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SCREENING FOR ANTIBACTERIAL ACTIVITES OF ALGA *ULVA* SP. EXTRACTS

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ABSTRACT: *In vitro screening of organic solvent (Methanol, ethyl acetate, dichloromethane, butanol and water) extracts of Ulva sp. was carried out against pathogenic bacteria. Butanol extract showed highest activity against both Gram positive and Gram negative bacteria such as Bacillus subtilis strain-AN5 (+++), Bacillus cereus strain-AN19 (++), Streptococcus sp.(++) and staphylococcus epidermis (++), Escherichia coli DH5á (+++), Escherichia coli 107 (++). Both water and ethyl acetate extracts showed moderate activity against tested bacteria. While dichloromethane extract inhibited the growth of Escherichia coli 107 (+) and Bacillus subtillus strain-AN5 (+).The result of qualitative detection of chemical constituency showed presence of alkaloids, tannin, saponin, steroid, terpenoid, flavonoid and cardic glycoside and absence of phlobatannin.*

Key words: *Anibacterial activity; Ulva sp.; seaweeds; Pathogenic bacteria.*

INTRODUCTION

Pharmaceutical industries are giving importance to the compounds derived from traditional sources (soil and plants) and less traditional sources like marine organisms (McGee, 2006). The biodiversity of marine ecosystem provides an important source of chemical compounds, which have many therapeutic applications such as antiviral, antibacterial, anti-fungal and anticancer activities (Caccamese and Azzolina, 1979; Perez *et al.,* 1990; Harada and Kamei, 1997; Siddhanta *et al.,* 1997; Pereira *et al.,* 2004). The ability of seaweeds to produce secondary metabolites of potential interest has been extensively documented (Faulkner, 2002). There are numerous reports of compounds derived from macro-algae with a broad range of biological activities, such as antibiotics (antibacterial and antifungal properties), antiviral diseases (Trono, 1999), antitumors and anti-inflammatories (Scheuer, 1990) as well as neurotoxins (Kobashi, 1989).

The revolutionized therapy of infectious diseases by the use of antimicrobial drugs has certain limitations due to changing patterns of resistance in pathogens and side effects they produced. These limitations demand for improved pharmacokinetic properties, which necessitates continued research for the search of new antimicrobial compounds for the development of drugs. Seaweeds are used in traditional remedies in many parts of the world. The production of inhibitory substances by seaweed was noted as early as in 1917. Since then, numerous studies have been carried out to find and extract antimicrobial compounds from marine algae.

The aim of the present study was to assess the antimicrobial activity of *Ulva sp.* Extracts of dichloromethane, ethyl acetate, n-butanol and water against pathogenic bacteria.

MATERIALS AND METHODS

1- Plant materials

The algae "seaweeds" were harvested in the spring of 2010 from an exposed rocky side near the western edge of Abu-Qir Bay, Alexandria, Egypt. Botanical identification was done using standard literature and taxonomic keys, all seaweeds were identified following Abbot and Hollenberg (1976), Taylor (1985) and Aleem (1993). Algal samples were cleaned of epiphytes, extraneous matter, and necrotic parts were removed. The samples were also washed successively with tap water, distilled water and air dried under shade for 2 weeks. The air dried samples were cut into small pieces and powdered in a mixer grinder.

2- Chemical extraction and fractionation

The alga powder (25 gm) was soaked with 75ml of 70% methanol for 48h at room temperature with occasional shaking .The extract was filtrated through a glass funnel with whatman No1filter paper. The remains of plant material were extracted with 70% methanol towice again to obtain the total extract. Finally total extract was obtained. The filtrate was evaporated to dryness at 40°C at oven.

The crude extract was dissolved in 50 ml distilled water. Aqueous extract fractionation by different solvents in 500 ml conical shape separating funnel. Dichloromethane, ethyl acetate and n-batanol were used in extract fractionation, three times and equal volumes of solvents were used sequentially in a similar+ manner. The fractions were evaporated to dryness at 40°C at oven. The crude extracts were stored at 4°C until further use. For antibacterial activity, extracts obtained with organic solvent and water extracts were prepared at concentration of 50 mg/1 ml of 2%Dimethylsulfoxide (DMSO).

3- Microorganisms

The strains used in this study *E.coli DH5á, E-coli 107, Bacillus subtilis strain-An5, Bacillus cereus strain -An19, Streptococcus sp.,* and *staphylococcus epidermis* were isolated from different sources(sewage, soil, patient) and kept on nutrient agar slants at 4°Cin our bacteriology lab until further use, Microbial Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), Minufiya University, characterized and identified to 16s rDNA level and deposited in the GenBank (NCBI) under accession numbers listed in Table (1). The bacterial strains were grown and maintained on nutrient agar slants.

4 - Antibacterial activity Test

The antibacterial activity assay was carried out following Bloor and England (1991). Antibacterial activity was achieved by Hole or Well technique. The cultures were grown in Lauria Bertani (LB) broth and incubated at 37°C for 24h. The O.D of the concentrated cultures was diluted and adjusted to 0.2 at 630 nm using spectrophotometer with sterile LB broth, 25µl for each plate from the adjusted culture were added to the sterile nutrient agar media before solidified, mixed well, poured in to sterile petri dishes and allowed to dry for 30 minutes. A disc of agar is punched out with a cork-borer or suitable instrument and the hole filled with 70 µl of algal extracts. Each test was prepared in triplicate. Negative control was prepared by using 2%Dimethylsulfoxide (DMSO). The plates were incubated for 24h at 37°C. After incubation, a clear zone around the well was evidence of antibacterial activity.

5-Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration (MIC) was performed according to the standard reference method (NCCLS, 2002). The cultures were grown in nutrient broth and incubated at 37°C for 24h. The optical density (O.D) of the cultures was adjusted to 0.2 at 630 nm using spectrophotometer with sterile LB broth. 20 ml of sterile nutrient agar media were poured in to sterile Petri plate and allowed to solidify. 25µl of adjusted culture were spread on the top of solidified media and allowed to dry for 30 minutes. A disc of agar is punched out with a cork-borer or suitable instrument.

The required concentration of the extract was dissolved in water + Dimethylsulfoxide (DMSO) (2%) and diluted to give serial two-fold dilutions that were added to each medium well. The plates were incubated for 24h at 37°C.The Minimum Inhibitory Concentration (MIC) for bacteria was determined as the lowest concentration of the compound inhibiting the visual growth of the test cultures on the agar plate. Three replications were maintained.

Table (1). The list of tested bacterial isolates used in the present study.

No	organism	Gram stain	Source/accession No
1	*Bacillus subtillus* strain-AN5	positve	JQ746509
2	*Bacillus cereus* strain-AN19	positve	JQ746508
3	*Streptococcus sp.*	positve	JX131631
4	*staphylococcus epidermis*	positve	*JX131632*
5	*Escherichia coli DH5á*	negative	Fermentas Cop.
6	*Escherichia coli* 107	negative	Fermentas Cop.

6- Qualitative Detection of the chemical constituency

Chemical tests were carried out on the aqueous extract and on the powdered specimens using standard procedures to identify the constituents as described by Sofowara (1993), Trease and Evans (1989) and Harborne (1973). The plant samples were air-dried and ground into uniform powder. The aqueous extract of each sample was prepared by soaking 100g of dried powdered samples in 200 ml of distilled water for 12h. The extracts were filtered using Whatman filter paper No1.

Test for tannins

About 0.5 g of the dried powdered samples were boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration.

Test for phlobatannins

Deposition of a red precipitate when an aqueous extract of each plant sample was boiled with 1% aqueous hydrochloric acid was taken as evidence for the presence of phlobatinins.

Test for saponin

About 2 g of the powdered sample were boiled in 20 ml of distilled water in a water bath and filtered. 10ml of the filtrate were mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

Test for flavonoids

5 ml of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract followed by addition of concentrated sulphoric acid . A yellow colouration observed in each extract indicated the presence of flavonoids. The yellow colouration disappeared on standing.

Test for terpenoids (Salkowski test)

Five ml of each extract were mixed in 2 ml of chloroform, and 3 ml concentrated sulphoric acid were carefully added to form a layer. A reddish brown colouration of the inter face was formed to show positive results for the presence of terpenoids.

Test for cardiac glycosides (Keller-Killani test)

Five ml of each extracts were treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

RESULTS AND DISCUSSION

The yield of total extract of plant material with 70% methanol was 1.5g. The yield of fractionations were: dichloromethane extract (0.4g), ethyl acetate extract (0.4g), butanol extracts (0.2g) and water extract (0.5g).

The extracts of *Ulva sp*. were tested against bacteria (*E.coli DH5á, E.coli 107, Bacillus subtilis strain-An5, Bacillus cereus strain-An19*, *Streptococcus sp*., and *staphylococcus epidermis*). The results of primary screening tests are summarized in Table (2), which showed that the extracts of *Ulva sp*. possessed antibacterial activity. The results are represented in Table 2 and Fig.1.

Results of Ulva extracts that tested as fractions against bacteria showed that butanol extract hold a promising antibacterial activity. Ethyl acetate extract showed activity against bacteria such as *E.coli DH5á*, *E.coli 107, Bacillus subtilis strain-An5* and *Bacillus subtilis strain-An19* and did not show any activity against *Streptococcus sp*., and *staphylococcus epidermis*. The results are represented in Table 3 and Fig. 2, 3, 4, 5 and 6.

Water extract inhibited the growth of tested bacteria moderately. Dichloromethane showed low activity against *E.coli DH5á* and *Bacillus subtilis strain-An5*. Maximum inhibition zone was observed in butanol extract.

Table 2: The antibacterial activity of the total extract of *Ulva* sp.

Test organism	Zone of inhibition
	50mg/2%DMSO
E.coli DH5á	+++
E.coli 107	++
Bacillus subtilis strain-An5	+++
Bacillus cereus strain - An19	++
Streptococcus sp.	++
staphylococcus epidermis	++

- No activity,
+ Low activity (7-10mm halo),
++ high activity (10-15 mm halo),
+++ to++++ highest activity (25-100mm halo)
Ciprofloxacin standard antibacterial agent (positive control)
2% DMSO negative control

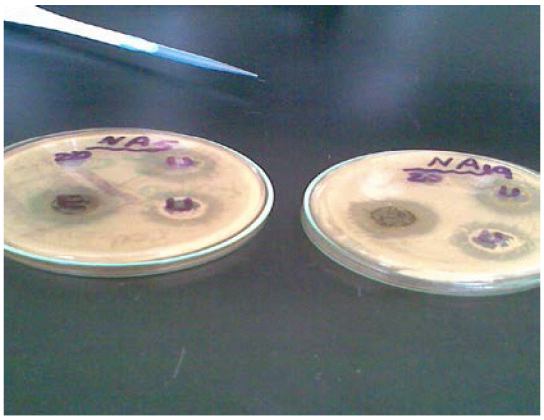

Fig.1. Antibacterial activity of total extracts on *Bacillus subtilis* strain-An5 and *Bacillus cereus* strain - An19.

Table 3. Antibacterial activity of the fractions extracted from *Ulva sp.*

fractions	Zone of inhibition					
	E.coli DH5á	*E.coli 107*	*B.subtilus strain-An5*	*B.cereus strain An19*	*Strep.sp.*	*Staph. epidermis*
Ethyl acetate	++	+	++	++	-	-
Dichloromethane	-	+	+	-	-	-
Butanol	++	+++	+++	++	++	++
Water extract	+	+	++	+	++	+

- No activity,
+ Low activity (7-10mm halo),
++ high activity (10-15 mm halo),
+++ to++++ highest activity (25-100mm halo)
Ciprofloxacin standard antibacterial agent (positive control)
2% DMSO negative control

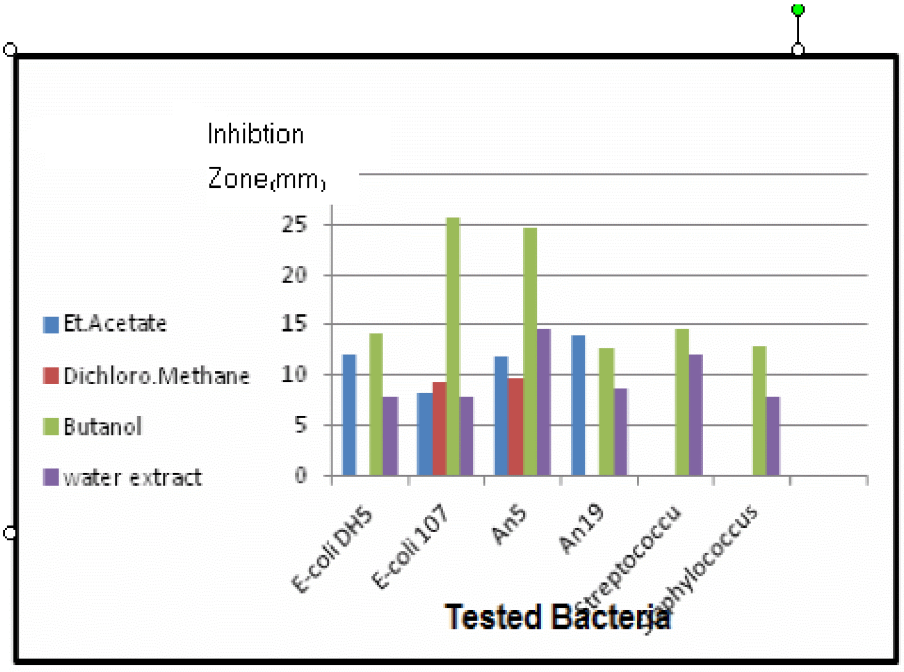

Fig.2. The antibacterial activity of the fractions extract of Ulva sp.

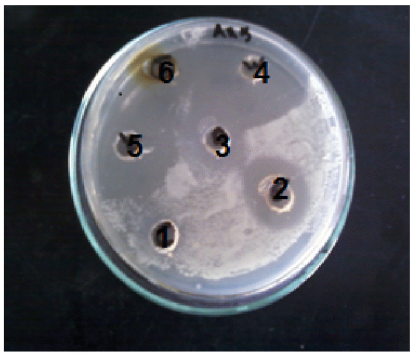

Fig.3. The antibacterial activity of the fractions extract on *Bacillus subtillus strain-An5*

1 Negative control(DMSO)
2 Ethyl acetate extract
3 Dichloromethane extract
4 water extract
5 Butanol extract
6 Positive control(ciprofloxacin)

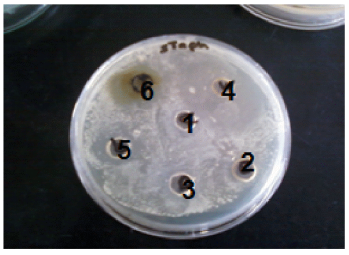

Fig.4 The antibacterial activity of the fractions extract on *staphylococcus epidermis.*

1 Negative control(DMSO)
2 Ethyl acetate extract
3 Dichloromethane extract
4 water extract
5 Butanol extract
6 Positive control(ciprofloxacin)

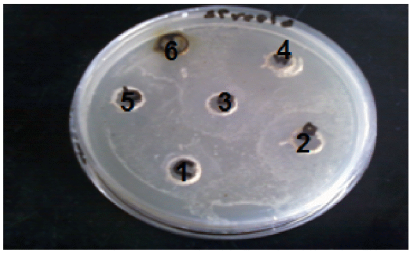

Fig.5 The antibacterial activity of the fractions extract on

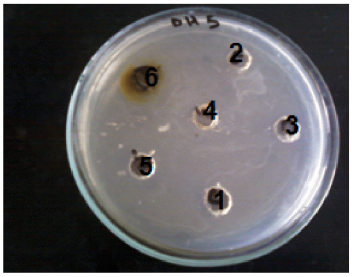

Fig.6 The antibacterial activity of the fractions extract on *E.coli DH5α*

1 negative control(2% DMSO)
2 Ethyl acetate extract
3 Dichloromethane extract
4 Water extract
5 Butanol extract
6 Positive control(ciprofloxacin)

The results of MIC tests are summarized in Table 4. The lowest MIC value of 2 mg/ml was obtained for *E.coli* 107 and *Bacillus subtilis* strain-An5.

The *Ulva* sp. was tested for presense of medicinally active constituents. The results of qualitative detection are summarized in Table 5.

Seaweeds are considered as such a source of bioactive compounds as they are able to produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities. Compounds with cyto static, antiviral, anthelmintic, antifungal and antibacterial activities have been detected in green, brown and red algae (Lindequist and Schweder, 2001; Newman *et al*., 2003).

Lipid-soluble extracts from marine macroalgae have been investigated as a source of substances with pharmacological properties. Moreover, several different organic solvents have been used to screening algae for all antibacterial activity (Mahasneh *et al*., 1995; Sukatar, 2006)

Algae antibiotics were first found in cultures of the green algae *Chlorella vulgaris* in 1948 and finally identified as fatty acid called chlorellin (reviewed by Jones, 1988). Later in 1959, the first marine phytoplankter which produced an antibiotic was found. This microalga, *Phaeocystis pouchetii*, produced an antibiotic called acrylic acid which inhibited both Gram-positive and Gram-negative bacteria (Sieburth, 1960). Subsequent surveys of antibacterial activity in algae have been reported all over the world. For example, there have been reports from the Mediterranean (Caccamese *et al*.,1980; 1981; 1985; Bernard &Pesando, 1981), Britain (Hornsey& Hide, 1974 and1976), India (Sreenivasa Rao& Parekh, 1981; Sreenivasa Rao&Shelat, 1982; Sreenivasa Rao *et al*., 1988), Australia (Reichelt &Borowitzka, 1984), Chile (Henriquez *et al.,* 1979) and Brazil (Campos-Takaki *et al.,*1988).

In the current study, butanol was the best solvent for extracting the bioactive compounds, meanwhile it gave the highest antibacterial activity against the selected pathogens. These results differ with those of Wefky and Ghobrial (2008) and Fareed and Khairy(2008), they found that acetone was the best solvent for extracting the bioactive compound . In contrast Tüney *et al.* (2006) demonstrated that diethyl ether yields higher antimicrobial activity than methanol, acetone and ethanol when extracting 11 seaweeds species from the coast of Urla.

Table 4. Values of MIC (mg/ml) of the crude extract of *Ulva sp.*

Test pathogen	MIC(mg/ml)
E.coli DH5á	4
E.coli 107	2
Bacillus subtilis strain-An5	2
Bacillus cereus strain - An19	4
Streptococcus sp.	6
staphylococcus epidermis	4

Table 5. The qualitative analysis of the phytochemicals of Ulva sp.

Chemical constituents	*Ulva* sp.
Alkaloids	+
Tannin	+
Saponin	+
Steroid	+
Phlobatannin	-
Terpenoid	+
Flavonoid	+

+ Presence of constituent
- Absence of constituent

Conclusion

The *in* vitro antibacterial activity of the marine green algae *Ulva lactuca* was examined against Gram-positive bacteria and Gram-negative bacteria. The butanol extract of algae exhibited a broad-spectrum of antibacterial activity. This result confirms the potential use of seaweed extracts as a source of antibacterial compounds or as a health-promoting food for aquaculture (Hae Kim *et al.*, 2007).

The present investigation concluded that butanol and ethyl acetate extracts showed activity against bacteria. They are potential sources of bioactive compounds and should be investigated for natural antibiotics.

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دراسه لنشاط مستخلصات طحلب ulva كمضاد للبكتريا

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الملخص العربي

تهدف الدراسه الى دراسه تاثيرمستخلصات طحلب Ulva على البكتريا الممرضه وأظهرت نتيجه الفحص المختبرى للمستخلصات العضويه (الميثانول ، إيثيل أسيتات، ثنائي كلورو ميثان و البيوتانول) والمائيه من مستخاص طحلب .ulva sp ضد البكتريا الممرضه

E.coli DH5α,

E-coli 107, Bacillus subtilis strain-An5, Bacillus cereus strain -An19, Streptococcus sp., staphylococcus *epidermis*

وأن مستخلص البيوتانول يعطى اعلى نشاط ضد البكتريا موجبه وسالبه الجرام وتظهر النتيجه أن (Bacillus subtilis strain-AN5 (+++), Bacillus cereus strain-AN19 (++), Streptococcus sp. (++) and staphylococcus epidermis (++), Escherichia coli DH5α (++), Escherichia coli 107(+++)). بينما يعطى مستخلص الماء ومستخلص الايثيل أسيتات نشاط معتدل ضد البكتريا المختبره. فى حين أن مستخلص ثنائى كلورو الميثان يثبط نمو E-coli 107(+) و Bacillus subtillus strain-AN5(+). وظهر نتيجه الاظهار الوصفى للمحتويات الكيميائيه وجود القلويات والتانين والستيرويد سابونين و Terpenoid و الفلافونيد و cardic glycoside وغياب phlobatannin.

ومن خلال النتائج السابقه تبين وجود فاعليه لمستخلصات طحلب Ulva كمضادات لبعض انواع البكتريا الممرضه(*E.coli DH5α,E -coli 107, Bacillus subtilis strain-An5, Bacillus cereus strain -An19, Streptococcus sp., Staphylococcus epidermis)*