993) and was also compared with the reference herbaria found in the Marine Botany Lab at Faculty of Science, Suez Canal University. It is worthy to note that these herbaria were confirmed in 1992 at the Museum of Natural History at Paris.

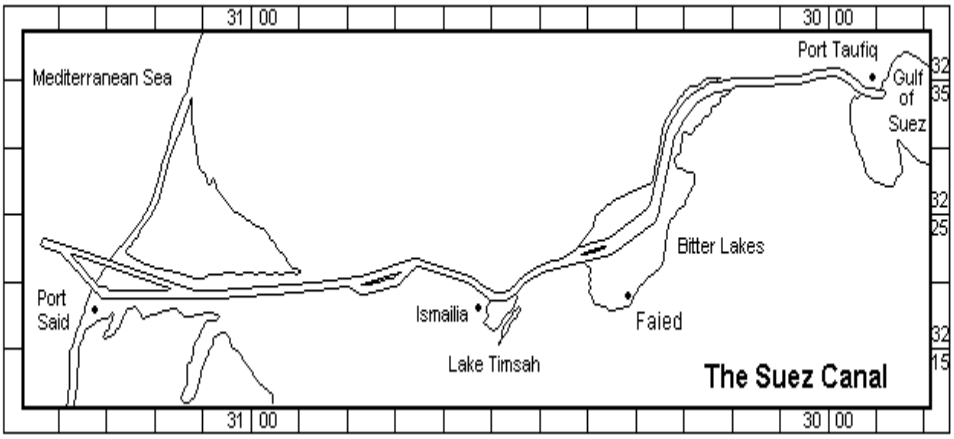

Figure (1): The Suez Canal and its lakes.

2- Phytochemical study

Dried powder of *Ulva lactuca* (500g) was exhaustively extracted with ethanol (80%). The concentrated extract (47.25g) was fractionated into light petroleum (3.5g), chloroform (4.60g) and ethyl acetate (0.22g). About 3.5g of light petroleum extract was subjected to saponification by refluxing with 60mL of 10% alcoholic KOH according to El-Said and Amer (1965) and Williams (1966) to give the unsaponifable matter (USM) 0.85g. The residue was subjected to acetylation according to Vogel (1966) and Williams (1967) to afford 1g, and those subsequently subjected to GLC and GC/MS. The alkaline aqueous layer remained after extraction of the unsaponifable matter was acidified with conc. HCl and the fatty acids were extracted with successive partition of ether (5×200ml) and evaporated to afford 2g. About 0.1g of fatty acid content was subjected to methylation according to Vogel (1966) and Williams (1967) to give about 0.15g of fatty acid methyl esters. GLC analysis of fatty acid methyl ester was established by comparing the retention times with those of authentic. Qualitative estimation was carried out by peak area measurements followed by normalization.

Gas liquid chromatographic (GLC) analysis of methyl esters of the total fatty acids carried on GCV pye Unicam gas chromatograph under the following operating conditions: detector temp was 300^{o}C, injector temp was 270^{o}C, recorder was dual channel recorder, column temp was 70^{o}C-190^{o}C (8^{o}C min^{-1}), column package was diatomite 100-120 mesh, liquid phase was 10% polyethyleneglycol (PEG7), column dimension was 1.5m×4mm, carrier gas was nitrogen 3ml.min^{-1}, hydrogen flow was 330ml.min^{-1}, chart speed was cm.min^{-1}. Authentic fatty acids methyl esters were preceded in the Central Research Lab, Fac of Agriculture; Cairo University.

The GC-MS of the unsaponifable matter (USM) was carried out on Varian 3400 gas chromatograph equipped with a fused silica column (DBS, 30m × 0.25µm film thickness), J% PP/N: 122-5032 under the following conditions: carrier gas was helium, flow rate was 2ml.min^{-1}, detector was FID, detector temp was 300^{o}C, injector temp was 250^{o}C, split ratio was 1:10, oven temp program was (initial temp 50^{o}C for 4 min, 50-90^{o}C at 4^{o}C min^{-1} then hold for 10 min).

For GC-MS analysis, GC conditions were the same as mentioned above, and the capillary column was directly coupled with a quadruple mass spectrophotometer (Finnigan MATSSQ 7000) and (El-MS were recorded at 70 $_{e}$V). Thin layer chromatographic (TLC) screening of the unsaponifable matter was carried out using silica gel GF_{254} chromato-plates and different developing solvent systems. The developed chromatograms were visualized by anisaldehyde sulfuric acid spray reagent.

3- Microorganisms

Antimicrobial susceptibility tests were performed using clinical pathogens and laboratory control strains. Pathogens were two of gram negative strains (*Escherichia coli* and *Sarcina lutea*), two gram positive strains (*Bacillus subtilus* and *Staphylococcus aureus*) and four fungi (*Candida albicans*, *Aspergillus niger*, *Fusarium* sp. and *Chrysosporium* sp.). Two media were used, the nutrient agar and Czapek's Dox medium. The former composed of beef extract (1g), yeast extract (2g), peptone (5g), sodium chloride (5g), Agar-Agar (15g) in a liter of distilled water, and adjusted at pH 7.0. Czapek's Dox medium consisted of sucrose (30g), $NaNO_3$ (3g), KH_2PO_4 (1g), KCl (0.5g), $MgSO_4$ (0.5), $FeSO_4$ (0.01g), agar-agar (20g) in a liter of distilled water.

4- Antimicrobial activity assay using filter paper disc method

Bacterial isolates were grown on nutrient liquid medium for a day and fungi grown on Czapek's agar for 7 day. For screening of the different algal extract, 1 ml of isolate suspension was injected into Petri dishes under aseptic condition and seeded with agar medium after solidification. Filter paper discs, of 4 mm diameter, were loaded with the algal extract and transferred to the surface of the inoculated plates according to (Tomas *et al.,* 1980). The plates left for 24 hours in a refrigerator for diffusion, after which the plates were incubated at 37^{o}C for 24 hours to bacteria and 7 days for the fungi and the growth inhibition zones were estimated.

Results and Discussion

1- Algal aspects

Taxonomy is the hidden but important foundation when seaweeds would be exploited for any of their natural products (Abbott and Norris, 1985; Frederic *et al.*, 1996). According to van Den Hoek *et al.* (1995), *Ulva lactuca* (L.) belongs to division Chlorophyta, family Ulvaceae and has no synonym. It forms green to dark-green broad and crumpled frond, translucent and membranous, fixed to solid substrata by discoid holdfast, and varies in size from three to 30cm across but rarely reached to 50-100cm in length especially in sheltered eutrophic areas. The cross section is two-layers of cells with parietal chloroplasts. This species is used as the Type specimen for other species of the genus *Ulva*.

The biomass of the exploited species is another important basis for evaluation of algal potentiality. So, it is necessary to know where and when seaweeds can be found in large quantity. The algal samples were obtained from February through April 2003. This was the luxuriant growth period of *Ulva lactuca*. The species dominated the shore of the Suez Canal near Timsah Lake (Figure 1) where its biomass was 760 g/m^2. These species were also found in other sites but showed reduced biomass. Although *Ulva lactuca* was found in

appreciable amount at Faied (440 g/m^2), it was not collected for the purpose of this work because the sewage effluents are discharged there.

Seaweeds, including the *Ulva*, grow in the intertidal, near-shore or offshore waters; and their growth and chemical constitutes are dependant on ecological requirements (Yasumoto, 1993; Norambuena, 1996; Hurtado *et al*., 2001). *Ulva lactuca* occurs at all levels of the intertidal zone and may extend deeper into the shallow sublittoral zone when water is clear. Thalli grow on stones, rocks, and mollusks. Plants characterize the water of low salinity, variable in size, the larger plants may be floating or fixed on rocks, the smaller ones live as epiphytes on other larger seaweeds. In very sheltered conditions, plants that have become detached from the substrate can continue to grow, forming extensive floating communities.

2- Phytochemical constituents

The total alcoholic extract of *Ulva lactuca* was fractionated to light petroleum, chloroform and ethyl acetate extracts. The light petroleum extract was subjected to saponification and subsequently to extraction of fatty acids content. Table (1) showed the results of GLC analysis for the unsaponifable fraction. Six hydrocarbon constituents were identified with concentrations summed to 67.44%. C_{28} was the major constituent as its concentration was 63.76%.

Table (1): GLC analysis of hydrocarbons from the unsaponifable matter of *Ulva lactuca*

	Retention time	Number of carbon	Concentration %
1	**10.6**	**Unidentified**	**2.569**
2	**13.2**	**C : 20**	**1.405**
3	**14.5**	**C : 21**	**0.377**
4	**15.3**	**C : 22**	**0.772**
5	**18.5**	**C : 26**	**0.914**
6	**19.9**	**C : 28**	**63.767**
7	**21.4**	**C : 30**	**0.212**
8	**25.5**	**Unidentified**	**1.114**

Table (2) showed the results of GC/MS analysis for the unsaponifable matter (USM). Eight hydrocarbon constituents were identified. 3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol, Isoheptadecanol and Pentadecanoic acid 14 methyl-methyl ester were the major constituents of the unsaponifable matter.

Their concentrations were 40.85, 17.90, and 12.87 % of the total concentration, respectively.

Table (2): GC/MS analysis of unsaponifable acetate of light petroleum extract of *Ulva lactuca*

	Rt	Mw	Chemical name	Percent conc.	B-b	Mass Fragments
1	**27.42**	**256**	**Isoheptadecanol**	**17.908**	**55**	**(55,69,83,97,111,125, 139,154,238)**
2	**30.13**	**252**	**5-octadecene**	**4.109**	**55**	**(55,57,83,97,135,177, 224)**
3	**31.21**	**268**	**2-petadecanone 6,10,14-trimethyl**	**6.318**	**58**	**(58,71,85,109,124,149, 191)**
4	**33.08**	**270**	**Pentadecanoic acid 14 methyl-methyl ester**	**12.876**	**74**	**(74,87,97,143,185,227, 240)**
5	**34.27**	**284**	**Hexa decanoic acid ethyl ester**	**6.413**	**88**	**(88,101,157,213,256)**
6	**34.45**	**256**	**Hexa decanoic acid**	**4.741**	**73**	**(73,83,157,213)**
7	**38.46**	**296**	**3,7,11,15-tetramethyl-2-hexadecen-1-ol**	**40.856**	**68**	**(68,123,137,179,208, 278)**
8	**41.38**	**296**	**Cyclotetra decanol 1,7,11-Trimethyl-4-1-methyl ethyl**	**6.776**	**57**	**(57,84,95,121,140,213, 276,294)**

GLC analysis of fatty acids from *Ulva* (Table 3) revealed 12 fatty acids. The saturated fatty acids were Caprylic acid, Capric acid, Lauric acid, Myristic acid, Palmitic acid, Margaric acid and Stearic acid. They constituted about 77 % of the total percentage of fatty acids. Palmitic acid was the major, as its concentration reached 70.34%. Margaric acid was 2.26% and myristic acid was 1.65%, while other saturated acids were very low. On the other hand, the unsaturated fatty acids represented only by oleic acid, which reached to 20.9 % of the total fatty acids.

Table (4) represents the results of TLC analysis for the unsaponifable matter after the visualization by anisaldehyde/sulfuric acid. The usage of solvent system I revealed three main spots with R_f of 0.86, 0.66, and 0.48, respectively. Solvent system II also revealed three main spots with R_f of 0.82, 0.63, and 0.39, respectively.

Table (3): GLC analysis of fatty acids methyl ester of *Ulva lactuca*

	RT	Trivial name	No. of carbon	Systemic name	Conc.
1	6.3	Caprylic acid	8:0	—	0.180
2	6.8	Unidentified	Unidentified	—	0.403
3	7.3	Unidentified	Unidentified	—	0.079
4	7.7	Unidentified	Unidentified	—	0.785
5	9.3	Capric acid	10:0	Decanoic acid	0.416
6	12.1	Lauric acid	12:0	Do decanoic acid	0.325
7	14.6	Myristic acid	14:0	Tetra decanoic acid	1.658
8	15.8	—	15:0	Pentadeconoic	0.679
9	18.0	Palmitic acid	16:0	Hexa decanoic acid	70.344
10	19.5	Margaric acid	17:0	Hepta decanoic	2.264
11	21.3	Stearic acid	18:0	Octa decanoic acid	0.841
12	22.2	Oleic acid	18:1	Cis-9-octa decanoic acid	20.9

Meanwhile the solvent system III gave only two spots at R_f of 0.89 and 0.72. Preparative TLC using system II afforded decortinol and 3-O-β-D glycopyrannosl- stigmasta-5, 25 dien. The structures of these substances were established by Ahmad *et al.* (1992) and Awad (1998) using mass and IR.

Table (4): Results of TLC of unsaponifable matter from *Ulva lactuca*

Spot No. / Solvent system	1	2	3
I. Light petroleum : Ethyl acetate (7:3)	0.86	0.66	0.48
II. Light petroleum : Chloroform : Methanol (15:15:1)	0.82	0.63	0.39
III. Chloroform : Methanol (9.5:0.5)	0.89	0.70	0

3- Antimicrobial activity

Tables 5 and 6 showed that almost all organic extracts had broad spectrum effect against most species under investigation. The effects on fungi were more pronounced as antifungal than as antibacterial. Ethyl acetate extract had no effect on *Escherichia coli* and *Sarcina lutea* but affected *Bacillus subtilus* and *Staphylococcus aureus* as the inhibition zones were 1.5 and 1.1 cm, respectively. Unsaponifable acetate had no effect on *Escherichia coli* and *Staphylococcus aureus* but affected *Sarcina lutea* and *Bacillus subtilus* as the inhibition zones were 1.0 and 0.9 cm, respectively. *Bacillus subtilus* was found to

be sensitive to all solvent extracts. The efficiencies of different organic solvents on this species were ascendingly arranged as unsaponifable acetate < fatty acid methyl ester < petroleum < chloroform < ethyl acetate.

Table (5): The effect of algal extracts on different species of bacteria

Bacteria / Extracts	*Escherichia coli*	*Sarcina lutea*	*Bacillus subtilus*	*Staphylococcus aureus*
Petroleum extract	**0.9**	**1.0**	**1.2**	**1.2**
Chloroform extract	**1.0**	**1.1**	**1.4**	**0.9**
Ethyl acetate extract	**-**	**-**	**1.5**	**1.1**
Unsaponifable acetate	**-**	**1.0**	**0.9**	**-**
Fatty acid methyl ester	**0.7**	**1.0**	**1.0**	**1.1**

Inhibition zones are indicated in cm.

Table (6) showed that all extracts exhibited antifungal activity. *Aspergillus niger*, *Chrysosporium* and *Fusarium* were more susceptible (1.0-2.2 cm) than *Candida albicans* (0.8-1.0 Cm). Petroleum extract had the more prominent effect on *Aspergillus niger* followed by the extract of fatty acid methyl ester on *Chrysosporium* sp. Unsaponifable acetate extract had also a good effect on *Fusarium* sp.

Table (6): The effect of algal extracts on the different fungal species

Fungi / Extracts	*Aspergillus niger*	*Fusarium* sp.	*Chyresosporium* sp.	*Candida albicans*
Petroleum extract.	**2.2**	**1.5**	**1.0**	**1.0**
Chloroform extract	**1.1**	**1.3**	**1.2**	**1.0**
Ethyl acetate extract	**1.7**	**1.8**	**1.3**	**0.8**
Unsaponifable acetate	**1.5**	**2.0**	**1.2**	**0.9**
Fatty acid methyl ester	**1.6**	**1.7**	**2.1**	**1.0**

Inhibition zones are indicated in cm.

The previous data is concomitant with Bandara *et al*. (1988) who found that 26 species from 35 species of seaweeds exhibited antibacterial and antifungal activities; where the more prominent species was *Ulva lactuca* (L.). Moreover, Mesmar *et al.* (1991) found that 11 marine algae exhibited antibacterial activity with special reference to *E. coli* and *Staphylococcus aureus*. Smyrniotopoulos, *et al*. (2003) isolated sixteen secondary metabolites from some green-algae with antimicrobial activity against some bacteria and some microalgae with potent clear inhibition zone, using petroleum extract, especially for *Enterobacter*

aprogenes, Klebsiela pnellmoniae, Psudomonas aeruginosa, Salmonella typhi, Salmonella cholerae-suis, Serratia marcescens, Vibrio cholerae, Bacillus subtilus and *Staphylococcus aureus.* Such results manifested that the nature of substances induced inhibition zones are lipid soluble substances, which have been investigated by many other authors e.g., Vitor *et al. (*2002).On a similar manner Olessen *et al*. (1963) investigated the antimicrobial activity of chloroform extract of *Eulhenbergia billebrandii* against *Staphylococcus aureus*.

Also Sastry and Rao (1994), found that antibacterial activity against Gram-positive and Gram-negative bacteria was related to successive extraction with benzene, chloroform and methanol. Likewise, Mahasneh *et al*. (1995) have shown that antimicrobial activity was indicated in organic extracts of 6 species of marine algae.

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الفحص الفيتوكيميائي والنشاط الضد ميكروبى لطحلب خس البحر

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جمعت عينات خس البحر خلال فبراير- أبريل 2003 بحيث تضمنت مراحل العمر المختلفة للطحلب (يولفا لكتيوكا) وبيئات متباينة فى قناة السويس وتم تجهيز العينات واستخلاصها بالكحول وفصل هذا المستخلص لعدد من المكونات تبعا لعدد من المذيبات العضوية. وتم التعرف على المكونات الكيميائية بها ونشاطها الضد ميكروبى لعدد من الميكروبات الفطرية والبكتيرية. وقد أسفرت النتائج عن نشاط ضد فطرى أكثر منه ضد بكتيرى. وقد كان تأثير المستخلص البترولى أكثر وضوحا على فطر الأسبرجلس نيجر. وقد أظهر مستخلص الميثيل استر تأثيرا أكثر على الكريزوسبوريم، بينما أثر مستخلص أستات الإثيل غير المتصبنة على فطر الفيوزاريوم وبكتريا ستافيلوكوكس. وبدت بكتريا بسلس سبتالس متأثرة بمكونات كل المذيبات المستخدمة فى هذا البحث. وباستخدام طرق التحليل GLC, GC/MS, and TLC تم فصل وتعريف 12 حمض دهنى على رأسها حمض بالمتك وأوليك ومارجريك وكانت نسبهم المئوية 70.34 ، 20.9 ، 2.26 على التوالى. أم الهيدروكربونات غير المتصبة فكان أعلاها تركيزا هو C-28 الذى بلغ 63.76٪.