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Full Length Research Paper

Screening for antibacterial activities in some marine algae from the red sea (Hurghada, Egypt)

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Methanolic and ethyl acetate extracts from eight different seaweeds collected from the red sea Hurghada, Egypt (June, 2009) were screened for their antibacterial activities against both gram positive bacteria (*Staphylococcus aureus* NCIMB 50080 and *Bacillus cereus*) and gram negative bacteria (*Escherichia coli* NCIMB 50034, *Enterococcus feacalis* NCIMB 50030, *Salmonella* sp. and *Pseudomonase aeruginosa*). The antibacterial activities were expressed as zone of inhibition and minimum inhibitory concentrations (MIC). The seaweeds belong to Phaeophyaceae (*Cystoesira myrica, Cystoesira trinodis, Padina gymnospora, Sargassum dentifolium* and *Sargassum hystrix*); Rhodophaceae (*Actinotrichia fragilis*) and Chlorophyceae (*Caulerpa racemosa* and *Codium fragile*). Ethyl acetate extracts of *C. racemosa*, *C. fragile* and *P. gymnospora*; methanolic extracts of *P. gymnospora* and *C. fragile* showed higher antibacterial activities than other members of the tested algae. The most resistant bacteria was *E. feacalis* against both solvents extracts of *S. dentifolium*, *C. myrica* and *A. fragilis* while, *Salmonella* sp. and *P. aeruginosa* were resistant to methanolic extracts of *C. racemosa*, *S. dentifolium* and *A. fragilis*. On the other hand, *B. cereus*, *S. aureus* and *E. coli* were the most sensitive to all seaweed extracts. Our conclusion confirmed that susceptibility of gram positive bacteria to the algal extracts (zone of inhibition up to 19 mm) was more than those of gram negative bacteria (zone of inhibition up to 14 mm). The activities of ethyl acetate extracts were higher than those of methanolic extracts and the most powerful inhibitory extract was ethyl acetate extract of *C. racemosa*.

Key word: Antibacterial, ethyl acetate extracts, marine algae, methanolic extracts, minimum inhibitory concentration, seaweeds.

INTRODUCTION

Bacterial infection causes high rate of mortality in human population and aquaculture organisms (Kandhasamy and Arunachalam, 2008). For example, *Bacillus cereus* is responsible for causing food borne diseases (Wijnands, 2008). *Enterococcus faecalis* is the causative agent of inflammatory bowel disease (Balish and Warner, 2002). *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* cause diseases like mastitis, abortion and upper respiratory complications, while *Salmonella* sp. causes diarrhea and typhoid fever (Jawetz et al., 1995). *P. aeruginosa* is an important and prevalent pathogen among burned patients capable of causing life-threatening illness (Kandhasamy and Arunachalam, 2008).

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 necessitate continued research for new antimicrobial compounds for the development of drugs (Al-Haj et al., 2009). So accordingly, pharmaceutical industries are giving importance to the compounds derived from traditional sources (soil and plants) and less traditional sources like marine organisms (Solomon and Santhi, 2008). Hence, the

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Abbreviations: MIC, Minimum inhibitory concentrations; **MRSA,** methicillin-resistant *Staphylococcus aureus*

Table 1. Species / class of the collected algae.

Class / species*
Phaeophyceae
A- Cystoesira myrica
B- Cystoesira trinodes
C- Padina gymnospora
D- Sargassum dentifolium
E- Sargassum hystrix
Rhodophyceae
F- Actinotrichia fragilis
Bryopsidophyaceae
G- Caulerpa racemosa
H- Codium fragile

interest in marine organisms as a potential and promising source of phar-maceutical agents has increased during recent years (Kim and Lee, 2008).

Marine algae or seaweeds are rich and varied source of bioactive natural products so it has been studied as potential biocidal and pharmaceutical agents (Rangaiah et al*.,* 2010). There have been a number of reports of antibacterial activity from marine plants (Al-Haj et al., 2010) and special attention has been reported for antibacterial and/or antifungal activities related to marine algae against several pathogens (Kolanjinathan and Stella, 2009).

Seaweeds are considered as 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 (Cox et al*.,* 2010) with antiviral, antibacterial and antifungal activities (Del Val et al., 2001) which acts as potential bioactive compounds of interest for pharmaceutical applications (Solomon and Santhi, 2008). Most of these bioactive substances isolated from marine algae are chemically classified as brominated, aromatics, nitrogen-heterocyclic, nitrosulphuric-heterocyclic, sterols, dibutanoids, proteins, peptides and sulphated polysaccharides (Kolanjinathan et al*.,* 2009).

The antibacterial activity of seaweeds is generally assayed using extracts in various organic solvents, for example, acetone, methanol-toluene, ether and chloroform-methanol (Cordeiro et al., 2006). Using of organic solvents always provides a higher efficiency in extracting compounds for antimicrobial activity (Tüney et al., 2006). Several extractable compounds, such as cyclic polysulfides and halogenated compounds are toxic to microorganisms, and therefore, responsible for the anti-biotic activity of some seaweeds (Wrattens and Faulkner, 1976). Cox et al. (2010) revealed that the extraction of antimicrobials from the different species of seaweeds was solvent dependent; Methanol was a good solvent for extraction of antimicrobials from brown seaweeds whereas acetone was better for red and green species.

Screening of organic extracts from marine algae and other marine organisms is a common approach to identify compounds of biomedical importance. However, reports on antimicrobial activity of seaweed extracts from Egypt are very limited. Hence, the present work aimed to screen and evaluate the efficiency of methanol and ethyl acetate extracts as antibacterial agents from the Egyptian seaweeds and to select the most active species against the most common pathogenic bacteria, all were in focus as much as possible.

MATERIALS AND METHODS

Algae collection and extract preparation using two different solvents

Eight marine algae were collected by hand picking from the red sea in Hurghada, Egypt during june 2009 (Table 1 and Figure 1). Algal samples were cleaned from epiphytes, extraneous matter and necrotic were removed. Samples were collected in sterilized polyethylene bags, and put in an ice box, then transferred to the laboratory immediately until the experimental work was done at the same day. Samples were washed thoroughly with sea water then sterile distilled water, air dried, cut into small pieces and then ground in a tissue grinder (IKA A 10, Germany) until reach fine powder shape.

10 g of each dried sample were extracted in two different solvents: (100 ml of methyl alcohol or ethyl acetate) under stirring condition (50 rpm) for 7 days at room temperature. The solution was filtered through Whatman No. 1 sterile filter paper. The filtrates then were dried using desiccator (Cole- parmer instrument, Chicago). The dried precipitates were dissolved in the above two solvents to give 50 mg/ml extracts, then stored in airtight bottles in a refrigerator before testing. These crude extracts were screened against common pathogenic bacteria as shown in Table 1.

Antibacterial activity

Bacterial source and culture condition

The bacteria used in this study were *E. coli* NCINB 50034, *S. aureus* NCINB 50080, *E. feacalis* NCINB 50030, *Salmonella* sp., *B. cereus* and *P. aeruginosa*. These bacterial strains were maintained on suitable medium at 4°C and subcultured on Mueller Hinton Broth at 37°C for 18 h before testing.

Antibacterial assay

Antibacterial activity was determined against the above bacteria using the paper disk assay method (El-Masry et al*.,* 2000). Whatman No. 1 filter paper disk of 6-mm diameter was sterilized by autoclaving for 15 min at 121°C. The sterile disks were impregnated with different extracts (50 mg/ml). Agar plates were surface inoculated uniformly from the broth culture of the tested microorganisms. In all cases, the concentration was approximately $1.2X10^8$ CFU/ml. The impregnated disks were placed on the Muller Hinton medium suitably spaced apart and the plates were incubated at 37°C for 24 h. Methanol and ethyl acetate were used as a negative control while commercial antibiotic discs (chloromophenicol, 10 mg/disc

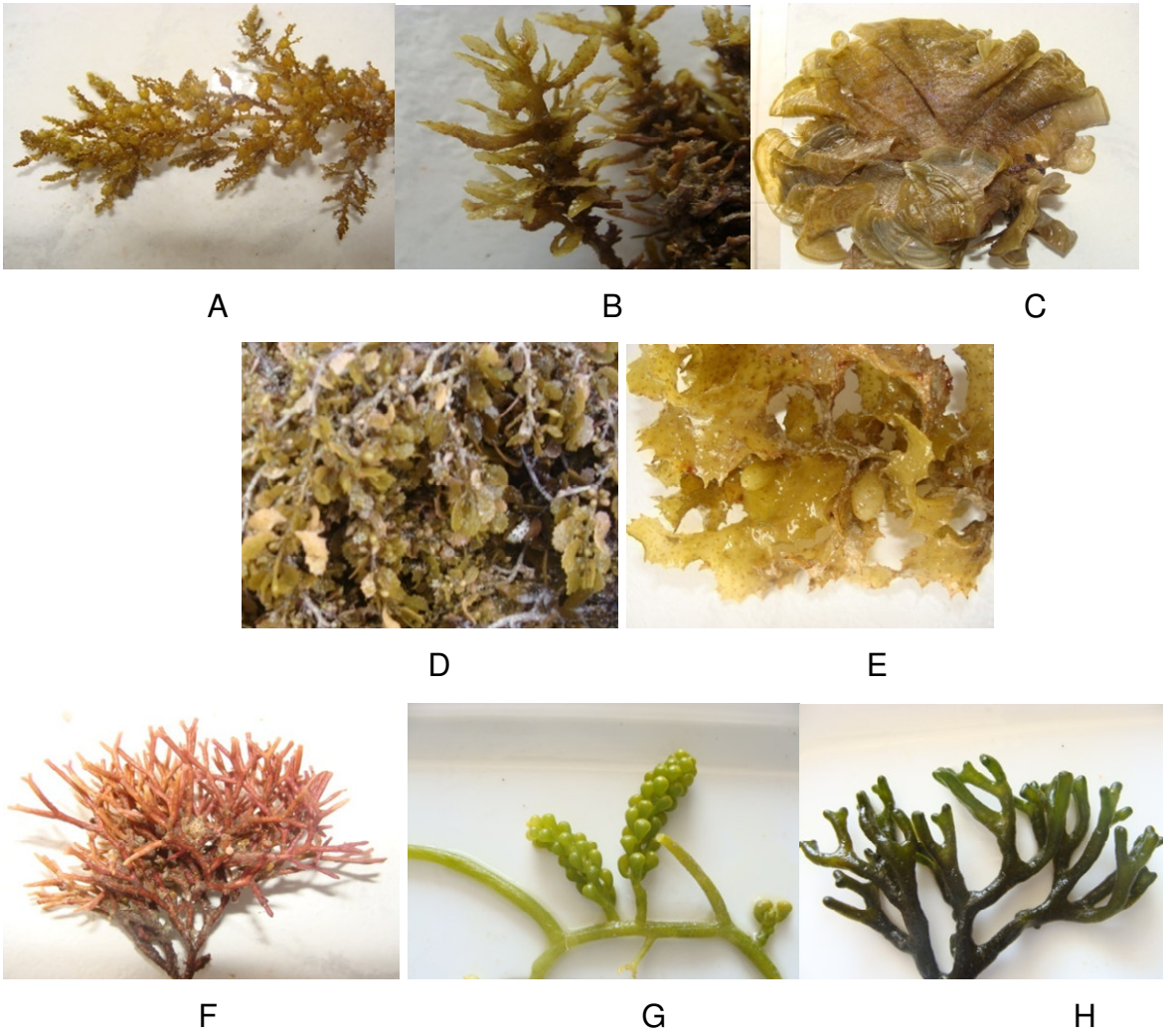

Figure 1. Marine algae collected from the red sea (Hurghada, Egypt).

and tetracycline, 30 mg/disc) were used as a positive control. The diameter of the growth inhibition halos caused by the methanolic and ethyl acetate extracts of marine organisms was measured by a ruler and expressed in millimeter. All the assays were carried out in triplicate.

Minimal inhibitory concentration (MIC)

Sensitivity of bacteria to methanol and ethyl acetate extracts of marine algal suspension can be measured by using a tube dilution technique, which determines the MIC of seaweeds used in this study *in vitro*. These tests were done to determine the lowest concentration of algal extracts that inhibit the growth of bacteria. The test was performed in 96 well microtitre plates, so that several replicates of each sample can be run. All isolates were grown in Mueller Hinton Broth at 37°C and diluted in Mueller Hinton Broth supplemented with 2% NaCl to a concentration of 50 mg L^{-1}. Then the suspension of the bacterial cultures was added into 96 well microtitre plates containing diluted samples of algal extract (50, 20, 10 and 5 mg L^{-1}).

The 96-well microtitre plate containing different diluted samples of algal extracts and bacteria was then incubated overnight at 37°C with constant shaking on the shaker. On the next day, the diluted sample of the bacteria with the algal extracts in the 96 well microtitre plates were plated out onto the Muller-Hinton agar plate. The plates were then incubated at 37°C for 24 h in the incubator. Finally, the number of bacteria colonies developed on each agar plates was counted to determine the lowest concentration of algal extracts that inhibit the growth of bacteria (MIC).

RESULTS

Disc diffusion test

Methanol and ethyl acetate extracts from eight marine algae were assayed for antibacterial activity by using agar diffusion method. Antibacterial activity of these extracts is shown in Table 2.

Most of the algal extracts exhibited antibacterial activity against all the tested bacterial species. Among gram positive bacteria, *S. aureus* was the most sensitive to all the seaweed extracts. The higher antibacterial activity (indicated as zone of inhibition) was recorded for ethyl

Table 2. Inhibition halo diameter and MIC of methanolic and ethyl acetate extracts of some marine algae against gram positive and gram negative bacteria.

Seaweed extracts	Inhibition halo diameter (mm) *						MIC (mg/ ml)					
	S. aureus	*B. cereus*	*E. coli*	*Salmonella* sp.	*P. aeruginosa*	*E. faecalis*	*S. aureus*	*B. cereus*	*E. coli*	*Salmonella* sp.	*P. aeruginosa*	*E. faecalis*
Control												
Chloromphenicol	13.5±0.7	23.5±0.5	12.5±0.5	13.5±0.5	8.5±0.5	10±1	10	10	10	10	10	10
Tetracycline	23.5±1	19.5±1.2	15±0.4	7.5±0.3	12.5±0.4	-ve	30	30	30	30	30	-ve
Ethyl acetate extracts												
S. hystrix	11.8±0.8	9±1	8.5±0.5	7.5±0.5	8±0.8	7±1.5	20	20	10	50	50	20
C. racemosa	19.2±1.2	13.3±0.6	9.5±0.2	10.5±1	14.3±0.3	8±1.7	50	5	50	50	20	50
S. dent.	14.3±0.5	10.8±0.8	12.3±0.3	11±0.6	9.8±0.4	-ve	50	10	50	20	50	-ve
C. myrica	10±0.5	9.3±1.3	11±1	-ve	9±0.5	-ve	50	20	10	-ve	50	-ve
P. gymnospora	17.8±0.8	11.3±0.3	11.3±0.8	9.5±1.2	10±1.2	9±0.5	20	50	10	50	10	50
C. fragile	10±0.2	9.8±0.4	11.8±0.3	10±0.5	9±0.8	9±0.7	20	10	50	50	50	20
A. fragil.	9±1	8±0.5	10.5±0.5	10±1	9.7±0.7	-ve	20	50	50	50	50	-ve
C. trinodis	11±0.4	9.5±0.2	7±0.5	10±0.4	-ve	13±1	20	50	50	50	-ve	50
Methanolic extracts												
S. hystrix	13.8±0.8	9±0.5	11.3±0.3	-ve	15±1	7±1	50	10	20	-ve	20	50
C. racemosa	19.8±0.4	10.5±0.3	9.5±0.3	-ve	-ve	8±0.8	5	20	50	-ve	-ve	50
S. dent.	18±0.5	10±0.8	13.3±1.2	-ve	-ve	-ve	50	20	10	-ve	-ve	-ve
C. myrica	17±1	6.5±0.5	8±1	8.5±0.5	8±0.7	-ve	10	50	50	50	50	-ve
P. gymnospora	11.5±0.5	12.8±0.8	13.3±0.3	15±1	-ve	7±0.5	20	20	50	10	-ve	20
C. madagasc.	11.3±1.3	15.5±0.5	14.5±1	6±0.6	7.5±0.5	9±1	20	5	50	50	50	50
A. frail.	7.8±0.6	8.5±0.7	12±1	-ve	-ve	-ve	50	50	50	-ve	-ve	-ve
C. trinodis	11.5±0.5	11.8±0.7	10±1.2	10±0.4	9±0.3	12±2	20	10	50	50	50	50

* measured as mm, mean ± SD, n=3.

acetate extracts of *C. racemosa*, *S. dentifolium* and *P. gymnospora* (19.2, 14.3 and 17.8 mm) respectively; methanolic extracts of *S. hystrix*, *C. racemosa*, *S. dentifolium* and *C. myrica* (13.8, 19.8, 18 and 17 mm) respectively, while the inhibition zone of chloromophenicol was 13.5 mm (Table 2). Hence, the susceptibility of *S. auerus* to algal extracts was more pronounced when compared to the antibiotic chloromophenicol. Next to *S. aureus*, *B. cereus* and *E. coli* were very susceptible to all the algal extracts used.The most observed antibacterial activity was recorded for methanolic extract of *C. fragile* against *E. coli* (16% increase) compared to chloromophenicol. On the other hand, *P. aeruginosa* was resistant to the methanolic extracts of *C. racemosa, S. dentifolium, P. gymnospora* and *A. fragilis*; ethyl acetate of *C. trinodis*, while it was sensitive to the other extracts. It is worthy to mention that the clear zone caused by methanolic extract of *S. hystrix* and ethyl acetate extract of *C. racemosa* nearly double the inhibition zone caused by chloromophenicol disc.

Methanolic extracts of *S. hystrix*, *C. racemosa,*

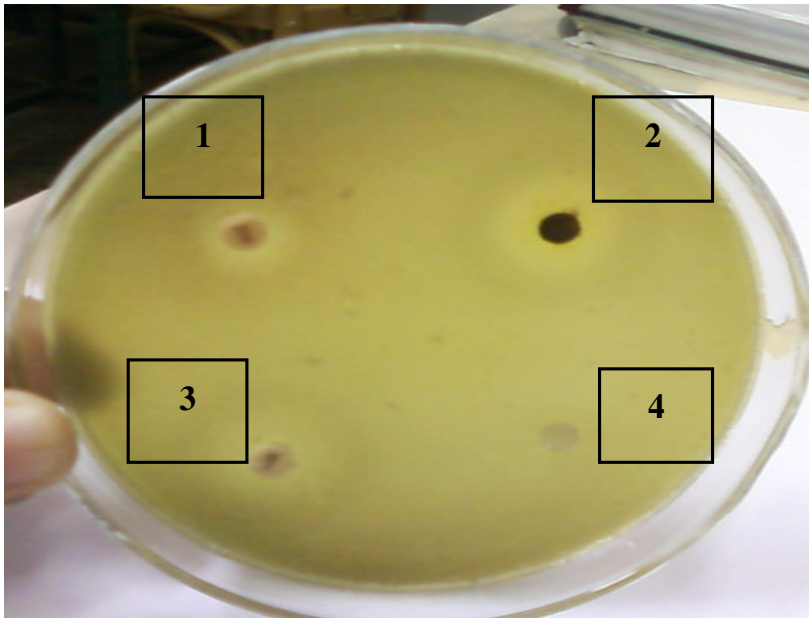

Figure 2. Inhibition halo zone of ethyl acetate extracts of S. dentifolium against Salmonella Sp. 1, tetracycline; 2, ethyl acetate extract of S. dentifolium; 3, chloromophenicol; 4, ethyl acetate.

S. dentifolium, A. fragilis and ethyl acetate extract of *C. myrica* had no antibacterial effect on *Salmonella* sp. while the other extracts showed higher clear zones when compared to tetera cycline (Table 2). *E. feacalis* was the most resistant bacteria; it did not show any inhibition zones with any extracts from three marine algae, *S. dentifolium*, *C. myrica*, and *A. fragilis*.

Minimum inhibitory concentration (MIC)

Table 2 and Figure 2 showed the MIC of seaweed extracts. MIC of the tested marine algal extracts was ranging from 5 mg/ml to 50 mg/ml. Lowest MIC value was recorded for the methanolic and ethyl acetate extract of *C. racemosa*; methanolic extract of *C. fragile* (5 mg/ml) followed by methanolic and ethyl acetate extracts of *S. dentifolium*, *P. gymnospora,* ethyl acetate extracts of *S. hystrix*, *C. myrica*, *C. fragile* and methanolic extract of *C. trinodis* (10 mg/ ml). Consequently, *C. racemosa* was considered as the ideal algal extract (inhibition zone 19.8 mm and MIC 5 mg/ml).Among the three groups (Phaeophyaceae, Rhodophaceae and Chlorophyceae) of seaweeds, maximum activities were recorded in Chlorophyceae species (*C. racemosa*) and Phaeophyaceae species (*P. gymnospora* and *S. dentifolium*) which the most effective seaweeds and minimum activities were recorded in Rhodophaceae species (*A. fragilis*).

The efficiency of extraction of antibacterial natural products from marine macroalgae was higher with the organic solvent ethyl acetate and the above results confirm the broad antimicrobial effect of marine algae by using ethyl acetate extract.

DISCUSSION

Antibacterial activity of red, brown and green algae against both Gram positive and Gram negative bacteria has been established by several scientists (Kolanjinathan et al*.,* 2009). But variation in antibacterial activity may be due to the method of extraction, solvent used in extraction and season at which samples were collected (Kandhasamy and Arunachalam, 2008). Several different organic solvents have been used to screen algae for antibacterial activity (Marasneh et al*.,* 1995). Early, Sastry and Rao (1994) showed antibacterial activity against Gram-positive and Gram-negative pathogenic strains after successive extraction with benzene, chloroform and methanol. Kim and Lee (2008) used methanolic extracts of *Esiena bicyclis* (B32) and *Sargassum* sp. (B36) which showed strong antibacterial activities against Methicillin-resistant *Staphylococcus aureus* (MRSA) strains, *Vibrio parahemolyticus* and *Edward tarda*. Kolanjinathan and Stella (2009) indicated that acetone was the best solution for extracting the effective antimicrobial materials from *Sargassum myricystum*, *Turbinaria*

conoides, *Hypnea musiformis*,n *Gracilaria edulis* and *Halimedia gracilis*; whereas, Karthikaidevi et al. (2009) used seven different solvents including methanol and ethyl acetate for extraction of antibacterial substances from *Codium adherens, Ulva reticulata* and *Halimeda tuna*.

In our study, eight different marine algae collected from the red sea, Hurghada, Egypt were screened for their antibacterial activities using methanolic and ethyl acetate. The results showed that, *C. racemosa* member of green algae, *P. gymnospora* and *S. dentifolium* members of brown algae were more active compared to other groups of algae tested. Similar results were also obtained by Kandhasamy and Arunachalam (2008) and Karthikaidevi et al. (2009). These strong activities related to brown algae may be due to the phenolic compounds such as phlorotannins, eckol and eckol- related compounds that have strong bactericidal activity (Nagayama et al., 2002).

Antibacterial activities of seaweeds varied with the species from different division; Caccamese et al. (1985) have reported that brown algal extracts showed higher activity than the extracts of red algae which was in accordance with our results, whereas Viachosi et al. (2001) reported that extracts of the Phaeophyta exhibited the highest level of antibacterial activity, followed by the Rhodophyta and then the Chlorophyta. In contrast, Yi et al. (2001) reported that species of Rhodophyta showed the highest antibacterial activity. The reason for this variation was not explained by these workers but it was suggested that more species have to be screened before coming to a definite conclusion (Vallinayagam et al. 2009).

Of the two solvents tested, ethyl acetate was determined to be the best solvent for isolation of antimicrobial compounds from the tested marine algae followed by methanol (Table 2). These results were in close agreement with those obtained by Patra et al. (2008). It was revealed that the chloroform and ethyl acetate extracts *Enteromorpha compressa, Chaetomorpha linum* and *Polysiphonia subtilissima* were active against most of the pathogens whereas methanol and ethanol extracts were active only against *Shigella flexneri* (Patra et al., 2009). This may indicate that the extraction method had definite effects on the isolation of bioactive principles. Some authors showed that methanol extraction yielded higher antimicrobial activity than n-hexane and ethyl acetate which in contrast to our results (Manilal et al., 2009 and Rangaiah et al., 2010). This difference in results may be firstly due to difference in species used, time and place of sample collection, secondly; there may also be differences in the capability of the extraction protocols to recover the active metabolites and finally, differences in the assay methods that would result in different susceptibilities of the target strains.

It is worthy to mention that, in some species (such as *S. dentifolium* and *A. fragilis*) the inhibitory activity was only observed in the extract obtained with one kind of solvent but not in the extract obtained in other solvents. This result could be related to the presence of bioactive metabolites present in this species of algae, which are not soluble in one solvent but they can be soluble in the other. Karthikaidevi et al. (2009) who obtained the same results suggested that a particular solvent is required to extract some antimicrobial substances within the algal plant and therefore the inhibitory activity will go up when several solvents are used in the screening.

All the seaweeds extracts inhibited both gram positive and gram negative bacteria except *E. feacalis* which was resistant to *S. dentifolium*, *C. myrica* and *A. fragilis* extracts; *Salmonella* sp. which was resistant to methanolic extracts of *S. hystrix*, *C. racemosa, S. dentifolium, A. fragilis*, ethyl acetate extract of *C. myrica* and *P. aeruginosa* which was resistant to the methanolic extracts of *C. racemosa, S. dentifolium, P. gymnospora, A. fragilis* and ethyl acetate of *C. trinodes*. Thus the susceptibility of gram positive bacteria to the algal extracts was more than those of gram negative bacteria. Many authors made similar observations (Demirel et al., 2009 and Ibtissam et al., 2009). The more susceptibility of Gram-positive bacteria to the algal extract was due to the differences in their cell wall structure and their composition (Taskin et al., 2007). In Gram-negative bacteria, the outer membrane acts as a barrier to many environmental substances including antibiotics (Tortora et al., 2001). The presence of thick murine layer in the cell wall also prevents the entry of the inhibitors (Kandhasamy and Arunachalam, 2008).

According to our results, the higher antibacterial activity was recorded for ethyl acetate extracts of *C. racemosa*, *S. dentifolium, P. gymnospora*; methanolic extracts of *S. hystrix*, *C. racemosa*, *C. fragile*, *S. dentifolium* and *C. myrica* which also showed lowest MIC ranging from 5 to 10 mg/ml. Marine macro algae can inhibit the growth of some bacteria (Abd El Mageid et al., 2009). Many pervious authors (Kumar et al., 2008; Rajasulochana et al., 2009) reported the activities of *P. gymnospora*, *Padina* sp., *Sargassum wightii* and *Caulerpa* sp. against gram positive and gram negative bacteria. Rangaiah et al. (2010) investigated the antimicrobial potentiality of the marine Chlorophycean algae *Ulva lactuca, Caulerpa taxifolia* and *Spongomorpha indica* against six strains of Gram positive, Gram negative bacterial and fungal organisms that cause diseases and disorders in man, animals and plants. According to the later author and Patra et al. (2008), this indicates the presence of active constituents in the extractions of seaweeds which can be exploited for the production of lead molecules which are of use in pharmaceutical industry. In close agreement, Yamashita et al. (2001) attributed that to the high concentration of polysaccharides in these species which

are known to have antimicrobial properties.

C. racemosa was the most active against all the tested pathogens. This may be due to active components which are present in seaweed extract. However some seaweeds extracts were unable to exhibit antibacterial activity against tested bacterial strains (Vallinayagam et al., 2009). In study carried by Mtolera and Semesi (1996), the antimicrobial activity shown by *C. racemosa* attributed to caulerpin or caulerpein (Paul et al., 1987), or flexin and trifarin (Blackman and Wells, 1978) or by caulerpanyene (Amico et al., 1978). The noteworthy capability of caulerpales to produce antimicrobial activities has been also reported (Del Val et al., 2001).

Taking all of the previous results together, we conclude that all the crude extracts of seaweeds showed promising activity against the test pathogens. The highest activities were recorded for ethyl acetate extracts of *C. racemosa*, *S. dentifolium, P. gymnospora*, methanolic extracts of *S. hystrix*, *C. racemosa*, *C. fragile*, *S. dentifolium* and *C. myrica*. *C. racemosa* was the ideal seaweed extract used against the tested pathogenic bacteria. Among marine the algal extracts tested, some appeared to be specific in their activity against several test bacteria. This point may be important for the development of specific antibiotics, and further work is needed to identify the compounds causing the activity, to evaluate specific antimicrobial activity against pathogenic bacteria especially those causing the human diseases. Finally, we can recommend that, macroalgae from the red sea Hurghada, Egypt are potential sources of bioactive compounds and should be investigated for natural antibiotics.

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