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Antibacterial Activity of Extracts from Selected Marine Algae in Bahrain

النشاط البيولوجي المضاد للبكتيريا لبعض الطحالب البحرية في البحرين

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Abstract: In this study, the bioactivity of some algal extracts were evaluated *in vitro* against different bacterial strains. Five commonly occurring benthic algae, namely *Cystoseira myrica, Digenea simplex, Hormophysa triquetra*, *Sargassum cervicorne* and *Sarconema filiforme*, were collected from Bahrain's coastline in July 2007. A total of six chemical extracts were derived from those algal species, four of which were prepared by Soxhlet (petroleum ether, chloroform, ethyl acetate, methanol), aqueous and crude methanol. Their bioactivity was assessed against four testing bacterial strains using the agar well diffusion assay and expressed as the diameter of inhibition zone (mm). Results revealed that the petroleum ether extract of *D. simplex* exhibited the highest inhibition zone (24.3 mm) against *Pseudomonas aeruginosa* while the aqueous extract of *C. myrica* exhibited the lowest inhibition zone (13.3 mm) against *Staphylococcus aureus*. The Soxhlet extracts of all the algal species were biologically active only against *P. aeruginosa*. The aqueous extracts showed an inhibitory activity against *S. aureus* only. The crude methanolic extract was biologically broadly active on a wide range of tested bacteria, *P. aeruginosa*, *Escherichia coli* and *Bacillus subtilis*. The bacterium *P. aeruginosa* was the most susceptible microbe whereas *S. aureus* was the most resistant. On the average, The gram-negative bacteria tested were more sensitive than the gram-positive bacteria towards the extracts of the algal species used. The algal species that belong to the red algae class Rhodophyceae were more biologically active than algal species that belong to the brown algae class Phaeophyceae.

Keywords: *Algae, antibacterial Activity, Bahrain*.

المستخلص: هدفت هذه الدراسة إلى تقييم النشاط البيولوجي المضاد للبكتيريا لمستخلصات بعض الطحالب البحرية. فقد جمعت عينات لخمسة أنواع من الطحالب القاعية الشائعة من سواحل البحرين وهي *Cystoseira myrica. Digenea simplex. Hormophysa triquetra. Sargassum cervicorne* و *Sarconema filiforme* في يوليو 2007. و قد تم تحضير ستة مستخلصات كيميائية مختلفة من هذه الطحالب أربع منها باستخدام جهاز الاستخلاص (Soxhlet) وهي الايثر البترولي و الكلوروفورم و الايثيل اسيتيت والميثانول بالإضافة إلى المستخلص المائي و مستخلص الميثانول الخام. أختبر النشاط البيولوجي لهذه المستخلصات ضد أربعة أنواع من البكتيريا و ذلك باستخدام طريقة الانتشار في الآجار. و تم التعبير عنه بقياس قطر المنطقة

المثبطة لنشاط البكتيريا. تشير النتائج إلى أن مستخلص الايثر البترولي لطحلب *D. simplex* أظهر أعلى نسبة نشاط ضد بكتيريا *P. aeruginosa* حيث بلغ قطر المنطقة المثبطة 24.3 مم، بينما سجل المستخلص المائي لطحلب *C. myrica* أقل نسبة نشاط ضد *S. aureus* (قطر المنطقة المثبطة ب 13.3 مم). في المقابل وجد أن جميع مستخلصات Soxhlet لكل الطحالب المستخدمة كانت نشطة بيولوجيا ضد بكتيريا *P. aeruginosa* فقط. من جانب آخر لوحظ ان جميع المستخلصات المائية كانت فعالة ضد بكتيريا *S. aureus* فقط , بينما اثبت مستخلص الميثانول الخام فعاليته ضد مجموعة واسعة من البكتيريا المستخدمة في الدراسة *E. coli*، *P. aeruginosa* و *B. subtilis*. بشكل عام لوحظ أن بكتيريا *P. aeruginosa* السلبية التصبغ الجرامي كانت أكثر الأنواع حساسية بينما بكتيريا *S. aureus* الايجابية التصبغ الجرامي كانت أكثرها مقاومة للنشاط البيولوجي لهذه الطحالب. أما بالنسبة لأنواع الطحالب، فتشير النتائج إلى أن الطحالب الحمراء كانت أكثر فعالية في نشاطها البيولوجي من الطحالب البنية.

كلمات مدخلية : *الطحالب، النشاط البيولوجي المضاد للبكتيريا، البحرين.*

INTRODUCTION

Macroalgae, generally termed seaweeds, produce a variety of natural products with economic value. These constituents are used as food, hydrocolloids, pharmaceuticals, cosmetics, and as agricultural product additives. Arabian Gulf algae are rich in primary and secondary metabolites. Primary metabolites include photosynthetic pigments, reserve carbohydrates that provide energy, structural carbohydrates and lipids, plus nitrogen-containing compounds. Secondary metabolites, in contrast, are defined here as compounds that mediate communication among living organisms and other 'nonessential' compounds (Rizk, *et al*. 1999).

Iinterest in marine organisms as a potential and promising source of pharmaceutical agents has increased recently (Lindequist and Schweder, 2001, and Newman, *et al*. 2003). Seaweeds are considered 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 cytostatic, antiviral, antitumor, antihelminthic, antifungal, and antibacterial activities have been detected in green, brown and red algae (Lindequist and Schweder; 2001 and Newman, *et al*. 2003). There are numerous reports concerning the inhibiting activities of macroalgae against human pathogens, fungi and yeasts, but few contain data about effects against fish pathogens Sridhar and Vidyavathi, 1991; Mahasneh, *et al*. 1995; Choudhury, *et al*. 2005).

Due to the evolving resistance of microorganisms to existing antibiotics, there is an increasing need for new antibiotics, not only for humans, but also for veterinary medicine. Competition for space and nutrients led to the evolution of antimicrobial defense strategies in the aquatic environment. Therefore, aquatic organisms such as seaweeds offer a particularly rich source of potential new drugs and antibiotics. (Bansimer, *et al*. 2004).

Many species of marine algae have now been screened and tested against common disease-causing bacteria, fungi and protozoans. In these studies, the test organisms included gram-positive bacteria such as *Staphylococcus aureus* and *S. pneumoniae,* gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*, fungi such as *Trichophyton mentagrophytes,* the yeast *Candida albicans,* as well as the *protozoan, Trichomonas foetus* (Mshigeni ,1991).

These studies have revealed that many species of seaweeds are biologically active against many common disease pathogens. Among the Green Algae (or the Chlorophyta), biologically active members include species of the genera *Codium, Halimeda, Ulva, Cladophoropsis Caulerpa* and *Enteromorpha*. Among Phaeophyta (Brown Algae) taxa with antimicrobial activity include species of *Dictyopteris, Zonaria, Ecklonia, Durvillea, Dictyota, Sargassum* and *Turbinaria*. Amongst the Rhodophyta (Red algae) microbial activity has been detected within species of *Chondria, Digenia, Laurencia, Caloglossa, Grateloupia, Hypnea* and *Murayella* (Mshigeni, 1991).

The marine environment is a wide and largely unexplored environment. Eighty percent of all life forms on earth are present only in the oceans. In addition to the biodiversity of the marine environment, there is a large chemical

diversity and genetic uniqueness. Marine organisms often incorporate halogens (F, Cl, Br, I) into their chemical structures , a phenomenon rarely seen in terrestrials (Newman, 2004; Carte, 1996). Occasionally, a large number of brown and red macroalgae grow on the seashores around Bahrain. Their bioactivity has never been studied so there are no reports about their antimicrobial activity. This paucity of knowledge initiated the current investigation. Five common macroalgae widely distributed around Bahrain in the summer season were selected on which to conduct preliminary screening for antibacterial activity. The objective of the study was to assess antibacterial activity of some extracts of the most common algal species in the summer season from the coast of Bahrain against some gram negative and gram positive bacteria using the agar well diffusion assay.

MATERIAL AND METHODS

Collection of Algae

Based on availability, abundance and purity, five common algae were collected from different intertidal areas around Bahrain (Figure 1) during July, 2007 at mid low tide (Table 1). Each algal species was collected from at least five locations, approximately 15 to 20 meters apart, then combined in one composite sample. The composite sample was placed in a clean polyethylene bag, properly labeled and immediately refrigerated at 5°C until processing, usually within a week. The algal material was identified according to Basson, *et al* (1989). Representative voucher specimens were preserved in 5% formalin solution (Fluka, Purum, Flpt. 64°C) and deposited in the Dr. Qaher Mandeel Laboratory, Department of Biology, University of Bahrain, Sakhier. Acquisition code numbers are listed in Table (1).

Table 1. Data of the algal species used in the antibacterial assay.

Sampling code	Algae name	Phyla	Color at collection	Collection site	Collection code[a]	Date
S1	*Sarconema filiforme*	Rhodophyceae	Red	Arad	HAQ108	08 July 2007
S2	*Sargassum cervicorne*	Phaeophyceae	Olive	Karbabad	HAQ107	07 July 2007
S4	*Cystoseira myrica*	Phaeophyceae	Brown	Askar	HAQ110	10 July 2007
S5	*Digenea simplex*	Rhodophyceae	Brownish orange	Al Dor	HAQ210	10 July 2007
S7	*Hormophysa triquetra*	Phaeophyceae	Brownish green	Zallaq	HAQ101	01 July 2007

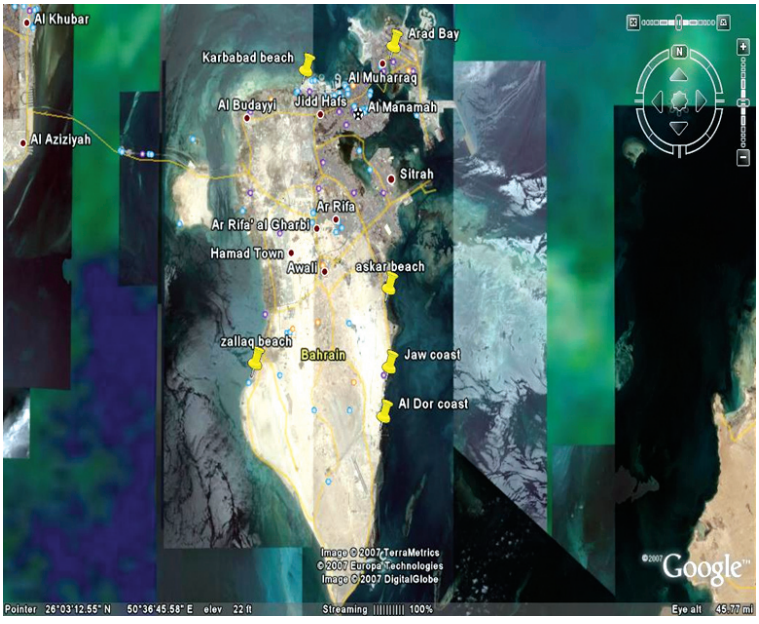

Fig. 1. A satellite image of Bahrain. Pins show the sites of algae collection around Bahrain's coastline.

Preparation of Algae Samples for Extraction

Each composite algal sample was washed and cleaned from extraneous materials like substrate and shells at least three times using distilled deionized water. Algal parts mostly thallus from the same species were separated. Water was strained from Algae using clean paper towel then dried in a drying cabinet at 50±1°C on clean, sterilized paper towels for 48 hours. The dried samples were later ground into a fine powder using a household blender and sieved using 450 mm screen mesh.

The Extraction of Agae Samples

Six types of extracts were prepared from each composite processed algal sample. These were aqueous extracts, crude methanolic extracts and four Soxhlet extracts. The prepared Soxhlet extracts were petroleum ether (Fluka, 60-80°C), chloroform (Fluka, Purum), ethyl acetate (Fluka, Purum) and methanol (Fluka, Purum) (Figure 2).

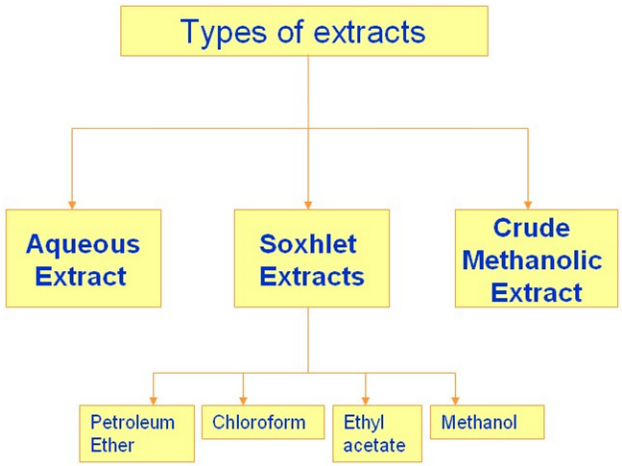

Fig. 2. A flowchart showing the types of the extracts prepared and used in the study.

Preparation of Soxhlet Extracts

Pulverized material was extracted sequentially using a range of solvents from the least polar solvent to the most polar solvent with a Soxhlet. The solvents were arranged in the following order: petroleum ether, chloroform, ethyl acetate and methanol. The extraction procedure by each solvent was carried out following the method of Mandeel and Taha's method (2005), slightly modified. Five grams of dried pulverized material of each alga was extracted in Soxhlet in 250 ml of each solvent. Consequently, each extract was filtered through Whatman® filter paper No. 4. The filtrate was dried by complete evaporation of the solvent in a vacuum rotary evaporator (Buchi) at 40-43°C using a water bath. The dried solid residue for each alga was weighed to calculate the percentage yield (Table 2). All solid residues were each redissolved in 10 ml ethyl acetate, except the methanol extracts which were redissolved in 10 ml ethanol. A volume equivalent to 15 mg was collected from each extract and its volume adjusted to 3 ml to adjust the concentration to 0.5 mg/0.1ml (Figure 3).

Preparation of Crude Methanolic Extracts

For the crude methanolic extract, a 2 g sample of algae powder was soaked in 40 ml of methanol for 2 hours in a water bath at 40°C. The mixture was left overnight at room temperature then filtered through Whatman® filter paper No. 4. The solvent was evaporated in *vacuo* at 40°C in a water bath. The dry solid residue was dissolved in 10 ml methanol. A volume equivalent to 40 mg was collected from each extract and its volume adjusted to 2 ml to adjust the concentration to 2 mg/0.1ml. The extract was leater dried again under vacuum in a dessicator. The dried extract was redissolved in 2 ml of dimethylsulphoxide (DMSO) (BDH, Analar).

Preparation of Aqueous Extracts

In preparation of the aqueous extract, 2 g of algae powder was soaked in 50 ml of deionized water for two hours in a water bath at 45°C. The mixture was then cooled and filtered through Whatman® filter paper No. 4. The filtrate was freeze-dried using a LABCONCO© freeze dryer (-86 °C, 0.1 mbar). A volume equivalent to 30 mg was collected from each extract and its volume diluted with sterile distilled water to 3 ml to adjust the concentration to 1 mg/0.1ml. Preliminary attempts to dissolve the dried aqueous extracts in ethanol and DMSO proved unsuccessful.

Table 2. Percentage of total yield from the various algal extracts [a].

Algal species	Extract types					
	Petroleum ether	Chloroform	Ethyl acetate	Methanol	Crude Methanol	Aqueous
Sarconema filiforme	0.404	1.872	0.696	0.0580	0.965	2.600
Sargassum cervicorne	0.804	2.748	0.360	0.0964	0.860	2.495
Cystoseira myrica	1.384	0.328	1.596	0.1618	2.000	4.845
Digenea simplex	0.920	1.370	1.818	0.0560	0.595	1.635
Hormophysa triquetra	1.230	2.286	1.136	0.1634	1.670	4.270

[a] % extract = (mass of final dried extract/mass of initial dry material) x 100.

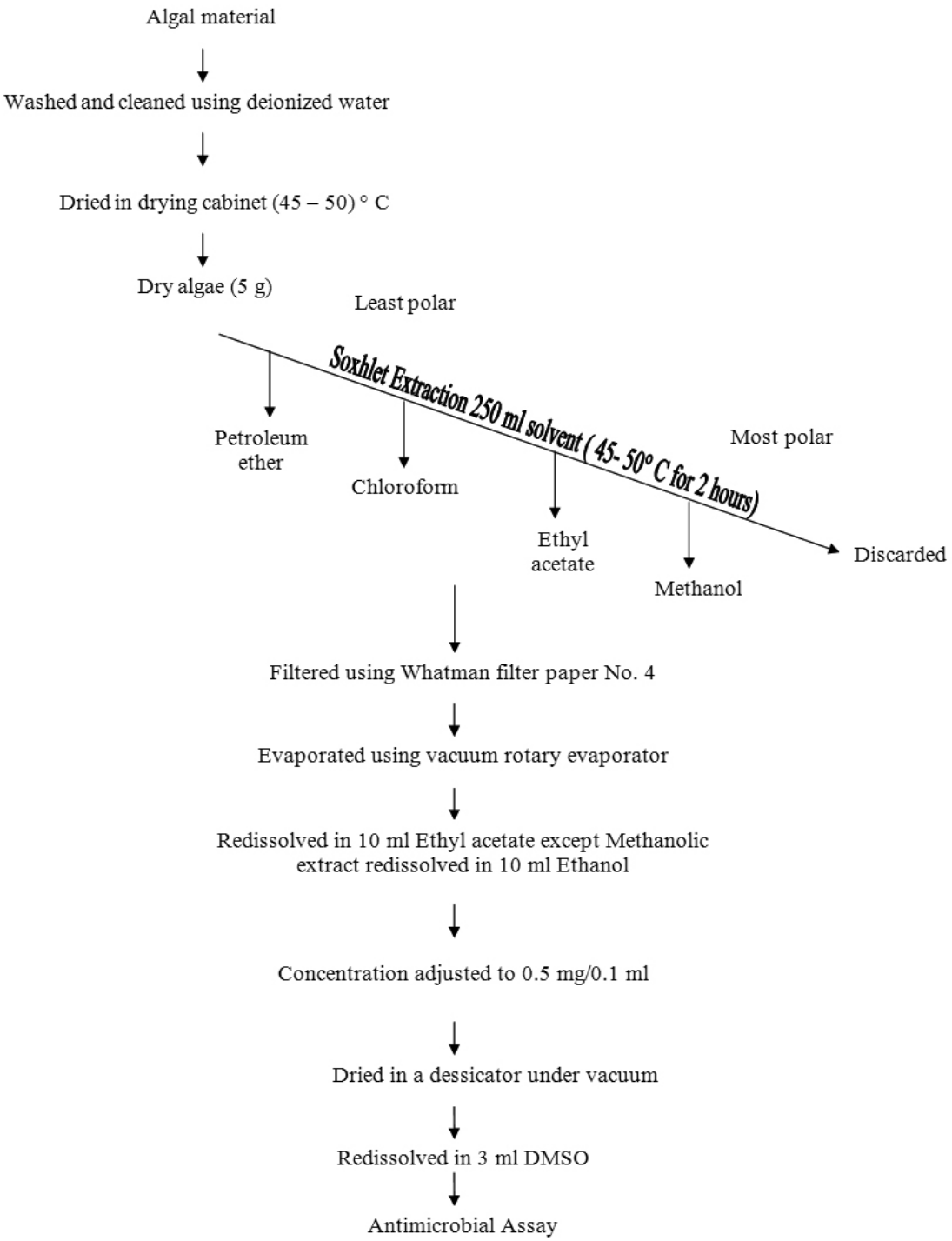

Fig. 3. Flowchart of the Soxhlet extraction technique.

Preparation of the Final Concentration

With the exception of the methanolic and crude methanolic algal extracts dissolved in 10 ml ethanol, all other chemical extracts were individually transferred to small vials and suspended in 10 ml ethyl acetate which was considered the stock solution of the extracts. The aqueous algal extracts were dissolved in sterile distilled water only. The crude methanolic and Soxhlet algal extracts were dissolved in DMSO to obtain the desired concentration. The final concentrations for the Soxhlet, aqueous and crude methanolic extracts were 0.5, 1 and 2 mg/0.1 ml, respectively. Initially 0.5 mg/0.1 ml was used for screening as a standard concentration for all the extracts. Since no bioactivity was observed for the aqueous and crude methanolic extracts, higher concentrations were used to observe bioactivity. Moreover, the above concentrations were the highest obtainable from the dried extract material.

Test Bacteria and Growth Conditions

The following four bacterial strains were used as testing microorganisms for antibacterial activity assay (Table 3). All bacteria were obtained from the American Type Culture Collection (ATCC) as lyophilizates in ampoules. Gram positive species included *Bacillus subtilis* (ATCC 6051) and *Staphylococcus aureus* (ATCC 6538). The Gram negative species included *Escherichia coli* (ATCC 11775) and *Pseudomonas aeruginosa* (ATCC 10145). Cultures of these bacteria were grown on 25 ml nutrient agar (Oxoid) plates and incubated for 24 hours at 37°C. For each experimental replicate, new cultures of lyophilized ampoules were used to assure the highest bacterial energy potential.

Antibacterial Activity Assay

The diluted extract of each of the six prepared algal extracts was employed to screen for antibacterial activity against previously named bacterial strains .The procedure used is essentially the modified agar well diffusion method) Mandeel and Taha .(2005 ,Overnight nutrient broth cultures of bacteria10^5) per 0.5 ml) were aseptically mixed with 20 ml of nutrient agar cooled down to 50°C in plastic Petri dishes. Plates were allowed to stand for 10 minutes at room temperature then wells of 7 mm in diameter were made in the solidified agar medium with a sterilized steel cork borer. Fifty μl of each algal extract was slowly loaded into the wells by micropipettes with sterile tips, then incubated for 24 hours at 37°C. Each experiment was repeated at least twice and the diameter of inhibition zone surrounding the agar well for each plate was averaged and expressed in mm.

Code numbers were used to maintain the blinded identity of the plates Appropriate treatments, including wells loaded only with sterilized deionized water or DMSO, were considered as negative controls. For comparative purposes, standard antibacterial disks served as positive controls. Each experiment was carried out in triplicate and repeated simultaneously at least twice. The results were recorded by measuring the zone of growth inhibition around the agar well and expressed in mm diameter. The mean of the three readings per zone was calculated and the standard deviation of all replicates was determined.

Table 3. Bacterial strains used in antimicrobial assay.

Bacterial strain	ATCC code No.	Gram stain	Biosafety level	Isolation status
Bacillus subtilis	ATCC 6051	Gram +ve	1	Not mentioned
Staphylococcus aureus	ATCC 6538	Gram +ve	2	Human lesion
Escherichia coli	ATCC 11775	Gram -ve	1	Urine
Pseudomonas aeruginosa	ATCC 10145	Gram -ve	2	Not mentioned

Reference: American Type Culture Collection web site www.ATCC.org

RESULTS

Results of the *in vitro* evaluation of various standard antibiotic disks against two Gram positive and two Gram negative bacteria are given in Table 4. Among Gram negative bacteria, *E. coli* showed the highest susceptibility level of 36.0 mm inhibition zone diameter to Cefoperazone 75 μg (CFP75) followed by *S. aureus* with 35.3 mm inhibition zone against the same antibiotic standard. In general, uise of the Cefoperazone 75 μg standard antibiotic resulted in the highest inhibitory activity against all the tested bacteria. On the other hand, the lowest inhibitory activity was found using Streptomycin 10 μg (S10) against *S. aureus* with 13.0 mm inhibition zone followed by Novobiocin 30 μg (NB30) against *P. aeruginosa* with 17.0 mm inhibition zone. The average inhibitory activity of other antibiotic standard among all other bacteria is 21.3 mm**.**

Data on the effect of crude methanolic and aqueous chemical extracts of five selected marine algae from Bahrain tested on four Gram-positive and Gram-negative bacteria as mean inhibition zone (mm) is presented in Table 5. Overall, the crude methanol extracts yielded higher antibacterial activity than aqueous extracts. For the aqueous extracts, the inhibitory effect was observed against *S. aureus* bacteria only. The highest antibacterial activity was observed for the algal species *D. simplex* aqueous extract against *S. aureus* with 15.7 mm inhibition zone followed by *S. filiforme* and *S. cervicorne* with 14.0 mm inhibition zone. While *C. myrica* aqueous extract resulted in the lowest inhibition zone (13.3 mm). No effect of *H. triquetra* extract was found against *S. aureus*.

Table (5) also depicts the effect of the crude methanolic extract of the five different algae against Gram positive and Gram negative bacteria. The inhibitory effect of the crude methanolic extracts varied from as low as 15.3 mm to as high as 19.0 mm inhibition zone. The crude methanolic extracts of all algal species exhibited no antibacterial activity against *S. aureus*. The bioactivity of the crude methanolic extracts was mainly restricted to *E. coli* and *P. aeruginosa* which are Gram negative bacteria, and *B. subtilis* which is a Gram positive bacterium. The highest bioactivity was observed for the crude methanolic extracts of *H. triquetra* against *E. coli* with 19.0 mm inhibition zone. The crude methanolic extracts of *C. myrica* and *S. cervicorne* exhibited a similar inhibitory effect (18.3 mm inhibition zone) against different bacteria, *P. aeruginosa* and *B. subtilis*. The inhibitory effect of the crude methanolic extracts of *C. myrica*, *H. triquetra*, *S. filiforme* and *D. simplex* was shown as (17.7, 17.3, 17.3 and 17.0 mm inhibition zones) against *E. coli*, *P. aeruginosa*, *E. coli* and *B. subtilis*, respectively (Table 5). Preliminary experiments performed using crude methanolic and aqueous extracts at a concentration of 0.5 mg/0.1 ml of all the algal species showed no biological activity against the tested bacterial strains.

The antibacterial activity of the five algal Soxhlet chemical extracts against two Gram-positive and two Gram-negative bacteria is presented in (Table 6.) Biological activity was limited only to *P. aeruginosa*. Bioactivity ranged from a 14.3 mm to a 24.3 mm inhibition zone. The highest bioactivity was observed with the petroleum ether extracts of *D. simplex*, *S. filiforme* and *C. myrica* with 24.3 mm, 23.7 mm and 20.0 mm inhibition zone diameter, in that order. While the lowest activity was found in chloroform and petroleum ether extracts of *H. triquetra* and *S. cervicorne*, 14.3 mm and 15.0 mm inhibition zone, respectively. It is interesting to note that the methanolic extract of three algal species, namely *C. myrica*, *D. simplex* and *H. triquetra*, yielded almost similar antibacterial activity with an average of 15.2 mm which is low compared to other Soxhlet extracts.

Figure (4) shows the mean antibacterial inhibition zone (mm) of the algal species among all the extracts evaluated and bacterial strains used in this study. The highest activity was shown by *D. simplex* with a 17.7 mm mean inhibition zone followed by a 16.9 mm inhibition zone for *C. myrica* and a 16.8 mm inhibition zone for *S. filiforme*. On the other hand, *H. triquetra* and *S. cervicorne* showed an almost similar mean inhibition zone (15.7 mm).

The antibacterial inhibition zone (mm) of each chemical extract of the algal species against all the tested bacteria is shown in (Figure 5). The greatest activity was indisputably revealed by the petroleum ether extract with 20.6 mm

mean inhibition zone while the least activity was observed in the aqueous extract with a 14.3 mm mean inhibition zone. The methanolic and crude methanolic extracts showed an almost similar inhibitory effect with an average mean inhibition zone of 15.7 mm.

Figure (6) presents the susceptibility and resistance of the bacterial strains tested in this study to all the algal species and all chemical extracts evaluated and expressed as mean inhibition zone (mm). Results show *P. aeruginosa* the most susceptible bacteria with a 17.4 mm mean inhibition zone followed by *E. coli* with a 17.1 mm mean inhibition zone. The least susceptible bacterium (most resistant) is *S. aureus* with a 14.3 mm mean inhibition zone.

A general comparison between the susceptibility of bacterial strains based on Gram stain characteristics - Gram-positive or Gram-negative - on the mean inhibition zone (mm) among the extracts of all the algal species used in this study is shown in (Figure 7). The figure clearly illustrate the gram-negative bacteria (17.25 mm) tested in this study were more susceptible to algal extracts than Gram-positive bacteria (15.4 mm).

Having classified the algal species used into Phaeophytes and Rhodophytes, their antibacterial activity against all bacterial species used is plotted as a mean inhibition zone (mm) in Figure (8). Results clearly show Rhodophyceae extracts had greater bioactivity (17.15 mm) than Phaeophyceae extracts (16.1 mm) mean inhibition zone.

Table 4. Antibacterial activity of some standard antibiotic disks as positive control against tested microorganisms (mean ± SD.).

Antibiotic standard [a]	Diameter of inhibition zone (mm)							
	E. coli		*P. aeruginosa*		*B. subtilis*		*S. aureus*	
	mean	SD	mean	SD	mean	SD	mean	SD
TM10	21.0	± 2.6	21.0	± 2.0	20.0	± 1.0	21.0	± 1.7
FD300	32.3	± 1.5	0.0	± 0.0	27.0	± 1.0	31.7	± 1.5
CC2	0.0	± 0.0	0.0	± 0.0	17.3	± 0.6	0.0	± 0.0
NB30	17.7	± 0.6	17.0	± 0.0	31.7	± 1.2	20.3	± 4.5
CFP75	36.0	± 1.0	33.3	± 1.2	26.3	± 0.6	35.3	± 0.6
T30	30.3	± 0.6	21.3	± 0.6	24.7	± 0.6	21.3	± 0.6
NA30	34.0	± 1.0	21.3	± 0.6	30.7	± 0.6	34.0	± 0.0
S10	19.3	± 0.6	0.0	± 0.0	21.7	± 0.6	13.0	± 1.0

[a] TM10 : Tobramycin 10 µg, FD300 : Nitrofurantoin 300 µg, CC2 : Glindomycin 2 µg, NB30 : Novobiocin 30 µg, CFP75 : Cefoperazone 75 µg, T30 : Oxytetracycline 30 µg, NA30 : Nalidixic Acid 30 µg, S10 : Streptomycin 10 µg.

Table 5. Antibacterial activity of crude methanolic and aqueous algal extracts against 2 Gram-negative and 2 Gram-positive bacteria (Mean ± SD).

Algal species	Mean inhibition zone diameter (mm)							
	Extract	*B. subtilis*		*S. aureus*		*P. aeruginosa*		*E. coli*	
Sarconema filiforme	CME	0.0	± 0.00	0.0	± 0.00	16.0	± 1.00	17.3	± 0.58
	AQU	0.0	± 0.00	14.0	± 1.00	0.0	± 0.00	0.0	± 0.00
Sargassum cervicorne	CME	15.3	± 0.47	0.0	± 0.00	18.3	± 0.58	16.3	± 0.58
	AQU	0.0	± 0.00	14.0	± 1.00	0.0	± 0.00	0.0	± 0.00
Cystoseira myrica	CME	18.3	± 0.47	0.0	± 0.00	15.3	± 1.15	17.7	± 0.58
	AQU	0.0	± 0.00	13.3	± 1.53	0.0	± 0.00	0.0	± 0.00
Digenea simplex	CME	17.0	± 0.82	0.0	± 0.00	15.3	± 0.58	15.3	± 0.58
	AQU	0.0	± 0.00	15.7	± 0.58	0.0	± 0.00	0.0	± 0.00
Hormophysa triquetra	CME	15.3	± 0.58	0.0	± 0.00	17.3	± 0.58	19.0	± 0.00
	AQU	0.0	± 0.00	0.0	± 0.00	0.0	± 0.00	0.0	± 0.00

Extract type: CME: Crude methanol or AQU: Aqueous.

Table 6. Antibacterial activity of four Soxhlet algal extracts against two Gram-negative and two Gram-positive bacteria (Mean ± SD).

Algal species	Mean Inhibition Zone Diameter (mm)								
	Extract	*B. subtilis*		*S. aeureus*		*P. aeruginosa*		*E. coli*	
Sarconema filiforme	MET	0.0	± 0.0	0.0	± 0.0	0.0	± 0.00	0.0	± 0.0
	ETH	0.0	± 0.0	0.0	± 0.0	17.7	± 0.58	0.0	± 0.0
	CHL	0.0	± 0.0	0.0	± 0.0	0.0	± 0.00	0.0	± 0.0
	PET	0.0	± 0.0	0.0	± 0.0	23.7	± 1.53	0.0	± 0.0
Sargassum cervicorne	MET	0.0	± 0.0	0.0	± 0.0	17.7	± 0.58	0.0	± 0.0
	ETH	0.0	± 0.0	0.0	± 0.0	16	± 1.00	0.0	± 0.0
	CHL	0.0	± 0.0	0.0	± 0.0	17.3	± 0.58	0.0	± 0.0
	PET	0.0	± 0.0	0.0	± 0.0	14.3	± 0.58	0.0	± 0.0
Cystoseira myrica	MET	0.0	± 0.0	0.0	± 0.0	15.0	± 1.00	0.0	± 0.0
	ETH	0.0	± 0.0	0.0	± 0.0	16.7	± 1.53	0.0	± 0.0
	CHL	0.0	± 0.0	0.0	± 0.0	19.3	± 0.58	0.0	± 0.0
	PET	0.0	± 0.0	0.0	± 0.0	20.0	± 1.00	0.0	± 0.0
Digenea simplex	MET	0.0	± 0.0	0.0	± 0.0	15.3	± 0.58	0.0	± 0.0
	ETH	0.0	± 0.0	0.0	± 0.0	16.3	± 0.58	0.0	± 0.0
	CHL	0.0	± 0.0	0.0	± 0.0	18.3	± 0.58	0.0	± 0.0
	PET	0.0	± 0.0	0.0	± 0.0	24.3	± 0.58	0.0	± 0.0
Hormophysa triquetra	MET	0.0	± 0.0	0.0	± 0.0	15.0	± 1.00	0.0	± 0.0
	ETH	0.0	± 0.0	0.0	± 0.0	0.0	± 0.00	0.0	± 0.0
	CHL	0.0	± 0.0	0.0	± 0.0	14.3	± 0.58	0.0	± 0.0
	PET	0.0	± 0.0	0.0	± 0.0	0.0	± 0.00	0.0	± 0.0

[a] Extract type: MET: Methanol; ETH: Ethyl acetate; CHL: Chloroform; PET: Petroleum ether.

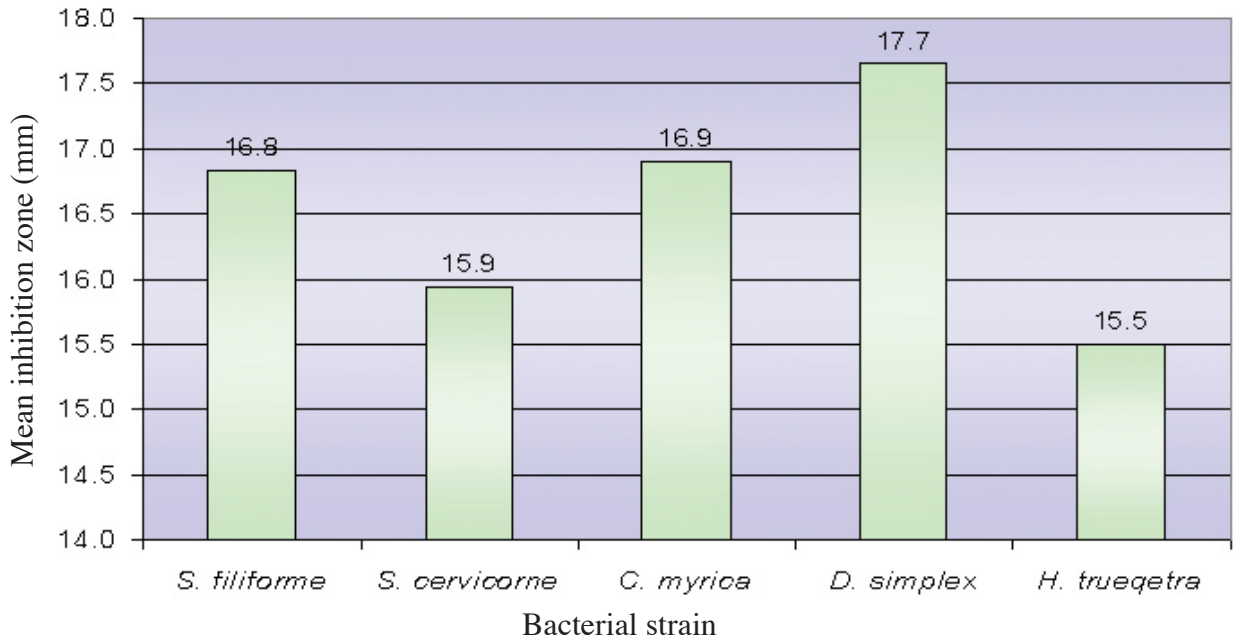

Fig. 4. Mean antibacterial inhibition zone (mm) of all algae extracts evaluated against each bacterial strain tested.

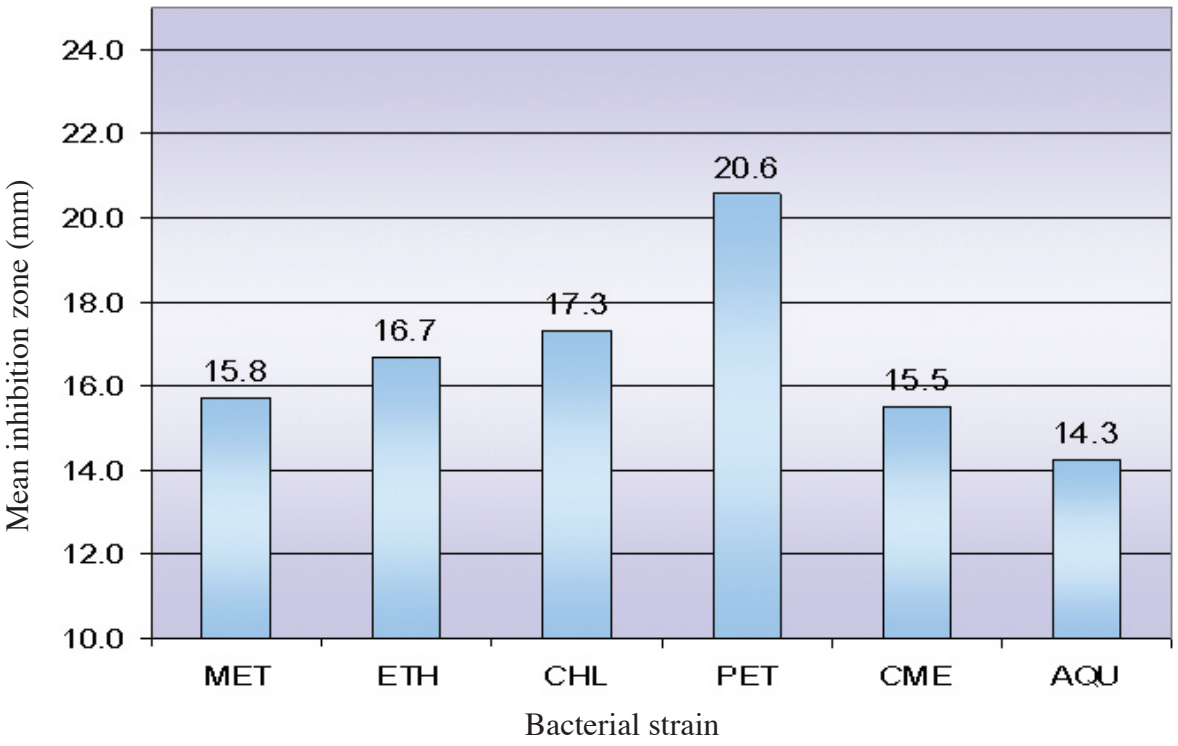

Fig. 5. Shows a comparison of the effects of chemical extracts for all algal species against all tested bacterial strains expressed as mean inhibition zone (mm). Extract type are: MET: methanol, ETH: ethyl acetate, CHL: chloroform, PET: petroleum ethe, CME: crude methanol and AQU: aqueous.

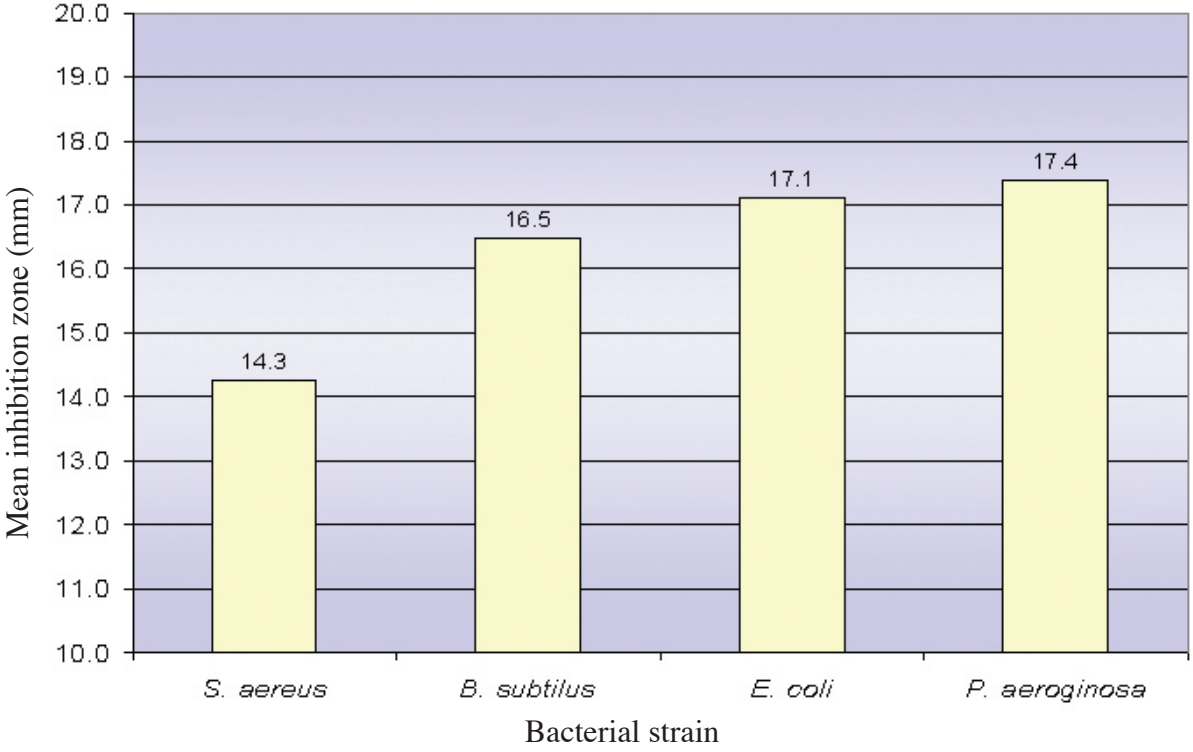

Fig. 6. shows a comparison between the mean inhibition zone (mm) against bacterial strains *S. aureus, B. subtilis, E. coli* and *P. aeruginosa* among the extracts of all algal species used..

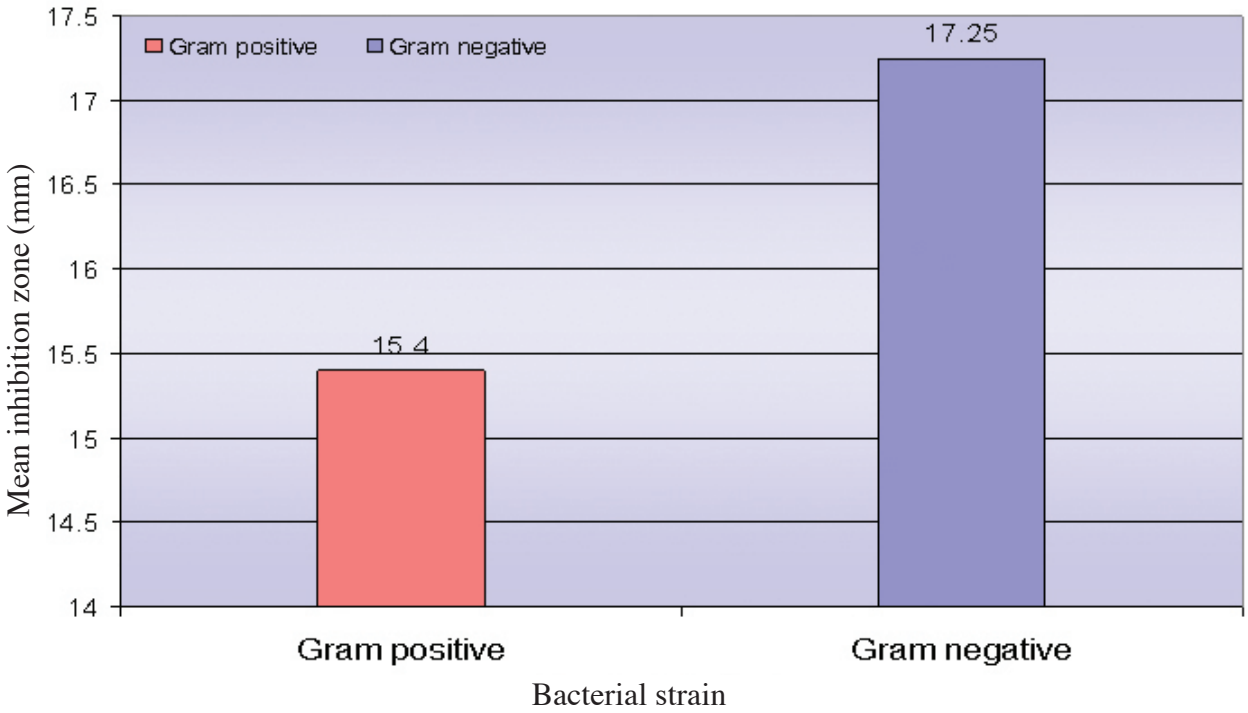

Fig. 7. Mean antibacterial inhibition zone (mm) based on Gram staining among the extracts of all algal species.

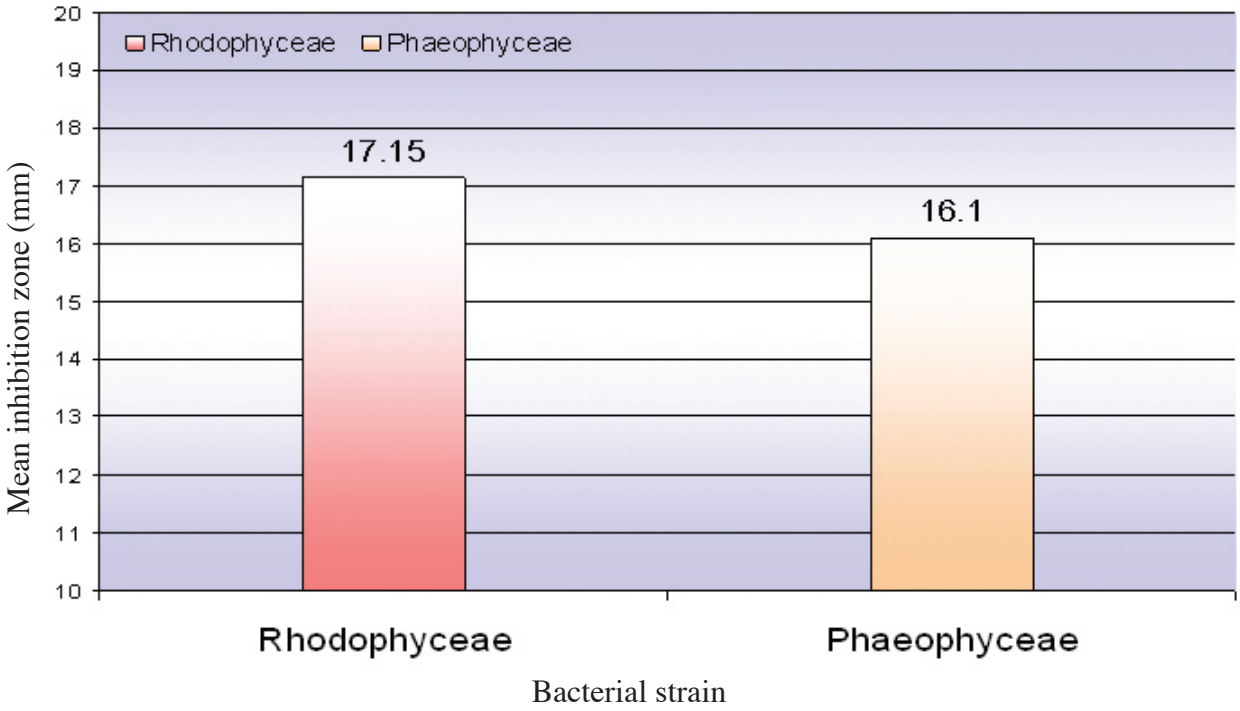

Fig. 8. Mean antibacterial inhibition zone (mm) of algal extracts of main classes of algae species.

DISCUSSION

This study clearly shows that the crude methanolic, aqueous and Soxhlet extracts of all five algal species in Bahrain possess varying yields of ingredients active against assayed microbes at tested concentrations (Tables5 and 6). The findings were compared with reference to standard antibiotic disks as a positive control treatment while sterilized deionized water and DMSO are considered to be negative controls.

Amongst all screened algal species of Bahrain, *D. simplex* exhibited the greatest inhibitory activity with a 17.7 mm mean inhibition zone (Figure 4). whereas amongst the various chemical extracts, petroleum ether showed the greatest inhibitory activity with a 20.6 mm mean inhibition zone (Figure 5). This figure also showed that the mean inhibitory activity possessed by Soxhlet extracts against tested bacteria increased from the most polar (methanol) to the least polar extract (petroleum ether). Petroleum ether, a well-known defatting agent, (Mcmurry, 1996) extracts all non-polar compounds, including fats and lipids.

The crude methanolic extract was the only extract to exhibit biological activity against more than one bacterial strain. It was mostly active against *E. coli* and *P. aeruginosa* which are Gram-negative and *B. subtilis* which is Gram-positive. In theory, the bioactivity found in the crude methanolic extract should be detected in one of the four Soxhlet extracts (petroleum ether, chloroform, ethyl acetate and methanol). At the tested concentration of the Soxhlet extracts (0.5 mg/0.1 ml) compared to the crude methanolic extract (2mg/0.1 ml), the activity was only observed against *P. aeruginosa* (Tables 5 and 6). This might indicate that the bacterium *P. aeruginosa* is highly sensitive at the assayed concentrations, compared to other bacteria. Similar findingswere observed elsewhere (Alam, *et al*. 1994), but without explanation.

The greatest biological activity was detected against *P. aeruginosa* with a 24.3 mm inhibition zone (Table 6). Five out of six chemical extracts exhibited bioactivity against the same bacteria. Clinically, *P. aeruginosa* is a well-known opportunistic pathogen that incites many disease processes (Todars, 2008). Moreover, the pathogenesis of *Pseudomonas* infections is multifactorial, suggested by the number and wide array of virulence determinants (Todars, 2008). Multiple and diverse determinants of virulence are anticipated in the wide range of diseases caused, including septicemia, urinary tract infections, pneumonia, chronic lung infections, endocarditis, dermatitisand osteochondritis (Todars, 2008).

In this study, the bacterium *S. aureus* was the most resistant bacterial strain with a 14.3 mean inhibition zone (Figure 6). The aqueous extract which is chemically polar was the only active extract against *S. aureus*. The observed activity against *S. aureus* may not provide a true quantitative measure of the activities of some components present in the extract such as polar and large molecules which have lesser mobility in the water agar media (Kumar *et al*, 1997).

The Gram-negative bacteria examined were more sensitive than gram-positive bacteria because the cell wall component of the former bacteria has an outer layer made up of lipopolysaccharide. The composition of this layer, consisting mainly of nonpolar compounds, interferes better with nonpolar extracts. As such, this cell wall layer may become more susceptible to nonpolar extracts. On the other hand, gram-positive bacteria have no lipopolysaccharide layer.

In this study, *P. aeruginosa* was the most susceptible bacterial strain while *S. aureus* was the most resistant strain (Figure 6). In a study conducted by Padmakumar & Ayyakkannu, (1997) the opposite was reported. This difference could be due to the types of extraction solvents and the extraction procedure for the assayed algae.

Some other reports also showed gram-positive bacteria were more sensitive to algal extracts than gram-negative as the latter have a more complex cell wall structure (Gonzalez, *et al*.2001; Salvador, *et al*. 2008). However, this also depends on the type of algal extract used, as most experiments which showed activity against gram-negative bacteria were mainly carried out using fractionated extracts. On the other hand, less or no activity against gram-negative bacteria was detected when crude extracts were used as they

contain inhibitory or antagonistic compounds that could mask other antimicrobial activities, especially those effective against gram-negative bacteria (Zubia, *et al*. 2008; Chowdhury, *et al*. 2005). This might indeed support our findings which showed gram- negative bacteria were more sensitive in our assay as we used fractionated, but not crude extracts.

Padmakumar and Ayyakkannu, (1997) conducted a large scale *in vitro* testing of antimicrobial activity which included 80 marine algae collected from the southern coast of India. They found the greatest antibacterial activity was associated with extracts of algal species belonging to the classes Rhodophyceae followed by Chlorophyceae and Phaeophyceae. Similar results were also reported by Salvador, et al. (2008). The results of our study showed Rhodophyceae exhibited a greater average bioactivity than Phaeophyceae with a mean inhibition zone of 17.15 mm and 16.1 mm, respectively (Figure 8). The elevated activity of red algae extracts may be attributed to their preference to inhabit warm water habitats like the Arabian Gulf Sea. Moreover, in Bahrain, red algae are more biodiverse and abundant than brown algae (Basson, *et al*. 1989). Thus, the better adaptability of the red algae to tolerate their stressful environment, especially high salinity and temperature, which may promote production of more efficient antibacterial compounds.

Caccamese, *et al* (1985) screened the lipid extracts for antimicrobial activity of 24 red and brown algal species collected from the southern Italian coast. These authors observed the greatest inhibition of active species occurs in the families Rhodomelaceae, Cystoseiraceae and Dictyotaceae. In this regard, the findings of this study agree with the above report that the chemical extract of the red algae *D. simplex* (Rhodomelaceae) is highly bioactive with a 17.7 mm mean inhibition zone (Figure 4). Moreover, the brown algal species extracts *C. myrica* and *H. triquetra* belonging to the family Cystoseiraceae also exhibited good activity (Figure 4).

The antibacterial activity of algae can be influenced by a number of factors including sample preparation methods. For example, it has been reported that lypholyzed samples have a greater activity than fresh samples. Also, the season in which the samples were collected can affect their activity as they tend to be more active in autumn and winter compared to summer (Salvador, *et al*. 2008).

Alam, *et al* (1994) conducted a preliminary screening of seaweeds collected from Papua, New Guinea for antibacterial and antifungal activity. The algae *Hormophysa* spp and *Sargussum* spp were included in this collection. The methanol and hexane extracts of these algal species were tested against *Klebsiella pneumoniae, P. aeruginosa*, *B. subtilis* and *S. aureus*. It was concluded that the methanolic extract of those algae exhibited high activity against the Gram-negative bacterium *P. aeruginosa*. In the current study, methanolic and the crude methanolic extracts of *H. triquetra* and *S. cervicorne* revealed activity against *P. aeruginosa* (Figures 6 and 7), which agrees with the study of Alam, *et al* (1994).

Razivi and Shameel, (2004) reported the algal extract of *Cystoseira indica* is the most active against four Gram-positive and seven Gram-negative bacteria in their survey including extracts of 26 species. In this study, extracts of *C. myrica* exhibited strong activity against the two gram-positive and two gram-negative bacteria that were tested. It is interesting to note that Zandi, *et al*. (2007) reported the aqueous extract of the brown alga *C. myrica* collected from the Arabian Gulf exhibited antiviral activity against herpes simplex virus Type 1. The same species in this study possessed a strong antibacterial activity (a 16.9 mm mean inhibition zone) (Figure 4).

Rizvi and Shameel, (2004) reported the algal extract of *S. filiforme* exhibited strong antibacterial activity against the tested bacteria. In this study, *S. filiforme* was the third most bioactive algal species extract with a 16.8 mm mean inhibition zone (Figure 4). In addition, the ethyl acetate extract of *S. filiforme* possessed the highest inhibition zone (17.7 mm) against *P. aeruginosa* amongst all ethyl acetate extracts of the five algal species.

The chemical compounds responsible for antibacterial activity in algae have been variously identified. They include organic compounds, fatty acids, terpenes, carbonyls, bromophenols, halogenated aliphatic and sulfur-containing

heterocyclic compounds, isoprenylated and brominated hydroquinones, as well as phlorotannins (Rosell and Srivastava, 1987). It was also reported that brown algae contain high levels of phenolic compounds known to be germicidal and useful in formulating disinfectants (Zubia, *et al*. 2008).

The alga *D. simplex* is a well known antihelminthic agent (Lindequist and Schweder, 2001 and Newman, *et al*. 2003). A chemical study conducted on this alga reported the presence of algenic acid, a small amount of unidentified alkaloid, galactan, fucoidin, and iodine (Lindequist and Schweder, 2001). Kainic acid (2-carboxy 4-isopropenyl 3-pyrrolidine acetic acid) was identified as the *active* principle in this red alga, *D. simplex* (Grant, 1982). It is probable that the kainic acid might be the inhibitory organic acid in extracts of this alga.

El Hattab, *et al*. (2007) studied the volatile compounds from brown algae *Hormophysa cuneiformis* collected from Northwest coast of Qatar in May, 2002, using two extraction methods, conventional hydrodistillation of crude diethyl ether extract and super critical CO_2 extraction. Volatiles obtained both methods were compared after analysis by GC and GC/MS. The major constituents that were detected were squalene, fatty acids and corresponding esters such as ethyl arachidonate. In this study, the same algal species with a different synonym name *H. triquetra* was used. Thus, it is probable similar active metabolites could be responsible for the bioinhibitory activity. It is also probable the same compounds could be found in brown algae *C. myrica* species since it belongs to the same family of *Hormophysa* which is Cystoseiraceae.

The bioactive compounds found in our extracts are mainly non-polar. The most probable reason is living in water, a polar environment, makes the algae produce non-polar bioactive compounds to avoid dissolution in water in order to perform their defense function efficiently.

CONCLUSION

We conclude that some chemical extracts of the algal species collected from the Bahrain coastline possessed antibacterial activity against the microbes tested. Amongst algal species, the extract of *D. simplex* was the most active and the extract of *H. triquetra* was the least active. Among the chemical extracts, petroleum ether was the most active and aqueous extract the least active. The crude methanolic extract was the only extract to possess a wide range of bioactivity. The most sensitive bacterium was *P. aeruginosa* while the most resistant was *S. aureus*.

Future Work

This study produced an idea about antibacterial activity by some algae of Bahrain, but further analysis and extraction is needed to determine the exact structure of chemical compounds responsible for biological activity against the bacteria tested. This can be accomplished using GC/ GC-MS and NMR spectroscopy. In addition, a large- scale evaluation of bioactivity of the algal species is proposed, to include more species from additional classes with more seasonal variation.

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