JOURNAL OF PURE AND APPLIED MICROBIOLOGY, APRIL 2014. Vol. **8**(2), p. 1275-1282

Antimicrobial and Biochemical Properties of Selected Edible Brown and Red Marine Macroalgae

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(Received: 25 October 2013; accepted: 03 January 2014)

The in-vitro antimicrobial activity of methanol and acetone brown algal species extracts: Kombu (*Laminaria japonica*), Wakame (*Undaria pinnatifida*), Arame (*Eisenia bicyclis*) and a red algal sample Sushi Nori (*Porphyra tenera*) was determined in this study against gram positive and gram negative bacterial isolates; some are antibiotic resistant such as methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 12498 and *Pseudomonas aeruginosa* ATCC 27853, and against a yeast isolate *Candida albicans* ATCC 60193.The highest antimicrobial activity was noted mainly with the brown methanolic algal extracts compared to red algal extracts. FTIR infrared Spectrometer analysis together with High performance liquid chromatography provided a detailed description of the functional chemical constituents present in marine macroalgae particularly in brown seaweeds to be mainly of phenolic nature to which the potent antimicrobial activity is being attributed; nano particles measurement with zeta sizer for *Laminaria japonica* acetone extract could indicate a preliminary correlation between stability and efficacy of the algal extracts.

Key words: Kombu, Sushi Nori, Wakame, Arame, FTIR: infrared Spectrometer, HPLC: high performance liquid chromatography, Zeta sizer.

The prevention and treatment of microbial infectious diseases has considerably increased the demand for biodiversity in screening new therapeutic drugs from natural products. There is now a greater interest in marine organisms, especially algae as a potential and promising source of pharmaceutical agents. The seaweeds have touched new horizons like marine pharmacology and bioremediation. Seaweeds are classified as red algae (Rhodophyta), brown algae (Phaeophyta) and green algae (Chlorophyta) depending on their nutrient and chemical composition. It was noted that edible seaweeds, especially red and brown algae, contain a significant amount of protein, vitamins and minerals[9]. However, this nutrient composition varies and is affected by the species, geographical areas, seasons of collection and water temperature[17].

Moreover, the ability of seaweeds to produce secondary metabolites of potential interest has been extensively documented[26,8,7]. Most of seaweeds extract showed antimicrobial activity which was regarded as an indicator to detect the potent pharmaceutical capacity of macroalgae for the synthesis of secondary metabolites[11, 27].

Many compounds derived from macroalgae were reported to have broad biological activities such as antibacterial[16,4] antifungal[18] and the most widely studied activity the antioxidant effect[15].

The levels of antimicrobial activity of seaweeds secondary active metabolites have been found to vary according to the algal species[32], the efficiency of the extraction protocol[31] and the

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solvents being used. Cox[6] revealed that the extraction of antimicrobials from different seaweed species was solvent dependent. Moreover, extracts prepared from fresh seaweed samples showed negligible antimicrobial activity compared to that obtained from dried seaweeds[22]. It is clear then that the use of organic solvents always provides a higher efficiency in extracting antimicrobial activities, compared with water extraction[10].

In an effort to reveal the major chemical constituents of the edible seaweeds being tested and to possibly determine their antimicrobial effect stability by measuring the nano particles present in the algal extracts; infra red spectroscopy (FTIR) together with High Performance Liquid Chromatography (HPLC) and zeta sizer nano particles were performed correspondingly.

Fourier Transform Infrared (FTIR) spectroscopy allows the analysis of a relevant amount of compositional and structural nature of environmental samples[14]. These constituents; however, could be precisely analyzed and detected by HPLC; with their stability probably to be determined by nanoparticles measurement.

The present experimental research was conducted to reveal and study the biological and medicinal properties of four edible macroalgae varying between red and brown algae widely consumed by humans such as Sushi Nori (*Porphyra tenera*), kombu (*Lamiaria japonica*), Arame (*Eisenia bicyclis*) and wakame (*Undaria pinnatifida*). All of these algal samples were bought dried ready packed from Clearspring (UK) found in a local market Riyadh, KSA.

Methanol and acetone *Laminaria japonica, Eisenia bicyclis, Undaria pinnatifida and Porphyra tenera* extracts were tested on a yeast isolate *Candida albicans*, gram positive and gram negative bacteria; among which antibiotic resistant such as methicillin resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeuroginosa.*

Highest antimicrobial activity was observed by methanol algal extract mainly against *Staphlyococcus aureus*, MRSA and *Pseudomonas aeruginosa* respectively. Only *Laminaria* acetone extract showed potent inhibitory effect against *Candida albicans*.

Laminaria japonica and *Undaria pinnatifida* acetone extracts chemical analysis revealed that the highest peaks are phenolic constituents to which the potent antimicrobial activity is associated; nano particle measurement by Malvern zeta sizer of *Laminaria japonica* acetone extract gave a single peak which possibly interpreted as a good correlation between potent antimicrobial activity and high stability of this algal extract.

MATERIALS AND METHODS

Algal collection and preparation for extraction

Ready dried edible algal samples were bought packed from Clearspring (UK) in a local market, Riyadh, KSA, widely consumed by humans were as follows: one red macroalga, Sushi Nori (*Porphyra tenera*) and three brown seaweeds Arame (*Eisenia bicyclis*), Kombu (*Laminaria japonica*) and Wakame (*Undaria pinnatifida*).

All algal species were chopped and ground thoroughly to powder, 10 grams of each seaweed sample were extracted with 100ml of methanol and acetone separately.

Samples were incubated for three days in a rotator shaker (100 rpm)(Comecta, Spain) at 27°C. Prior to incubation samples were filtered aseptically with 0.45µm pore size filter unit (Millipore, USA) and stored in sterile Eppendorf tubes at -4°C for further use.

Microbial isolates collection

Pure cultures of standard microbial isolates tested in this study were obtained from King Khaled Hospital, Microbiology Laboratory, Riyadh, KSA.

Bacterial isolates include gram positive bacteria such as *Staphylococcus aureus* ATCC 25923, *Staphylococcus xylosus*, methicillin resistant *Staphylococcus aureus* MRSA ATCC 12498, *Enterococcus faecalis* ATCC 29212 and Bacillus subtilis ATCC 6633; whereas gram negative bacteria were *Escherichia coli* ATCC 25966, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella sp.*(clinical isolate) and *Klebsiella pneumoniae* ATCC 700603, in addition to one yeast isolate *Candida albicans* ATCC 60193.Fresh cultures of all microbial isolates were grown on nutrient agar plates and nutrient broth (Oxoid) for further use.

Small inoculums of the microbial isolates were suspended in 5 ml sterile nutrient broth test

tube for a microbial suspension preparation of 0.5 MacFarland turbidity.

Antimicrobial assay

Antimicrobial activity was assayed using well diffusion technique[2], small inoculums of each of the microbial suspension prepared were loaded on sterile Muller Hinton agar plates surface (Oxoid) with sterile cotton swabs. Loaded plates were then perforated equidistantly with a sterile 6mm diameter cork borer, 100µl of each algal extract methanol and acetone, were loaded respectively in the appropriate wells. Plates were kept to rest for 30 minutes at room temperature and then incubated at 37°C for 18-24 hrs.

Antimicrobial activity was determined by measuring the inhibition zone. All tests were performed in duplicates and means of inhibition zones were recorded in mm.

Standard antibiotic discs , Amoxicillin \ CLAV \ ACID (AMC)-30 µg , MEM-10 µg ,MXF-5 µg were used as a positive control and for comparing the efficiency of the four algal extracts in study.

High Performance Liquid Chromatography analysis

Laminaria acetone extract with the potent antimicrobial activity was selected for HPLC analysis, using a linear gradient elution of 90% water with 10 to 100% (v/v) methanol at a flow rate of 1.0ml per min for 45 minutes monitored at 230nm, studying the major chemical constituents to which the inhibitory activity could be related.

Fourier Transform Infrared Spectrometer (FTIR) analysis

A qualitative and preliminary analysis of the main functional groups for both *Undaria pinnatifida* and *Laminaria japonica* surface samples were prepared analyzed and recorded using a Nicolet 6700 FT-IR instrument (Thermo scientific). FTIR spectrum of each sample was obtained separately by KBr pellet method. About 0.1g of dried algal biomass was mixed with KBr (0.1 g) and compacted in pellet form. FTIR spectra were then recorded in wavelengths between 4000–5000 cm^{-1}. Data were plotted on standard software.

Zeta sizer nano particles measurement

Zeta sizer(Malvern, UK) measures particle and molecule size from below a nanometer to several microns using dynamic light scattering with NIBS optics; it allows particle concentration to be measured in the 10-2000nm range in liquid suspension. The stable nano particles measurement in this study observed for kombu (*Laminaria japonica*) acetone extract given by a single peak could be interpreted as a better extract antimicrobial stability activity.

RESULTS

Organic solvent extraction of dried red and brown seaweeds experimented, showed variable degrees of antimicrobial activity against tested microorganisms among which antibiotic resistant such as MRSA and *P. aeruginosa*. The highest antibacterial activity was observed mainly on *Staphylococcus aureus* exerted by all algal extracts methanol and acetone, MRSA growth was affected mainly by *Undaria* and *Eisenia* methanolic extract; followed by *Pseudomonas aeruginosa* which was inhibited by *Eisenia bicyclis* methanolic extract. However, *Candida albicans* was highly affected by *Laminaria japonica* acetone extract (Figs 1-2). It could be noted that algal extraction with methanol was more effective than that of acetone in agreement with other findings[10,16,23,25]; Sushi (*Porphyra tenera*) on the contrary showed no activity against all tested microorganisms (Table. 1).

Obtained results indicated the presence of active metabolites extracted from seaweeds exploited for the production of therapeutical alternative agents used in the treatment of most human pathogens.

HPLC data

HPLC chemical analysis of both *Undaria pinnatifida* and *Laminaria japonica* acetone extracts revealed the major phenolic constituents indicated by highest peaks to which the greater antimicrobial activity is highly associated (Figs 3- 4).

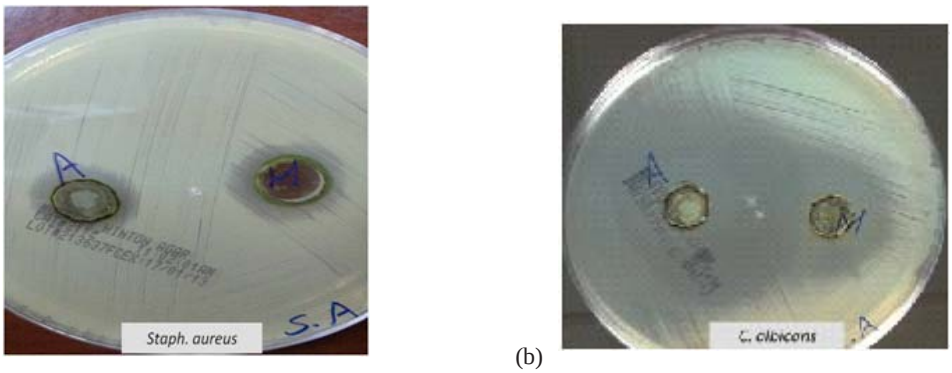
FTIR analysis

The infrared Spectrometer analysis experimented on *Undaria pinnatifida* and *Laminaria japonica* surfaces respectively at a wavelength between 4000 - 5000 cm^{-1} revealed that each peak is assigned to a functional group mainly a phenolic group, giving the most effective antimicrobial marine seaweeds activity (Figs 5, 6).

Table 1. Antimicrobial activity of different algal extracts using two different organic solvents methanol and acetone

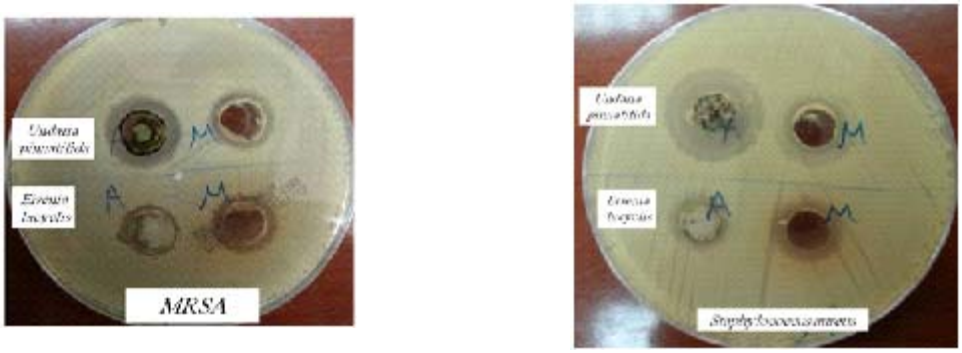
S. No	Pathogenic Bacterial Isolates	Algal species and Organic extractant								Antibiotic disc µg
		Porphrya tenera		*Laminaria japonica*		*Eisenia bicyclis*		*Undaria pinnatifida*		
		A	M	A	M	A	M	A	M	
1	*K. pneumoniae*	-	-	-	+	+	-	+	-	MEM 10
2	*E. coli*	-	-	-	+	++	+	++	-	MEM 10
3	*P. aeruginosa*	-	-	+	-	-	+++	-	-	MEM 10
4	*Salmonella sp.*	-	-	-	-	-	-	-	-	AMC 30
5	*E. feacalis*	-	-	-	+	++	-	++		AMC 30
6	*MRSA*	+	-	+	++	++	+++	+++	+++	MEM 10 MXF 5 AMC 30
7	*B. subtilis*	+	-	+ +	++	+	+	+++	++	MEM 10
8	*Staph. aureus*	-	-	++ +	+++	++	+++	+++	+++	MEM 10
9	*Staph xylosus* Yeast	-	-	+	++	-	-	+	+	MEM 10
10	*C. albicans*	-	-	+++	+ +	-	-	+	-	

(-) No activity, (+) low activity, (++) moderate activity, (+++) high activity.
A: acetone; M: methanol MRSA: methicillin resistant *Staphylococcus aureus.*



(a) (b)

Fig. 1. Effect of the organic solvents methanol and acetone *Laminaria japonica* extracts on (A) *Staphlococcus aureus* showing the zone of inhibition ; (B) *Candida albicans* showing a larger inhibition zone. (A: acetone; M: methanol)



A: acetone; M: methanol. MRSA: methicillin resistant *Staphylococcus aureus*

Fig. 2. Effect of the organic solvents extracts obtained from *Eisenia bicyclis* and *Undaria pinnatifida* on (A): MRSA showing an zone of inhibition around the well ; (B): *Staphlococcus aureus* indicating a good inhibitory activity around the well

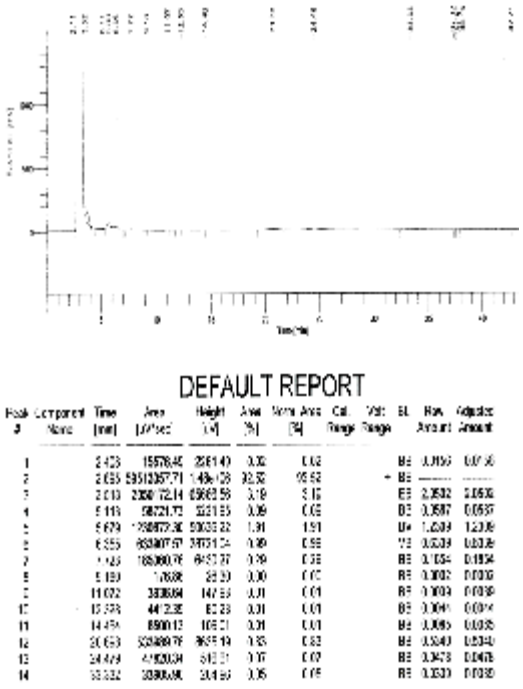


Fig. 3. HPLC analysis for *Undaria pinnatifida* acetone extract showing the highest peak related to a major chemical component of phenolic structure to which the potent inhibitory activity is being attributed

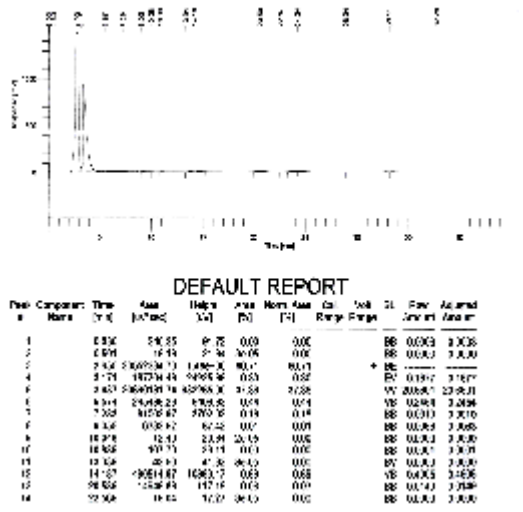


Fig. 4. HPLC analysis for *Laminaria japonica* acetone extract indicated the major chemical constituent to be of phenolic nature revealed by the highest peak

DISCUSSION

Antimicrobial activity

The production of antimicrobial activities, in the present work, indicated the capability of seaweeds to produce bioactive secondary metabolites mainly against gram positive bacteria. Previous screening studies of antimicrobial activities from macroalgae have detected that frequent potent antibacterial activity was reported more on gram positive than on gram negative bacteria[24, 1]. Taskin[27] and Tuney[31] reported that gram positive bacteria were more effectively controlled by the algal extracts used in their study compared to gram negative isolates.

The increased susceptibility of a particular bacterial group was due to the difference in cell wall structure and composition[21,29]. In gram negative bacteria, the outer membrane acts as a barrier to many environmental substances including antibiotics[30]. The presence of thick murine layer in the cell wall prevents the entry of inhibitory molecules[12].However; more emphasis should be pointed on the algal secondary metabolites mechanism of action.

Moreover, it was noted that brown algal extracts exhibited higher antimicrobial activity than red algal extract. Similar results were also reported by Kandhasamy[12] and Karthikaidevi[13]. These strong activities of brown seaweeds may be due to phenolic compounds that have strong bactericidal activity[20]. Viachosi[33] reported that Phaeophyta extracts exhibited the highest antimicrobial activity followed by Rhodophyta and Chlorophyta, in agreement with our findings.

Of the two organic solvents used, methanol was determined as the best solvent for the isolation of antimicrobial compound particularly from brown seaweeds, with the exception of *Laminaria* acetone extract which inhibited *Candida albicans*, hence supporting our findings where Manilal[16] and Rangaiah[23] indicated that extraction method had definite effects on the isolation of bioactive principles; the extract obtained having an inhibitory effect with one kind of solvent could not be obtained with another solvent, which may be related to the presence of bioactive metabolites present in this algal species, soluble in one solvent but not in the other[25], thus in addition to the therapeutical property of

macroalgal secondary metabolites; the use of a specific organic solvent as well as the extraction method will interfere in giving variable degrees of algal antimicrobial activity. Geographical locations and seasonal variations may alter algal extraction; however, it is still not clear how these variations could be used to optimize production in general.

Results obtained from HPLC analysis of *Laminaria japonica* and *Undaria pinatifida* indicated that their major chemical constituents

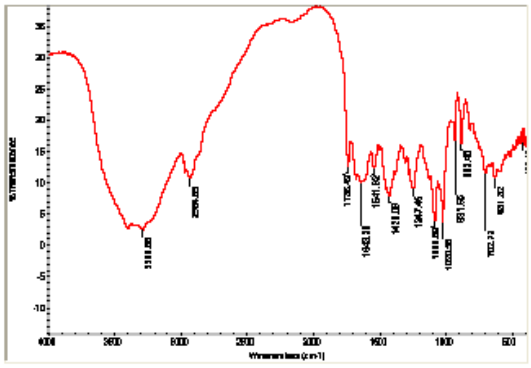


Fig. 5. FTIR spectra of *Undaria pinnatifida* representing the major chemical constituents of the brown algal species being experimented

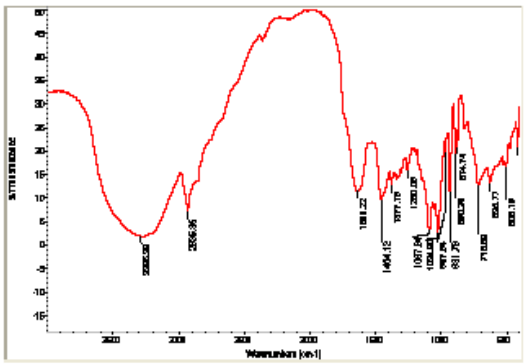


Fig. 6. *Laminaria japonica* FTIR spectrum showing the highest peak related to the major chemical constituent of the brown alga being tested

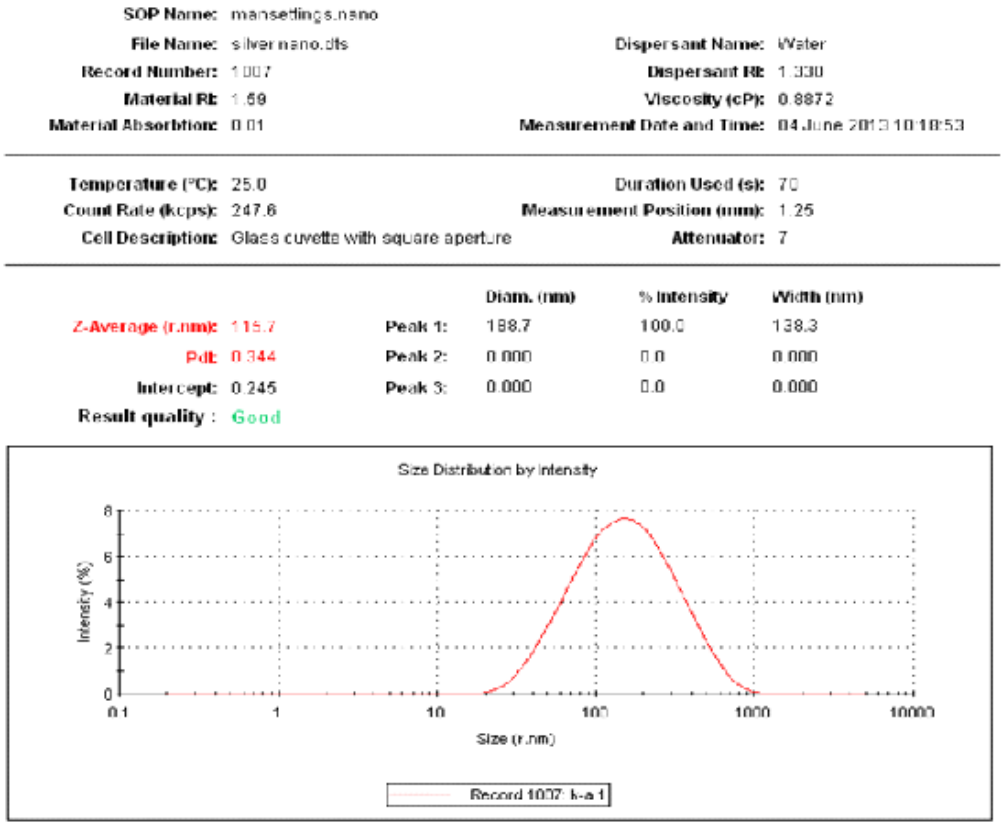


Fig. 7. *Laminaria japonica* nano particles measurement by Malvern zeta sizer showing one single peak indicating possibly stable antimicrobial activity

are of phenolic nature giving the highest antimicrobial activity[20].

FTIR spectral analysis for *Undaria pinnatifida* with peaks at 3266.66, 2934. 89 and 1736.49 cm-1, could possibly be [C-OH / alcohol (including phenol), C-C-H/ Alkane (saturated aliphatic) and C=O / non acid carbonyl (stretching of esters) respectively (Figure5). *Laminaria japonica* peaks observed at 3285.38, 2935.35 and 1635.22 cm-1 (Figure6) could be related to [C-OH / Alcohol (including phenol), C-C-H/Alkane (saturated aliphatic) and C-NH / Amine respectively results indicating that the major constituents of the selected brown seaweeds are mainly of phenolic nature (amide I) emphasizing the nutritional and therapeutic valuable effect of marine macroalgae, particularly brown macroalgae as reported by Cardenas[3] and Chauhan[5].

On the other hand, only *Laminaria japonica* (Kombu) acetone extract with nano particle measurement gave a single peak within 10 and 1000 nm interpreted to go well beyond simply measuring particle size but provide better understanding of sample's stability with a potent antimicrobial activity[19].

CONCLUSION

In this present investigation, all edible seaweeds methanolic extract precisely brown macroalgae, showed potent antimicrobial effect particularly on gram positive bacteria compared to the red alga *Porphyra tenera* and to acetone extracts, offering new promising opportunities for producing novel types of bioactive compounds used as alternatives to synthetic antibiotics.

ACKNOWLEDGMENTS

This research project was supported by a grant from the Research Center of the Female Scientific and Medical Colleges Deanship of Scientific Research, King Saud University.

REFERENCES

1. Ballantine DL, Gerwick WH, Velez SM, Alexander E and Guevara P, Antibiotic activity of lipid soluble extracts from Caribbean marine algae. Hydrobiologia, 1987: **151/152**: 463-469.
2. Berghe V.A and A.J Vlietinek, Screening methods for antibacterial and antiviral agents from higher plants. *Methods for Plant Biochemistry,* 1991; **6**: 47-48.
3. Cardenas, G.; Mirand, S. P., FTIR and TGA studies of chitosan composite films. *J. Chil. Chem. Soc.,* 2004; **49**(4), 291-295.
4. Chakraborthy K, Lipton AP, Paulraj R and Vijayan KK, Antibacterial diterpernoids of *Ulva fasciata* Delile from South –western coast of Indian Peninsula. *Food Chem.*,2010a; **119**: 1399-1408.
5. Chauhan, G. S.; Jaswal, S.; Verma, M., Post functionalisation of carboxtmethylated starch and acrylonitrile based networks through amidoximation for use as ion sorbents. *Carbohydra. Poly.*, 2006; **66** (4), 435-443.
6. Cox S, Abhu-Ghannam N and Gupta S, An assessment of the antioxidant and antimicrobial activity of six species of edible Irish seaweeds. *Int. Food Res. J.*, 2010; **17**: 205-220.
7. Ely R, Supriya T., Naik CG, Antimicrobial activity of marine organisms collected off the coast of South East India. *J. Exp. Biol. and Ecol.*2004; **309**: 121-127.
8. Faulkner DJ, Marine natural products chemistry: introduction. *Chem. Rev.* 1993; **93**: 1671-1673.
9. Fayaz K., K.N. Namitha, M.M. Chidambara, S.R. Mahadeva, S. K. Sarada and S. Ravishankar, Chemical composition Iron bioavailability and antioxidant activity of *Kappaphycus alvarezi* (Doty). *J. Agric. Food Chem.*, 2005; **53**: 792-797.
10. Febles CI, Arias A., Gill-Rodriguez MC, In vitro study of antimicrobial activity in algae (Cholorophyta, Phaeophyta and Rhodophyta) collected from the coast of Tenerife (in Spanish). *Anuario Del Estudios Canarios*, 1995; **34**: 181-192.
11. Gonzalez AD, Basilio PGA, Cabello A, Gorrochategul J, Suay I, Vicente F, Portillo E, Rio JDM, Garcia RG and Pelaez F, Screening of antimicrobial activities in red, green and brown macroalgae from Gran canaria (Canary Islands, Spain). *Int. Microbial.*, 2001; **4**: 3: 35-40.
12. Kandhasamy M and Arunachalam KD, Evaluation of in vitro antibacterial property of seaweed of Southeast coast of India. *African J. Biotech.*,2008; **7**(12):1958-1961.
13. Karthikaidevi G, Manivannan K, Thirumaran G, Anantharaman P, Balasubaramanian T, Antibacterial properties of selected green seaweeds from Vedalai coastal waters, Gulf of Mannar Marine Biosphere Reserve Global. *J. Pharmacol.*, 2009; **3**(2): 107-112.

14. Kögel-Knaber I. Analytical approaches for characterizing soil organic matter, *Org. Geochem.*, 2000); **31**: 609-625.
15. Le Tutour BF, Benslimane MP, Gouleau JP, Saadan B and Quemeneur F, Antioxidant and pro- oxidant activities of the brown algae, *Laminaria digitata, Himanthalia elongate, Fucus vesiculosus*, *Fucus serratus* and *Ascophyllum nodosum*. *J. Appl. Phycol.*, 1998; **10**: 121-9.
16. Manilal A, Sujith S, Sabarathnam B, Kiran GS, Selvin J, Shakir C, Ghandhimathi and Lipton A.P, Antimicrobial potential and seasonality of red algae collected from the Southwest coast of India tested against shrimp, human and phytopathogens. *Ann. Microbiol.*, 2009; **59**(2): 207-219.
17. Manivannan K, Karthikai devi G, Anantharaman P and Balasubramanian T, Antimicrobial potential of selected brown seaweeds from Vedalai coastal water, Gulf of Mannar. Asian Pacific *Journal of Tropical Biomedicine:* 2011 ; 114-120.
18. Marechal J.P, Guloli G, Hellio C, Thomas-Guyon H, Callow ME, Clare AS and Ortalo-Magne, Seasonal variations in antifouling activity of crude extracts of the brown alga *Bifurcaria bifurcate* (Cystoseiraceae) against Cyprids of *Balanus amphitrite* and the marine bacteria *Cobertia marina* and *Pseudoalteromonas haloplanktis*. *J. Exp. Mar. Biol. Ecol.*,2004; **313**: 47-62.
19. Mohanraj VJ, Chen Y, Nanoparticles Review. *Tropical Journal of Pharmaceutical Research*, 2006; **5** (1): 561-573
20. Nagayama K, Iwamura Y, Shibata T, Hirayama I, Nakamura T, Bactericidal activity phlorotannins from the brown alga *Ecklonia kurome*. *J. Antimicrob. Chemother.*,2002; **50**: 889-893.
21. Paz EA, Lacy RN, Bakhtian M, The blactum antibiotics penicillin and cephalosporin in perspective. Hodder strong, London.1995; 324p.
22. Rajasulochana P, Dhamotharan R, Krishnamoorthy P, Murugesan S, Antibacterial activity of the extracts of marine red and brown algae. *J. Am. Sci.*,2009; **5** (3): 20-25.
23. Rangaiah SG, Lakshmi P, Manjula E, Antimicrobial activity of seaweeds *Gracillaria, Padina* and *Sargassum* sps. on clinical and phytopathogens. *Int. J. Chem. Anal. Sci.*,2010; **1** (6): 114-117.
24. Reichelt JL, Borowitzka MA, Antimicrobial activity from marine algae: results of a large-scale screening program. *Hydrobiologia*, 1984; **116/117:** 158-167.
25. Salem WM, Galal H and Nasr El- deen F, Screening for antibacterial activities in some marine algae from the red sea (Hurghada, Egypt). *African J. Microbiol. Research*,2011; **5**(15): 2160-2167.
26. Scheuer PJ (ed), Bioorganic marine chemistry, vols 1-3. Springer, Berlin Heidelberg New York 1987.
27. Smit AJ, Medicinal and pharmaceutical uses of seaweed natural products. *A review. J.Appl. Phycol.*,2004; **16**: 245-262.
28. Taskin E, Ozturk M and Kurt O, Antibacterial activities of some marine algae from the Aegean Sea (Turkey). *Afr. J. Biotechnol.*,2001; **6**: 2746-2751.
29. Taskin E, Ozturk M and Kurt O, Antibacterial activities of some marine algae from the Aegean Sean (Turkey). *Afr. J. Biotechnol*, 2007; **6**: 2746-2751.
30. Tortora GJ, Funke BR and Case CL, Microbiology: an introduction. Benjamin Cummings. *San Francisco*,2001; **88**.
31. Tuney I Cadirci BH, Unal D and Sukatar A, Antimicrobial activities of the extracts of marine algae from the coast of Urla (Izmir, Turkey). *Turk. J. Biol.*,2006; **30**: 171-175.
32. Valchos V., Critchley AT, and Von Holy A, Antimicrobial activity of extracts from selected Southern African marine macroalgae. *South African J. Sci.*,1997; **93**: 328-332.
33. Viachosi V, Critchley AT, Holy AV, On the grass status of seaweeds observations on the association between antibacterial activity of ethanolic extracts and meta 1 levels present in selected seaweeds. *Bull. Mar. Sci. Fish., Kochi Univ.*,2001; **21**: 7-12.