ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Bioassay-guided isolation and identification of compound from *Sargassum ilicifolium* and investigation of antimicrobial activity.

Simpi Chandraraj C.[1*], Nagathan Channabasappa V. [2], Karajgi Santosh R.[3], Kenia Hasti. [4]

[1, 2.] Dept of Pharmacognosy, [3]Dept of Quality Assurance, [4]Dept of Pharmaceutical Chemistry
BLDEA SSM College of Pharmacy and Research Center Vijayapur 586101

Abstract

In many countries, seaweeds are used as traditional food which contains unusual types of secondary metabolites. The aim of this study was to perform bioassay-guided isolation of antibacterial metabolites from an Indian Ocean seaweed *Sargassum ilicifolium*. The seaweed *S. ilicifolium* was collected from the western sea cost of India. The plant was extracted by maceration with hexane saturated with methanol. The extract was partitioned by the Kupchan method to yield petroleum ether 80-120, petroleum ether 40-60, chloroform, ethyl acetate and methanol partitions. The most active partition found in the antibacterial assay i.e. methanol extract was further fractionated using medium pressure liquid chromatography methods to yield pure compound. The structures of the isolated compounds were elucidated using various spectroscopic methods. The antibacterial activities of all fractions were also tested. Methanol and petroleum ether 80-120 partitions exhibited higher and significant antibacterial activity against the *B. subtilis* with 45.92 mL/g and 44.8 mL/g respectively. The antibacterial effects of three fractions of the methanol extract against *Bacillus subtilis* were also ranging from 2.8 to 15.4 mL/g. From the spectral characteristics, the isolated compound from the extract was confirmed to be Di (2-ethylhexyl) phthalate, DEPH with moderate antibacterial activity.

Keywords: Antimicrobial, DEPH, *Sargassum ilicifolium*, Seaweed.

1. INTRODUCTION

Infectious diseases remain the major causes of morbidity and mortality in many developing countries.[1] In recent years, those pathogens bacterial turn out to be organisms resistant to multiple antibiotics, or also called the Multi Drug Resistant (MDR). There are much increasing numbers of victims have been reported.[2] Besides, these problems led to higher maintenance costs and the emergence of discovery of the newer antibiotics was needed for combating MDR strains.[3] Many studies have been reported that the natural resources from the sea have abundant potential bioactive compounds against human pathogens.[4] Marine algae, are considered as good source of novel pharmaceuticals and potent bioactive substances and are most unusually rich in sulfated polysaccharides.[5] Marine algae due to their abundance in bioactive compounds that may exhibit antitumor, cytotoxic antioxidant, anthelmintic, anticoagulant, antibacterial, antifungal, hepatoprotective effects and inhibits DNA polymerase and xanthine oxidase.[6] But the main problems were the limitations of the availability marine organisms. Exploration of bioactive compounds from marine organisms requires a lot of biomaterial that might cause over-exploitation and low levels of conservation.[7] Several bioactive metabolite studies have been reported that from *Sargassum spp*.[8] However, no attempt has ever been made to identify and isolate active principles responsible for unleashing *S. ilicifolium* for antibacterial activity. Identification and isolation of active principles from *S. ilicifolium* might prove promising antibacterial agents through foreseeable future endeavors. Therefore, current study aims at exploration to isolate bioactive potential metabolites for an antibacterial activity.

2. MATERIALS AND METHODS

2.1 General procedures:

NMR spectra were obtained on BrukerDRX-300 using TMS as internal standard. The IR analysis was obtained on Shimadzu FT-IR 8400S. Chromatographic separations were carried out using CombiFlash® Companion® with RediSep® silica cartridge. TLC studies were performed with Merck Millipore TLC Silica gel 60 and Alumina F_{365} TLC plate and spots were analyzed under UV light at 254 and 366 nm.

2.2 Algal material

The algae *Sargassum ilicifolium* Sargassaceae of the class Phaeophyceae was collected manually at the Devgad Island, near Karwar (14°49'12"N 74°7'12"E) of the Arabian Sea during May 2017. After carefully removing the associated algae, the material was washed with fresh water, dried in shade, and weighed (1.9 Kg). The herbarium specimen were carefully prepared and deposited in Department of Pharmacognosy, BLDEA College of Pharmacy, Vijayapur under the code No BLDE-2006-001. The taxonomy study was performed at the Department of Botany, Poona University Pune.

2.3 Preparation of hexane extract and liquid- liquid partition

According to Siddiqui et al., [9] some groups of antimicrobial compounds can be extracted by a single solvent. Therefore, Kupchan method of sequential fractional extraction was used to extract 1.8 Kg of powdered marine organisms. The dried *S. ilicifolium* algal material was powdered in a Wiley mill and the powdered was extracted three times with 5 L of hexane (saturated with methanol) at room temperature by maceration. The

extract was then filtered using Whatman No 1 filter paper and concentrated under vacuum using rotary evaporator (Buchi Rotavapor R-100) yielding 195 g of dried extract. A successive partition of hexane extract yielding petroleum ether 80-120 (38.80 g), petroleum ether 40-60 (4.67 g), chloroform (9.78 g), ethyl acetate (1.18 g) and methanol (57.40 g) fractions. These fractions were subjected to tests for antimicrobial activity.

*2.4 Source of microorganisms***:**
Staphylococcus aureus (NCL 2079), *Bacillus subtilis* (NCL 2063), *Proteus vulgaris* (NCL 2027), *Pseudomonas aeruginosa* (ATCC27853), *Escherichia coli* (NCL 2065), *Candida albicans* (NCIM3557), *Cryptococcus neoformans* (NCIM 3542), *Aspergillus niger* (NCIM 1269) were procured directly from National Chemical Laboratory (NCL) Pune and maintained by periodical subculturing on agar slant.

*2.5 Preparation of standard bacterial and fungal suspensions***:**
Before using, all bacteria cultures were subcultured on Mueller-Hinton agar and incubated for 24 h at 35^0C. Bacterial suspensions were prepared by transferring a few colonies of bacteria to Mueller-Hinton broth. The density of bacteria was adjusted spectrophotometrically using McFarland standard 0.5 (optical density: 0.08-0.10) to give a homogenous 1 x 108 CFU/mL. Necessary dilution was carried out to get an eventual concentration of 5 x 105 CFU/mL and dilution factor required was determined.
All fungal organisms were subcultured on potato dextrose agar (PDA) and incubated at 37^0C but at different incubation time. *C. albicans* was incubated for 24 h, while *C. neoformans* and *A. niger* were incubated for 48 h. A few colonies of the fungus were transferred to Sabouraud Dextrose Broth. For *C. albicans* suspensions, optical density was adjusted to get range within 0.12 to 0.15 at 530 nm. The optical densities values of 0.09 to 0.11 at 530 nm are adjusted for of *A. niger* and *T. mentagrophtes*. The concentration of the fungus within the range was 1 x 106 - 5 x 106 CFU/mL. Further dilution was done to obtain the necessary concentration of fungus. The final concentration of the *C. albicans* was 0.5- 2.5 x 103CFU/mL, that of *C. neoformans* was 0.5- 2.5 x 104 CFU/mL, and those of *A. niger* was 0.5-2.5 x 105 CFU/mL.

*2.6 In vitro antimicrobial activity test for MeOH extract***:**
The cup plate agar diffusion method was adopted to access the antimicrobial activity of MeOH extract. 0.5 mL of bacterial stock suspensions was carefully mixed with 60 mL of sterile nutrient agar. 20 mL of the agar were added into each sterile Petri dishes. After setting up of agar media at room temperature, 4 cups of 6 mm in diameter were punched using a sterile cork borer allowing equidistance space between adjacent wells and the agar discs were removed. Fixed volumes of the MeOH extract (1000, 500-250 µgmL-1) were then introduced into each wells using microtitre pipette and allowed to diffuse at room temperature for 2 h. In separate wells, 30 µg of Ciprofloxacin was added as positive controls whereas 10% DMSO was taken as negative control. The plates were then incubated at 37°C for 24 h. Then diameter of growth inhibition zones were measured; averaged and the mean values were recorded. Antifungal activity was carried out by using Sabouraud agar media using Fluconazole (1 mg/mL) as positive control.

2.7 Determination of Minimum Inhibitory Concentration (MIC)
Micro broth dilution method was used to determine the minimum inhibitory concentration (MIC) of extracts using 96-well plates against test pathogens.[10,11] Serial dilutions of the extracts were carried out in Mueller-Hinton broth medium to make 500 µg mL^{-1} final concentration, this was then two-fold serially diluted by adding to the broth media in a 96-wells microtitre plates to obtain 2500, 1250, 625, 313, 156, 78.1, 39.0 and 20.0 µg mL^{-1}. Then 100µl inoculum was added to each wells. To confirm the sterility of the broth medium, Mueller-Hinton broth medium with a volume of 100 µL was tested separately. Bacterial suspension was used as negative control while broth containing ciprofloxin (128 µl/ml) was used as standard control. After covering the plate with a lid, the plates are incubated at 37ºC for 24 h. 20 µL of 0.4 mg/ml p-iodonitrotetrazolium violet (INT) was further added to all wells after incubation. The MIC was noted as the lowest concentration of solvent in which there is no microbial growth; indicated by the absence of color change.

2.8 Total Activities of Extracts
Total antimicrobial activities of the extracts were calculated as the total mass (mg/g) of each of the extract divided by its MIC value (mg/mL). This value indicates the volume to which the extract obtained from 1 g of sample material can be diluted still maintaining its antimicrobial property.[12]

2.9 Identification and isolation of antibacterial compound from MeOH extract:
The bioassay-guided fractionation was carried out only on the methanol extract as it displayed the highest antimicrobial activity. Seven grams of extract was subjected to silica gel flash chromatography fitted with a 750 g RediSep® silica cartridge, in which the normal phase silica cartridge was packed with silica gel 60 (35 to 70 µm). Initial the column was eluted with hexane (100%) mobile phase, after which binary mixture with ethyl acetate which increased at every 5 % until it contained 40 % of ethyl acetate. Finally, the column was eluted with 100 % ethyl acetate. A total of one hundred twenty fractions with volumes of approximately 50 mL were congregated with a flow rate of 4.5 mL/min. The fractions were concentrated using a flash evaporator and then subjected to TLC. Fractions showing same Rf values were combined to produce twenty-four samples ("S1" to "S24"). All the samples were subjected to antimicrobial assay to track of the antimicrobial compounds which inhibited the growth of *S. ilicifolium*.
Based on the MIC values and results from Rf values of the fractions, "S10" to "S16", were combined and further

purification was carried out using silica gel flash chromatography fitted with 80g RediSep®. The initial mobile phase was a mixture of hexane-ethyl acetate system with increasing polarity (90:10 to 6:4 volume) and finally with 100% EtOAc. A total of fifty fractions each with a volume of 50 mL were congregated with a flow rate of 4.5 mL/min. The fractions were combined into seven samples ("T1" to "T7") based on the Rf values in TLC analysis. The samples were then subjected to antimicrobial assay.

The most active fractions, "T5" (208.0 mg) and "T6" (180.0 mg) were subjected to C_{18} reversed phase flash chromatography fitted with 26 g C_{18} cartridge (42-63 µm) particles. The initial C_{18} column was run with 100 % acidified water, followed by acidified water: methanol (50:50), 100 % methanol and finally with 100 % dichloromethane. All the fractions were subjected to antimicrobial assay. Ten fractions with volumes of 50 mL were collected at a flow rate of 4.5 mL/min from both purifications respectively. Total four fractions "U1" to "U4" were collected from combined samples of "T5" and "T6" and were subjected to antimicrobial assay. U3 which is a colorless liquid (12 mL), showed highest antimicrobial activity was further subjected for analytical and spectrophotometric analysis. Structure of antibacterial compound was elucidated through the integration of ^{1}H- and ^{13}C NMR spectra and comparison of their physical, chemical, and spectral data.

2.10 Characterization of isolated compound

The isolated compound was identified by spectroscopic analysis such as UV-Vis, FTIR, ESI-MS, H^1 NMR, COSY and HSQC. The result was compared with spectroscopic data previously published.

3. RESULTS

3.1 Antibacterial activity

The results of the antimicrobial activity of extracts of *S. ilicifolium*, which were obtained from successive extraction, are shown in Table 1. The petroleum ether 80-120 and petroleum ether 40-60, extracts produced the most dependable inhibition of bacterial growth with a mean MIC value of 0.625 mg/mL. All these extracts exhibited inhibition of growth on *E. coli* (range of mean MIC value= 1.52 to 2.50 mg/mL.) Growth of *S. aureus* was inhibited by ethyl acetate extract (mean MIC= 4.32 mg/mL) and methanol extract (mean MIC= 2.5 mg/mL). Ciprofloxacin was used as standard control for the antimicrobial tests. The total activity indicated that methanol extract had the most active antimicrobial activity towards *B. subtilis* and *P. vulgaris* as compared to other extracts. The total activity of methanol extract towards *B. subtilis* and *P. vulgaris* were 55.19 and 45.92 mL/g respectively. This suggests that the methanol extract prepared from 1 g of dried *S. ilicifolium* could be diluted to a volume of 45.92 mL and still retains activity against *B. subtilis*. Other than methanol, petroleum ether 80-120 extracts also exhibited total activity with a value of 44.8 mL/g towards *P. vulgaris*, *P. aeruginosa*, and *B. subtilis*. It gave a steady MIC value for three tests.

3.2 Antifungal activity

Extracts of *S. ilicifolium* were subjected to antifungal testing. Table 2 recapitulates the MIC values of the extract against four different fungal strains. All extracts exhibited inhibitory activity towards *C. neoformans* and *A.niger*. Mean MIC values ranged from 0.26 to 4.17 mg/mL. Ethyl acetate extract exhibited the strongest inhibitory activity towards *C. neoformans* with a mean MIC value of 0.26 mg/mL. However, the amount of EtOAc extract was the least as compared to others and it was insufficient for further fractionation. Only the petroleum ether 80-120 extract showed inhibition towards *T. mentagrophytes* with a mean MIC value of 3.33 mg/mL. No growth inhibition was observed on all crude extracts towards *C. parapsilosis*. The growth of *C. albicans* was inhibited only by petroleum ether 80-120, petroleum ether 40-60 and chloroform extracts with mean MIC values ranging from 2.50 to 5.00 mg/mL.

The total activity indicated that methanol extract showed the most active antimicrobial activity towards *A. niger* and *C. neoformans* as compared to other extracts. The extract prepared using 1g of freeze-dried *S. ilicifolium* may perhaps be diluted to a volume of 53.76 mL and 81.00 mL and still retain inhibitory activity towards *A. niger* and *C. neoformans* respectively.

Table 1: The mean MIC values (mg/mL) and total activity (mL/g) of extracts of *S. ilicifolium* towards five bacterial strains

Bacteria strains	Extracts of *S. ilicifolium*						
	PE 80-120	PE 40-60	$CHCl_3$	EtOAc	Methanol	Alcohol	Mean MIC of positive control (n=3), µg/mL
Mean MIC values (n=3), mg/mL							
S. aureus	-	-	-	4.32±1.33	2.5±0	-	8.00±0
P. aeruginosa	0.625±0	0.625±0	0.52±0.18	0.52±0.18	1.04±0.36	0.625±0	4.00±0
B. subtilis	0.625±0	0.625±0	0.625±0	0.42±0.18	1.25±1.08	0.83±0.36	4.00±0
P. vulgaris	0.625±0	0.625±0	0.625±0	0.625±0	1.67±0.72	1.46±0.95	8.00±0
E. coli	1.52±0.712	1.52±0.71	1.52±0.71	1.67±0.72	2.50±0	2.50±0	8.00±0
Total activity, mL/g							
S. aureus	-	-	-	0.27	22.96		NA
P. aeruginosa	44.8	7.47	18.80	2.26	55.19	82.08	NA
B. subtilis	44.8	7.47	15.64	2.80	45.92	61.80	NA
P. vulgaris	44.8	7.47	15.64	1.88	34.37	35.13	NA
E. coli	18.43	3.07	6.43	0.70	22.96	20.52	NA

"-" denotes no activity; and "NA" denotes not available

Table 2: MIC values (mg/mL) and total activity values (mL/g) of extracts of *S. ilicifolium* towards five fungal strains

Crude Extracts	*Candida albicans*	*Candida parapsilosis*	*Cryptococcus neoformans*	*Trichophyton mentagrophytes*	*Aspergillus niger*
Mean MIC values (n=3), mg/mL					
Fluconazole	0.002± 0	0.002 ± 0	0.001 ± 0	0.001 ± 0	0.004 ± 0
Pet ether 80-120	2.50 ± 0	-	0.63 ± 0	3.33 ± 1.44	4.17 ± 1.44
Pet ether 40-60	5.00 ± 0	-	1.04 ± 0.36	-	1.04 ± 0.36
Chloroform	4.17 ± 1.44	-	1.25 ± 0	-	0.52 ± 0.18
Ethyl acetate	-	-	0.26 ± 0.09	-	1.04 ± 0.36
Methanol	-	-	0.63 ± 0	-	1.25 ± 0
Total activity, mL/g					
Pet ether 80-120	12	-	47.62	9.01	7.19
Pet ether 40-60	0.96	-	4.60	-	4.60
Chloroform	2.46	-	8.21	-	19.73
Ethyl acetate	-	-	8.35	-	2.09
Methanol	-	-	7.70	-	3.88

"-" denotes no inhibition and "MIC" denotes minimum inhibitory concentration

3.3 Identification of compound isolated from Sargassum ilicifolium

The compound isolated form fraction U3 displayed a molecular ion peak [M+] at m/z 390, other major peaks at *m/z* 167 and *m/z* 149 (100) indicating the presence of phthalate moiety in the compound. The IR absorptions spectrum revealed an ester carbonyl band at 1719 cm^{-1} and strong C-O bands in the range 1039~1264 $cm^{-1}$and a characteristic band at 954 cm^{-1} refer to vinyl esters bond. ^{1}H NMR spectrum in *Acetone*-d6, CD_3COCD_3, displayed two multiplets at δ 7.76 and δ 7.68 ppm for H2 and H3 protons of the aryl ring. Multiplet signal at δ 4.38 ppm corresponds to H4 protons of the hexyl substituent of the ester. A multiplet at δ 1.42 ppm was due to H5 protons. The signals corresponding to H6, H7, H8 and H10 found in combination and appeared as a multiplet, at δ1.36 ppm. Chemical shifts observed between 0.9-2.0 are attributed to methine, methyl, and methylene protons of aliphatic chain of phthalate.^{13}C NMR (126 MHz, Acetone) spectrum of th e compound exhibited signals between δ = 14.7 and 29.85 ppm, which are attributed to aliphatic carbons. The chemical shift observed at δ = 69 ppm is evidence of a carbon involved in a C-O bond, and signals δ= 129-133 ppm are associated to aromatic ring carbons. Finally, signal at δ 167.88 is assigned for ester carbonyl carbon. From the mass spectrum, ^{1}H and ^{13}C NMR, the molecular formula was presumed to be $C_{24}H_{38}O_4$. The spectral data of this compound was matched with the type of compound in marine organisms has been previously reported[13] and established that this compound as Di (2-ethylhexyl) phthalate (DEHP) which has antimutagenic compound isolated from Octopus.[14]

Di (2-ethylhexyl) phthalate and other phthalates have been isolated from environmental pollutants and identified in sediments, soils, marine and terrestrial waters, and also in living organisms. DEHP has been isolated from terrestrial and other marine organisms including plants and marine algae.[15]

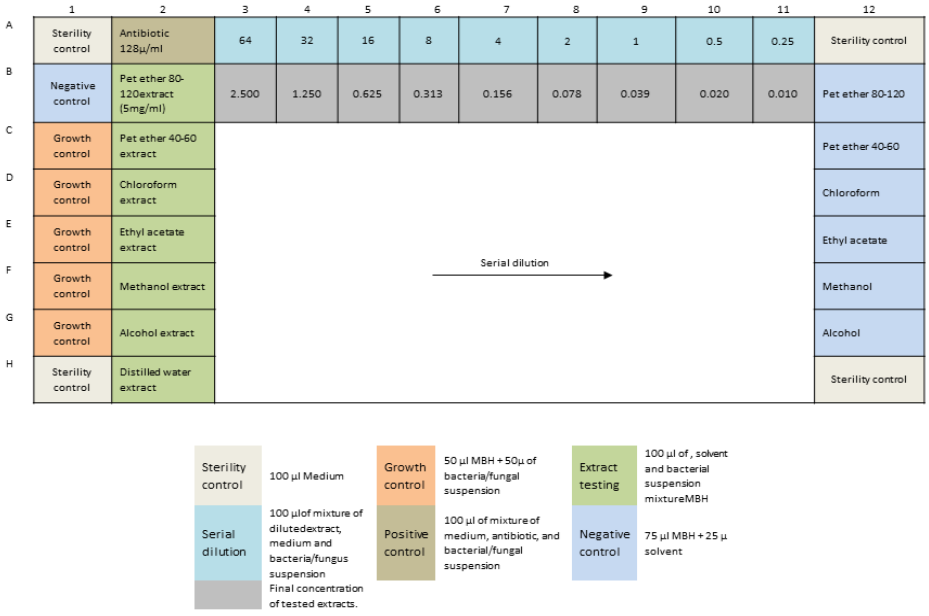

Figure 1: Design of the 96-well plate for colorimetric broth microdilution antimicrobial assay of crude extracts

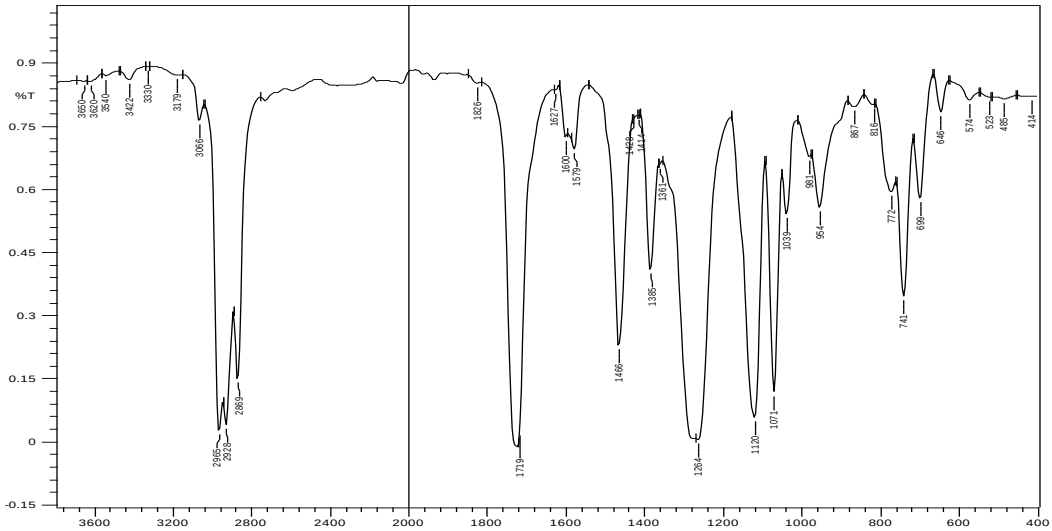

Figure 2: FTIR spectrum of the bioactive lead obtained from S. *ilicifolium.*

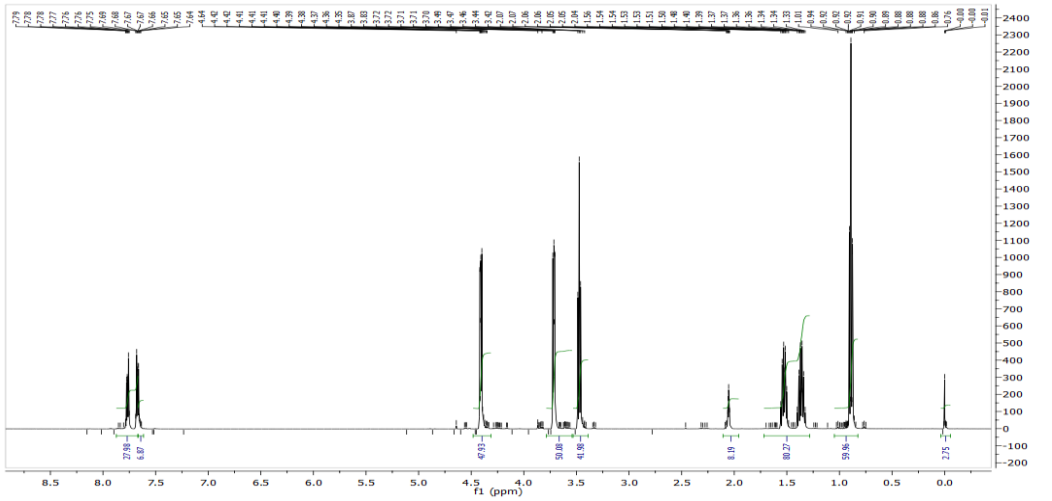

Figure 3: Proton ^{1}H NMR of the pure compound isolated from *S. ilicifolium*

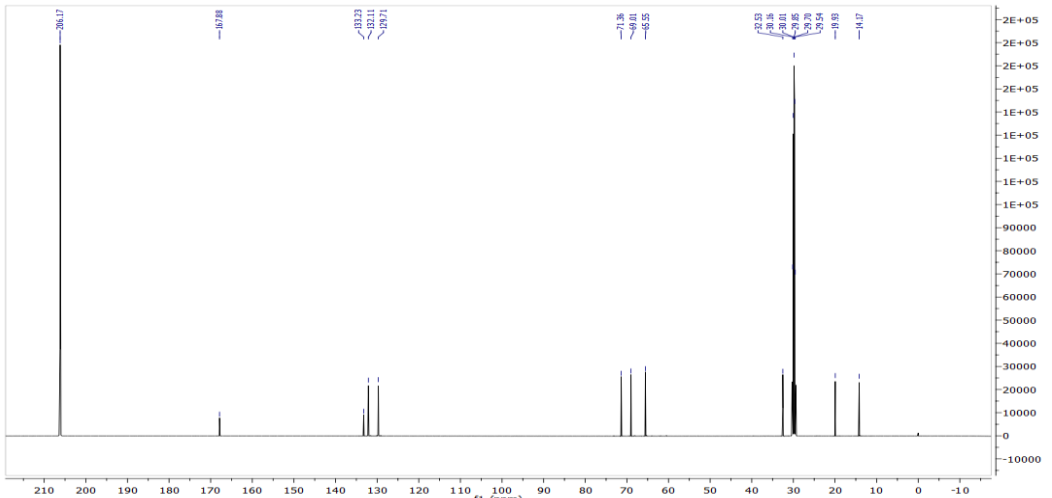

Figure 4: ^{13}C NMR of the bioactive compound isolated from *S. ilicifolium*

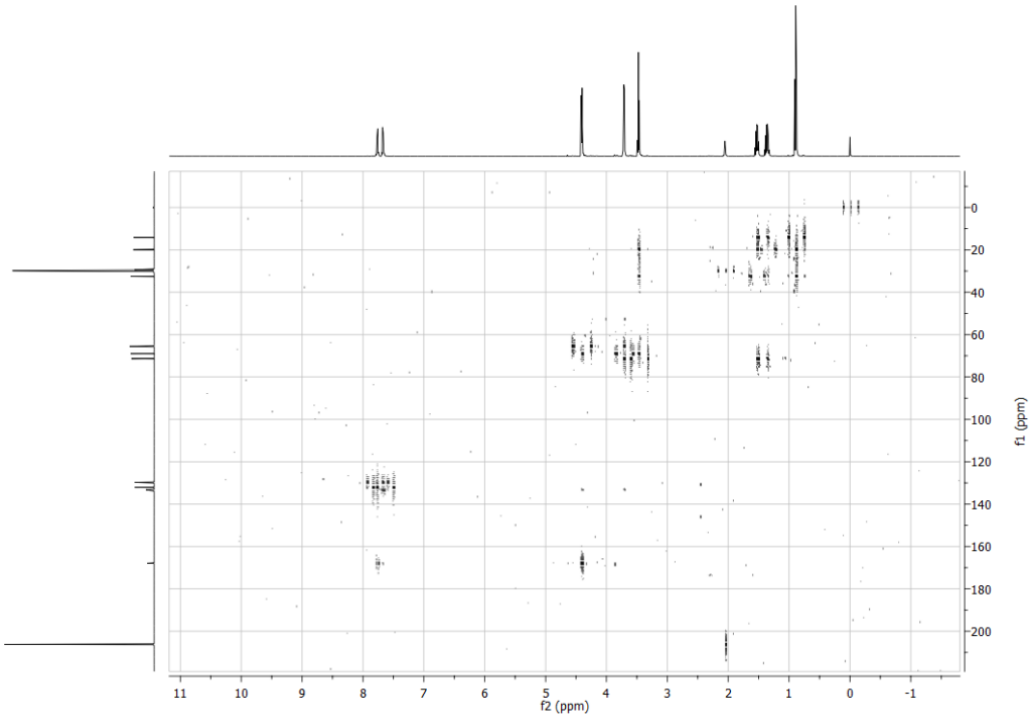

Figure 5: HMBC of the purified compound isolated from *S. ilicifolium.*

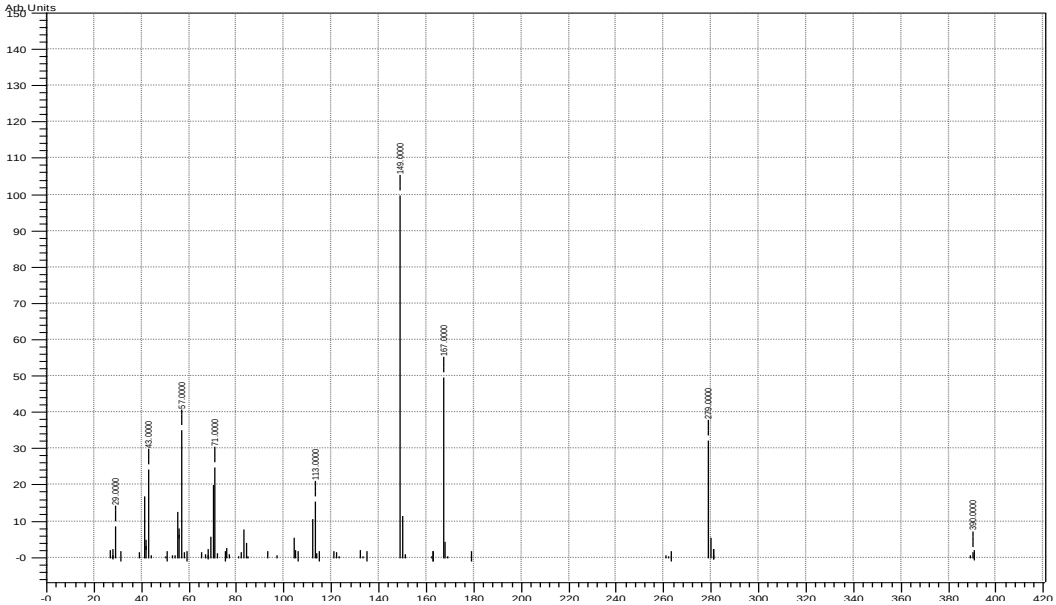

Figure 6: MS of the pure compound isolated from *S. ilicifolium.* with a molecular ion at m/z 391 (M+).

4. DISCUSSION:

During last four decades marine organisms have been in the focus of interesting discoveries, which have led to the discovery of important drugs for pharmaceutical industry. Seaweeds have emerged as a vast source of unique structures and bioactive metabolites which make them suitable to be used in medical applications. Our bioassay-guided fractionation of *Sargassum ilicifolium* has led to the isolation of Di-(2-ethylhexyl) phthalate which exhibited moderate antibacterial activity. The antimicrobial activity of all fractions from the hexane partition of the extract was evaluated and showed that polar partitions are more potent than non-polar ones. Further studies are necessary to isolate the active compounds in hexane partition. It can be concluded that the *Sargassum ilicifolium* could be a tremendous resource for production of bioactive metabolites.

Source of Funding: The authors gratefully acknowledge the financial support of Rajiv Gandhi University of Health sciences Bangalore Karnataka for funding this paper wide University notification number RGUHS: Adv.Research:2015-16 dated 17/03/2016 with project code P045.

Conflict of interest: None

Acknowledgements: The authors are thankful to Dr. B. B. Chaugule, Professor and Head of Department, Department of Botany, Poona University Pune for identifying the specimen samples of algae.

REFERENCES

(1) Devasahayam G, Scheld WM, Hoffman PS. Newer antibacterial drugs for a new century. *Expert Opin Investig Drugs* 2010;19(2):215-234.

(2) Kim JY, Inaoka T, Hirooka K, Matsuoka H, Murata M, Ohki R, et al. Identification and Characterization of a Novel Multidrug Resistance Operon, mdtRP (yusOP), of *Bacillus subtilis*. *J Bacteriol* 2009;191(10):3273-3281.

(3) Mayer AMS, Hamann MT. Marine Pharmacology in 2000: Marine Compounds with Antibacterial, Anticoagulant, Antifungal, Anti-inflammatory, Antimalarial, Antiplatelet, Antituberculosis, and Antiviral Activities; Affecting the Cardiovascular, Immune, and Nervous Systems and Other Miscellaneous Mechanisms of Action. *Mar. Biotechnol* 2004;6(1):37-52.

(4) Strahl ED, Dobson WE, Lundie J. Isolation and Screening of Brittlestar-Associated Bacteria for Antibacterial Activity. *Curr Microbiol* 2002;44(6):450-459.

(5) Renn D. Biotechnology and the red seaweed polysaccharide industry: status, needs and prospects. *Trends Biotechnol* 1997;15(1):9-14.

(6) Frestedt JL, Kuskowski MA, Zenk JL. A natural seaweed derived mineral supplement (Aquamin F) for knee osteoarthritis: A randomised, placebo controlled pilot study. *Nutr* 2009;8(1):1-8.

(7) Leal MC, Sheridan C, Osinga R, Dionio G, Rocha RJ, Silva B, et al. Marine Microorganism-Invertebrate Assemblages: Perspectives to Solve the Supply Problem in the Initial Steps of Drug Discovery. *Mar Drugs* 2014;12(7): 3929-3952.

(8) Simpi C, Nagathan C, Karajgi S, Kalyane N. Evaluation of marine brown algae *Sargassum ilicifolium* extract for analgesic and anti-inflammatory activity. *Pharmacogn Res* 2013;3(3):146-149.

(9) Siddiqui S, Verma A, Rather AA, Jabeen F, Meghvansi MK. Preliminary phytochemicals analysis of some important medicinal and aromatic plants. *Adv Biol Res* 2009;3(5-6):188-195.

(10) Eloff JN. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Med* 1998;64(8):711-713.

(11) Swenson JM, Killgore GE, Tenover FC. Antimicrobial Susceptibility Testing of *Acinetobacter spp.* by NCCLS Broth Microdilution and Disk Diffusion Methods. *J Clin Microbiol* 2004;42(11):5102-5108.

(12) Eloff JN. A proposal on expressing the antibacterial activity of plant extracts- a small first step in applying scientific knowledge to rural primary health care in South Africa. *S. Afr. J. Sci* 2000;96(3):116-118.

(13) Moushumi Priya A, Jayachandran S. Induction of apoptosis and cell cycle arrest by Bis (2-ethylhexyl) phthalate produced by marine *Bacillus pumilus* MB 40. *Chem. Biol. Interact* 2012;195(2):133-143.

(14) Cruz-Ramrez SG, Lopez-Saiz CM, Rosas-Burgos EC, Cinco-Moroyoqui FJ, Velzque C, Hernndez J, et al. Antimutagenic, antiproliferative, and antioxidant effect of extracts obtained from octopus (Paraoctopus limaculatus). *Food Sci. Technol* 2015;35:722-728.

(15) Namikoshi M, Fujiwara T, Nishikawa T, Ukai K. Natural Abundance (14)C Content of Dibutyl Phthalate (DBP) from Three Marine Algae. *Mar Drugs* 2006;4(4):290-297.