Indian Journal of Geo-Marine Sciences
Vol. 45(10), October 2016, pp. 1365-1371

Detection of antibacterial activity and its characterization from the marine macro-algae *Sargassum wightii* (Greville ex J. Agardh 1848)

Devanya Rosaline[1]., Sakthivelkumar S[2]., Rajendran K[3]. & Janarthanan S*[4]
[1]Department of Zoology & [3]Department of Botany, Thiagarajar College (Autonomous), Madurai 625009, India
[2 & 4]Department of Zoology, University of Madras, Guindy Campus, Chennai 600 025, India
*[E-mail: sundaram.janarthanan@gmail.com; janas_09@yahoo.co.in]

Received 07 April 2015; Revised 08 July 2015

Natural occurrence of a compound, mono-ethylhexylphthalate (MEHP) in one of the column fractions of marine macro-algae *Sargassum wightii* with promising antibacterial activity is examined. Infrared spectroscopy disclosed the presence of one acid group, one ester group, aromatic and aliphatic chains. ^{1}H and ^{13}C NMR spectra along with IR data predicted the structure of MEHP. Abundance of MEHP in the column fraction IV of *S. wightii* and its effective antibacterial activity points toward the potential utility of macro-algae *Sargassum wightii* as a source of novel biomedical compound.

[**Keywords:** Marine macro-algae, *Sargassum wightii,* Mono-ethylhexyl phthalate (MEHP), Antibacterial activity]

Introduction

Marine macro-algae or seaweeds are non-vascular and photosynthetic plants that inhabit the coastal regions within rocky intertidal or submerged reef-like habitats. Marine macro-algae are one of the most promising and richest sources of bioactive primary and secondary metabolites[1]. They are the extraordinary sustainable resources in the intertidal ecosystem that occupy an important place as a source of novel biomedical compounds[2]. They are known to produce a variety of secondary metabolites with broad spectrum biological activities. Many secondary metabolites with antifungal[3], cytostatic[4], nematicidal, fungicidal[5], antihelminthic and antioxidant[6,7], antimycobacterial[8], HIV antiviral[9], antiproliferative[10], antiviral[11] and antibacterial[12-17] properties have been identified from marine macro-algae. As our earlier study with crude acetone extract of *Sargassum wightii* exhibited good antibacterial activity to a wide array of human pathogenic bacteria[18], the present study was made to isolate and characterize active antibacterial molecule from the marine algae, *Sargassum wightii.*

Materials and Methods

Sargassum wightii Greville (brown algae - Phaeophyta) were collected from rocky and intertidal coast of Tuticorin, Tamil Nadu, India during the pre-monsoon (June - September) period. Dried algal powder of *S. wightii* was thoroughly soaked in hexane (100g / 300ml) at room temperature for 48 hours with occasional shaking in air-tight container. The extract was filtered and evaporated to dryness under pressure using rotary vacuum evaporator at 50°C. The residue was sequentially extracted using ethyl acetate followed by acetone and methanol using cold percolation method[19].

Crude acetone extract obtained from sequential extraction was adsorbed on to silica gel (Acme's 60–120 mesh) and chromatographed on a silica gel column (Acme's 100–200 mesh). The adsorbed acetone extract was fractionated with increasing polarity of solvents as per Table 1. Fractions pooled were stored at 4ºC in darkness until bioassay.

Pure cultures of human pathogenic Gram-positive (Methicillin resistant *Staphylococcus aureus, Bacillus subtilis* and *Enterococcus faecalis*) and Gram-negative (*Pseudomonas aeruginosa, Erwinia* sp., *Salmonella paratyphi-B, Enterobacter aerogenes, Proteus vulgaris, Klebsiella pneumoniae* and *Escherchia coli*) bacterial strains obtained from the Laboratory of

Microbiology, Christian Medical College, Vellore, Tamil Nadu, India were used for studies on antibacterial activity.

Antibacterial activity of all the column fractions against Gram-negative and Gram-positive bacteria was carried out using the disc-diffusion method[20].

Table 1—Column chromatography fractionation of crude acetone extract of *S. wightii* using sequential gradient partition with solvents

Eluent ratio of solvents	Fractions	Solvent ratio number
Hexane - 100%	No fractionation/separation in thin layer chromatography	1
Hexane: Ethyl acetate 95:5, 90: 10, 85:15, 80:20, 75:25	FRACTION I*	2-6
70: 30, 65:35, 60:40, 55:45, 50:50	FRACTION II*	7-11
45:55, 40:60, 35:65, 30:70, 25:75	FRACTION III*	12-16
20:80, 15:85, 10:90, 5:95	No fractionation/separation in TLC	17-20
Ethyl acetate - 100%	FRACTION IV*	21
Ethyl acetate : Acetone 95:5, 90: 10, 85:15, 80:20, 75:25	FRACTION V*	22-26
70: 30, 65:35, 60:40, 55:45, 50:50	FRACTION VI*	27-31
45:55, 40:60, 35:65, 30:70, 25:75	FRACTION VII*	32-36
20:80, 15:85, 10:90, 5:95	No fractionation/separation in TLC	37-40
Acetone - 100%	FRACTION VIII*	41
Acetone: Methanol 95:5, 90: 10, 85:15, 80:20, 75:25	No fractionation/separation in TLC	42-46
70: 30, 65:35, 60:40, 55:45, 50:50	FRACTION IX*	47-51
45:55, 40:60, 35:65, 30:70, 25:75, 20:80, 15:85, 10:90, 5:95	No fractionation/separation in TLC	52-60
Methanol - 100%		61

*TLC produced similar pattern of fractionation/profiling/separation with comparable Rf values were pooled

Three different concentrations (1000 µg/disc, 500 µg/disc and 250 µg/disc) of each column fractions were dissolved in 4% dimethyl sulfoxide (DMSO) and loaded on sterile discs (Hi-media) placed on solidified agar medium. Negative control was prepared using 4% DMSO. The plates were incubated for 24 hours at 37°C. Zones of inhibition were recorded in millimeters and the experiment was repeated thrice for concordant results. The antibacterial response of each of the fractions was compared with the Kirby-Bauer Standard Interpretative Chart[21]. All the data were statistically analyzed using Student's "t" test at 95% significant levels.

Fraction of *S. wightii* with highest antibacterial activity was further characterized using GC-MS (Shimadzu-QP2010 chromatography system with software version 2.53), IR (Shimadzu FT IR 8000 series in the region between 4000–500 cm^{-1}), ^{1}H NMR (at 23ºC in $CDCl_3$ using a Bruker 300 MHz spectrometer) and ^{13}C NMR (Bruker 300, 75MHz spectrometer in $CDCl_3$ as solvent). Molecular structure of the major sub-fraction of antibacterial substance was predicted based on the IR, ^{1}H and ^{13}C NMR spectra.

Results

Antibacterial assay performed for various column fractions revealed a significant activity by fraction IV for many of the bacterial pathogens in all its concentrations over other column fractions. The antibacterial activity determined by zone of inhibition (ZI) for different concentrations of fraction IV of *S. wightii* against *B. subtilis* were measured as 12.66±0.57 mm for 250µg, 16.33±0.57 mm for 500µg and 17.33±0.57 mm for 1000µg (Table 2; Fig. 1). *B. subtilis* was highly susceptible for all the concentrations of fraction IV when compared to standard interpretative chart for

positive control. Fractions II and VII of *S. wightii* also acted against the growth of *B. subtilis* but the antibacterial response were resistant when compared to the Kirby-Bauer Standard Interpretative Chart (Table 2). Column fractions I, II, IV, VI and VIII of *S. wightii* inhibited the growth of *E. faecalis* but fraction VI showed a ZI value of 13.33±0.57 mm with intermediary antibacterial response. Other fractions also showed ZI but were reported resistant.

The antibacterial response when compared to the Kirby-Bauer Standard Interpretative Chart revealed that 1000µg concentration of fraction III alone was sensitive to the growth of *P. aeruginosa*; 1000µg of fraction II and 500 µg of fraction III were intermediate in their antibacterial response (Table 3; Fig. 1). A sensitive antibacterial activity against *Erwinia* sp. was shown by fraction VII of *S. wightii* with a ZI value for 1000µg concentration as 14.33±0.57 mm. As per Kirby-Bauer Standard Interpretative Chart, 500µg concentration of fraction IV showed an intermediary antibacterial response against *E. aerogenes* but 1000µg concentration was sensitive

Table 2— Antibacterial activity of various column fractions of *Sargassum wightti* against human pathogenic Gram-positive bacteria

Fractions	Concentration (µg/disc)	Zone of inhibition (mm)		
		Methicillin resistant *Staphylococcus aureus*	*Bacillus subtilis*	*Enterococcus faecalis*
I	250	-	-	-
	500	-	-	8.33 ± 0.57*
	1000	-	-	10.33 ± 0.57*
II	250	-	-	-
	500	-	9.33 ± 0.57*	10.33 ± 0.57*
	1000	-	10.33 ± 0.57*	11.33 ± 0.57*
III	250	-	-	-
	500	-	-	-
	1000	-	10.33 ± 0.57*	-
IV	250	-	12.66 ± 0.57	-
	500	8.33 ± 0.57*	16.33 ± 0.57*	7.33 ± 0.57*
	1000	10.33 ± 0.57*	17.33 ± 0.57*	9.33 ± 0.57*
V	250	-	-	-
	500	-	-	-
	1000	-	-	-
VI	250	-	-	10.33 ± 0.57
	500	-	-	11.33 ± 0.57^{NS}
	1000	-	-	13.33 ± 0.57*
VII	250	-	9.33 ± 0.57	-
	500	-	10.33 ± 0.57^{NS}	-
	1000	-	11.33 ± 0.57*	-
VIII	250	-	-	9.33 ± 0.57
	500	-	-	10.33 ± 0.57^{NS}
	1000	-	-	11.33 ± 0.57*
IX	250	-	-	-
	500	-	-	-
	1000	-	-	-
4% DMSO	Negative Control	-	-	-
Streptomycin (10 µg)	Positive Control	22.33 ± 0.58		

Kirby-Bauer standard interpretative chart for positive control [21]:
1. Carbenicillin (10µg) for *P. aeruginosa* - Resistant (R) <13mm; Intermediate (I) 14-16mm; Sensitive >17mm
2. Streptomycin (10µg) for other bacteria - Resistant (R) <11mm; Intermediate (I) 12-14mm; Sensitive >15mm
* – Significant at 95% level, NS –Non-significant, ZI – Zone of inhibition, "-" No activity; Each value representing mean ± SD of 3 replicates

to the bacterium. Fraction V showed an antibacterial activity to *P. vulgaris* with ZI values of 8.33±0.57 mm (500µg) to 10.33±0.57 mm (1000µg) with a significant ($p<0.05$) increase in activity. The ZI for fraction IV for *E. coli* at 1000µg concentration was measured as 9.33±0.57 mm ($p<0.05$), but it was inferred as resistant. The studies on antibacterial activity of various fractions eluted from column chromatography revealed the fact that the bacterial species, *S. paratyphi*-B and *K. pneumoniae* were found to be resistant to all the column fractions of *S. wightii*.

Fraction IV of *S. wightii* possessing highest antibacterial activity was therefore selected for further characterization. GC-MS analysis of fraction IV revealed that mono-ethylhexyl phthalate (MEHP) was the major compound occupying an area of 97.41 % (Fig. 2A).

Table 3—Antibacterial activity of various column fractions of *Sargassum wightti* against human pathogenic Gram-negative bacteria

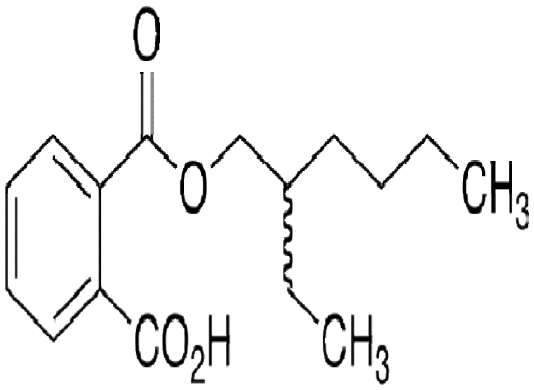
Fractions	Conc. (µg/disc)	Zone of inhibition (mm)						
		P. aeruginosa	*S. paratyphi-B*	*Erwinia* sp.	*E. aerogenes*	*P. vulgaris*	*K. pneumoniae*	*E. coli*
I	250	-	-	-	-	-	-	-
	500	9.33 ± 0.57*	-	-	-	-	-	-
	1000	11.33 ± 0.57*	-	-	-	-	-	-
II	250	-	-	-	-	-	-	-
	500	10.33 ± 0.57*	-	-	-	-	-	-
	1000	12.33 ± 0.57*	-	-	-	-	-	-
III	250	10.33 ± 0.57	-	-	-	-	-	-
	500	12.33 ± 0.57*	-	-	-	-	-	-
	1000	14.33 ± 0.57*	-	-	10.33 ± 0.57*	-	-	-
IV	250	-	-	-	10.33 ± 0.57	-	-	-
	500	-	-	-	11.33 ± 0.57^{NS}	-	-	-
	1000	-	-	-	15.33 ± 0.57*	-	-	9.33 ± 0.57*
V	250	-	-	-	-	-	-	-
	500	-	-	-	-	8.33 ± 0.57*	-	-
	1000	-	-	-	-	10.33 ± 0.57*	-	-
VI	250	-	-	-	-	-	-	-
	500	-	-	-	-	-	-	-
	1000	-	-	-	-	-	-	-
VII	250	-	-	10.33 ± 0.57	-	-	-	-
	500	-	-	11.33 ± 0.57^{NS}	-	-	-	-
	1000	-	-	14.33 ± 0.57*	-	-	-	-
VIII	250	-	-	-	-	-	-	-
	500	-	-	-	-	-	-	-
	1000	-	-	-	-	-	-	-
IX	250	-	-	-	-	-	-	-
	500	-	-	-	-	-	-	-
	1000	-	-	-	-	-	-	-
4% DMSO	NC	-	-	-	-		-	
STZ	PC	22.33 ± 0.58						

Kirby-Bauer standard interpretative chart for positive control [21]

1. Carbenicillin (10µg) for *P. aeruginosa* - Resistant(R) <13mm; Intermediate (I) 14-16mm; Sensitive >17mm
2. Streptomycin (10µg) for other bacteria - Resistant(R) <11mm; Intermediate (I) 12-14mm; Sensitive >15mm

* – Significant at 95% level, NS –Non-significant, ZI – Zone of inhibition, "-" No activity; Each value representing mean ± SD of 3 replicates
Conc. - Concentration; NC - Negative control; PC - Positive control; STZ - Streptomycin (10 µg)

IR spectrum of fraction IV indicated the presence of one acid group and one ester group (Fig. 2B). It also showed the presence of aromatic stretching frequency. According to the data, the extracted compound contained an acid ester, aromatic and aliphatic chains. The (^{13}C) NMR spectra indicated a value of 172, the carbonyls with 137 and 133 and both of them were aromatic ring carbons; 40 to 28 were aliphatic chain carbons (it was nearly 4 carbons) and 64 (showed 2^{O}carbon 19 and 15 were 2-methyl carbons). Proton (^{1}H) NMR spectra clearly indicated one aromatic ring (7.68 ppm and 7.06 ppm) and two methyl groups and 6 proton averages (Fig. 3). Based on IR and NMR (^{13}C &^{1}H) spectra, the structure of the major active anti-bacterial component in the fraction IV of *S. wightii* is:



IR: 3460, 2926, 2859, 1728, 1612, 1510, 1457, 1379, 1278, 1124, 1071, 742

^{1}H-NMR: 0.86-0.92 (6H,m,2CH_3), 1.01-1.40 (6H,m,3CH_2), 1.78 (4H,m,2 CH_2), 4.01 (1H,m,CH), 7.06-7.68 (4H,m,4CH).

^{13}C-NMR: 5.12 (CH_3), 15.9 (CH_3), 18.9 (CH_2), 25.8 (CH_2), 27.6 (CH_2), 31.8 (CH_2), 34.2 (CH_2), 83.0 (CH), 133.5-137.0 (Aromatic), 172.1 (C=O, -COO)

Molecular formula: C_{16} H_{22} O_4 ;Molecular weight: 278.15.

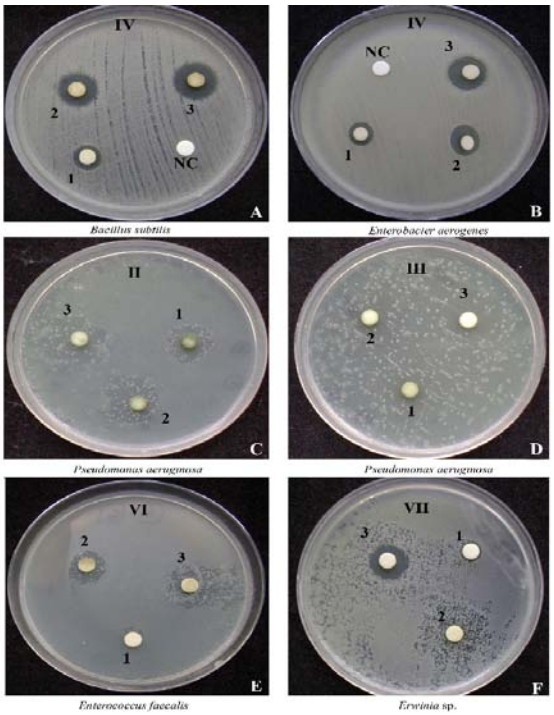
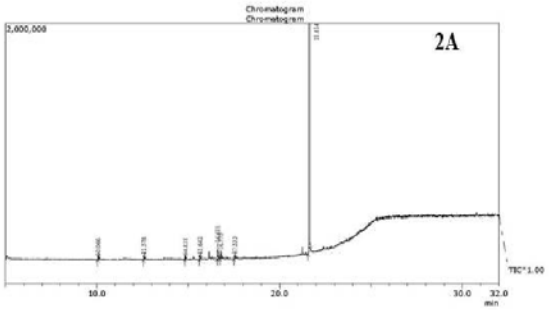


Fig. 1— Antibacterial activity of various column fractions (I - IX) of *Sargassum wightii* against different human bacterial pathogens. **A.** Antibacterial activity of the column fraction-IV of *S. wightii* against *Bacillus subtilis*; **B.** Antibacterial activity of the column fraction- IV of *S. wightii* against *Enterobacter aerogenes*; **C.** Antibacterial activity of the column fraction-II of *S. wightii* against *Pseudomonas aeruginosa*; **D.** Antibacterial activity of the column fraction-III of *S. wightii* against *P. aeruginosa*; **E.** Antibacterial activity of the column fraction-VI of *S. wightii* against *Enterococcus faecalis*; **F.** Antibacterial activity of the column fraction-VII of *S. wightii* against *Erwinia sp.* 1 - 250µg, 2 - 500µg and 3 - 1000µg of various column fractions; NC – negative control; No antibacterial activity was observed to any of the bacterial organisms for column fractions I, V, VIII and IX

Discussion

Many reports have been published on various isolated compounds from marine macro-algae with biological activity, demonstrating their ability to produce metabolites, with high complexity and unlimited diversity of pharmacological and biological properties[22]. Compounds with antiviral, antifungal and antimicrobial activities have been detected in marine macro algae[23,7]. Recent reports from natural sources also suggest that algae are a promising group to furnish novel biochemically active substances[24]. Present study on the marine algae *Sargassum wightii* clearly revealed the effectiveness of crude acetone extract of *S. wightii* in providing antibacterial activity

to a panel of eight target bacteria. Further, among various column fractions of *S. wightii*, fraction IV demonstrated highest antibacterial activity. The major compound in this fraction was identified as mono-ethylhexyl phthalate (MEHP).



Peak Report TIC

PEAK#	R.TIME	AREA	AREA%	NAME
1	10.066	41037	0.18	6-Methyl-1-heptanol
2	12.578	67142	0.30	1-Tridecene
3	14.831	48974	0.22	1-OCTADECENE
4	15.642	58219	0.26	1,2-BENZENEDICARBOXYLIC ACID, BIS (2-METHYLPROPYL) EST
5	16.611	223970	0.99	Dibutyl phthalate
6	16.775	98203	0.43	Octyl butyl phthalate
7	17.533	46900	0.21	Phthalic acid, cyclobutyl hexyl ester
8	21.614	22001774	97.41	Monoethylhexyl phthalate
		22586219	100.00	

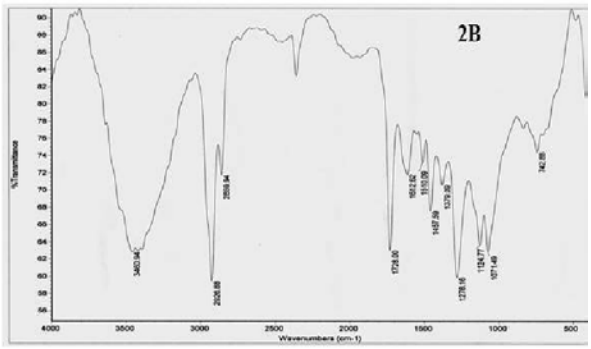


Fig. 2— A. Gas chromatography and mass spectroscopy (GC-MS) analysis of column fraction-IV of *S. wightii* and B. Infrared spectroscopy (IR) analysis of fraction-IV of *S. wightii*

Since MEHP is the most abundant component in the column fraction of *S. wightii* with antibacterial properties, it could be a potent biotic compound against pathogenic bacteria. Earlier reports revealed isolation of di-(2-ethylhexyl) phthalate (DEHP) from terrestrial and marine organisms including plants[25], marine algae[26-29], and fungal and bacterial culture broths[30, 31]. There were also reports of natural presence of phthalate esters [di-(2-ethylhexyl) phthalate and di-butyl phthalate] in the environment long before their commercial manufacture and release.

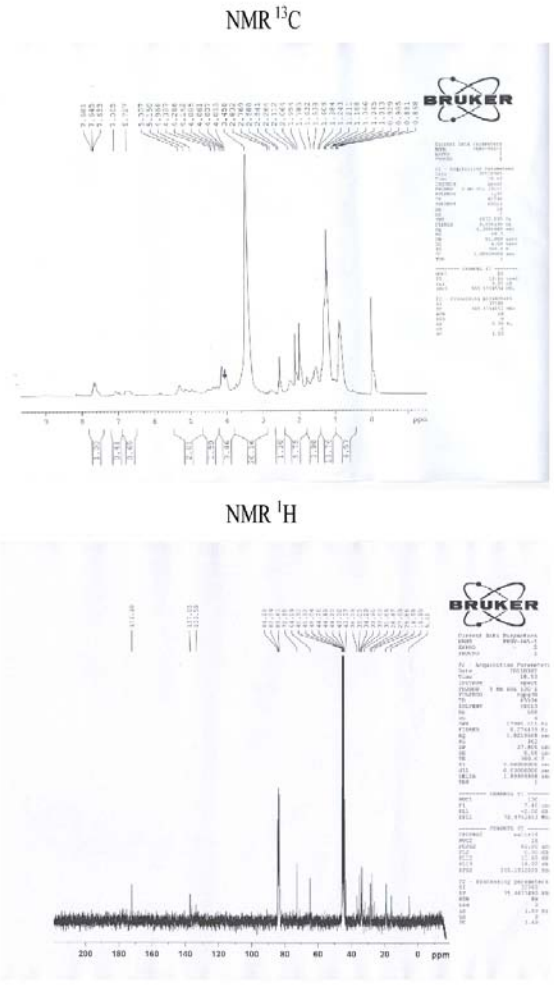


Fig. 3— ^{1}H and ^{13}C Nuclear magnetic resonance (NMR) analysis of fraction-IV of *S. wightii*

Conclusion

In our present investigation, mono-ethylhexyl phthalate (MEHP) was reported for the first time from *S. wightii* with a significant antibacterial activity against some selected human pathogenic bacteria. Since MEHP is the most abundant component in the column fraction of marine macro-algae *S. wightii* with antibacterial properties, the commonly present marine macro-algae*, S. wightii* could be a potent source of the biotic compound MEHP.

Acknowledgement

Authors thank the DST-PURSE, University of Madras (Grant No. 790/2010) for financial support.

References

1. Faulkner, D.J., Marine natural products, *Nat Prod Rep*., 19 (2002) 1-48.
2. Pallela, R., Na-Young, Y., Kim, S.K., Anti-photoaging and photoprotective compounds derived from marine organisms, *Mar Drugs* 8 (2010) 1189-1202.
3. Tariq, V.N., Antifungal activity in crude extracts of marine red algae, *Mycol Res*., 95 (1991) 1433-1440.
4. Harada, H., Kamei, Y., Selective cytotoxicity of marine algae extracts to several human leukemic cell lines, *Cytotechnology* 25 (1997) 213-219.
5. Ara, J., Sultana, V., Ehteshamul-Haque, S., Qureshi, S.A., Ahmad, V.U., Bioactivity of seaweed against soil borne plant pathogens, *Phytologia* 85 (1998) 292-299.
6. Metzger, P., Roger, M.N., Largean, C., Botryolins A ans B, two tetramethylsequalenetriethers from the green microalga *Botryoccus brauni*, *Phytochem*., 59 (2002), 839-843.
7. Chew, Y.L., Lim, Y.Y., Omar, M., Khoo, K.S., Antioxidant activity of three edible seaweeds from two areas in South East Asia, *LWT-Food Sci Technol*., 4 (2008) 1067-1072.
8. Copp, B., Antimycobacterial natural products, *Nat Prod Rep*., 20 (2003) 535-557.
9. Luescher-Mattli, M., Algae, a possible source for new drugs in the treatment of HIV and other viral diseases, *Curr Med Chem*., 2 (2003) 219-225.
10. Yuan, Y.V., Walsh, N.A., Antioxidant and antiproliferative activities of extracts from a variety of edible seaweeds, *Food Chem Toxicol*., 44 (2006) 1144-1150.
11. Vallim, M.A., Barbosa, J.E., Cavalcanti, D.N., *In vitro* antiviral activity of diterpenes isolated from the Brazilian brown alga *Canistrocarpus cervicornis*, *J Med Plants Res*., 4 (2010) 2379-2382.
12. Valdebenito, H., Bittner, M., Sammes, P.G., Silva, M., Watson, W.H., A compound with antimicrobial activity isolated from the red seaweed *Laurencia chilensis*, *Phytochem*., 21 (1982) 1456-1457.
13. Lindequist U., Schweder, T., Marine biotechnology. In Rehm, H.J. and Reed, G. (Eds.), Biotechnology, Vol. 10. Weinheim: Wiley-VCH, pp. 441-484, 2001.
14. Xu, N., Fan, X., Yan, X., Li, X., Niu, R., Tseng, C.K., Antibacterial bromophenols from the marine red alga *Rhodomela confervoides*, *Phytochem*., 62 (2003) 1221-1224.
15. Freile-Pelegrın, Y., Morales, J.L., Antibacterial activity in marine algae from the coast of Yucatan, Mexico, *Bot Mar*., 47 (2004) 140-146.
16. Taskin, E., Ozturk, M., Taskin, E., Kurt, O., Antibacterial activities of some marine algae from the Aegean Sea (Turkey), *Afr J Biotechnol*., 6 (2007) 2746-2751.
17. Kotnala, S., Garg, A., Chatterji, A., Screening for the presence of antimicrobial activity in few Indian seaweeds, *Pertanika J Trop Agrl Sci*., 32 (2009) 69-75.
18. Rosaline, X.D., Sakthivelkumar, S., Rajendran, K., Janarthanan, S., Screening of selected marine algae from the coastal Tamil Nadu, South India for antibacterial activity, *Asian Pacific J Trop Biomed*., (2012) S140.
19. Handa, S.S., Khanuja, S.P.S., Longo, G., Rakesh, D.D., Extraction Technologies for Medicinal and Aromatic Plants. ©United Nations Industrial Development Organization and the International Centre for Science and High Technology, ICS-UNIDO, AREA Science Park, Padriciano 99, 34012 Trieste, Italy, 2008.
20. Murray, P.R., Baron, E.J., Pfaller, M.A., Tenover, F.C., Yolken, R.H., Manual of Clinical Microbiology, Vol. 6. (Washington DC: ASM), pp. 441-484, 1995.
21. National Committee for Clinical Laboratory Standards.: Performance standards for antimicrobial susceptibility testing: Sixth informational supplement M100-S6. (NCCLS, Wayne, PA), 1995.
22. Kuda, T., Tsunekawa, M., Goto, H., Araki, Y., Antioxidant properties of four edible algae harvested in the Noto Peninsula, Japan, *J Food Comp Anal*., 18 (2005) 625-633.
23. Bansemir, A., Blume, M., Schröder, S., Lindequist, U., Screening of cultivated seaweeds for antibacterial activity against fish pathogenic bacteria, *Aquaculture* 252 (2006) 79-84.
24. Blunt, J.W., Copp, B.R., Munro, M.H., Northcote, P.T., Prinsep, M.R., Marine natural product, *Nat Prod Rep*., 22 (2005) 15-61.
25. Lee, K.H., Kim, J.H., Lim, D.S., Kim, C.H., Anti-leukemic and anti-mutagenic effects of di (2-ethylhexyl) phthalate isolated from *Aloe vera* Linne, *J Pharm Pharmacol* 52 (2005) 593-598.
26. Noguchi, T., Ikawa, M., Uebel, J.J., Andersen, K.K., Lipid constituents of the red algae *Ceramium rubrum.* A search for antimicrobial and chemical defense substances. In Hoppe, H. A., Levring, T., Tanaka, Y. (Eds.), Marine algae in pharmaceutical science, Walter de Gruyter & Co. New York, pp. 711-718, 1979.
27. Stefanov, K., Konaklieva, M., Brechany, E.Y., Christie, W.W., Fatty acid composition of some algae from the black sea. *Phytochem*., 27 (1988) 3495-3497.
28. Sastry, V.M.V.S., Rao, G.R.K., Dioctyl phthalate and antibacterial compound from the marine brown alga, *Sargassum wightii*, *J Appl Phycol*., 7 (1995) 185-186.
29. Chen, C.Y., Biosynthesis of di-(2-ethylhexyl) phthalate (DEHP) and di-n-butyl phthalate (DBP) from red alga *Bangiaatro purpurea*, *Water Resource* 38 (2004) 1014-1018.
30. Amade, P., Mallea, M., Bouaicha, N., Isolation, structural identification and biological activity of two metabolites produced by *Penicillium olsonii* Bainier and Sartory, *J Antibiotics*., 47 (1994) 201-207.
31. Al-Bari, M.A., Bhuiyan, M.S., Flores, M.E., Petrosyan, P., Garcia-Varela, M., Islam, M.A., *Streptomyces bangladeshensis* sp. nov., isolated from soil, which produces bis-(2-ethylhexyl) phthalate, *Int J Sys Evol Microbiol* 55 (2005)1973-1977.