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ORIGINAL ARTICLE

Novel phenolic compound from Southern Ocean microalgae *Chlorella* sp. PR-1 and its antibacterial activity

Nuevo compuesto fenólico antibacteriano aislado de la microalga *Chlorella* sp. PR-1 del océano sur

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ABSTRACT

Marine microalgae has been attracting the researcher's attention for centuries. Development on microalgal research is majorly favoured by its medicinal, pharmaceutical or cosmeceutical properties. The advancement in the investigation related to microalgal products have been concentrated in the coastal zones because of the greater supply of raw material. The Southern Ocean, highly productive and relatively poorly studied ecosystem, constitutes approximately 10% of the global volume of the oceans. In this study, marine microalgae *Chlorella* sp. PR-1 was isolated from the Southern Ocean (Indian Sector) for the identification of bioactive antibacterial compounds. The antimicrobial activity of this microalgal extracts was evaluated against three gram positive (*Staphylococcus aureus*, *Bacillus licheniformis*, and *Bacillus subtilis*) and three gram negative bacteria (*Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Escherichia coli*). The extract showing antibacterial activity was further purified by thin layer and column chromatography. The antimicrobial activity was again evaluated and confirmed with the purified fraction against the same bacteria. The identification of the functional groups in the purified fraction was performed by infrared spectroscopic analysis. Based on gas chromatographic and mass spectroscopic analysis, the principle bioactive compound was proved to be 2,4-bis (1,1-dimethylethyl)- phenol. Thus, the bioactive compound isolated from marine microalga of Southern Ocean origin may be a novel alternative source of antibacterials in the future.

Keywords: antibacterial activity, microalgae, phenolic compound, Southern Ocean.

RESUMEN

Las microalgas marinas han atraído la atención de los investigadores durante siglos. El desarrollo de la investigación de las microalgas se ve favorecido principalmente debido a sus propiedades medicinales, farmacéuticas y cosméticas. El avance de las investigaciones relacionadas con productos microalgales se ha concentrado en las zonas costeras debido a la mayor oferta de materias primas. El Océano Sur es un ecosistema extremo altamente productivo y relativamente poco estudiado, constituye aproximadamente el 10% del volumen global de los océanos. En este estudio, la microalga marina *Chlorella* sp. PR-1 fue aislada desde el Océano Sur (sector Indico) para la identificación de compuestos bioactivos antibacterianos. La actividad antimicrobiana de los extractos microalgales fue evaluada contra tres especies bacterianas gram

positivas (*Staphylococcus aureus*, *Bacillus licheniformis* y *Bacillus subtilis*) y tres gram negativas (*Pseudomonas aeruginosa*, *Salmonella typhimurium* y *Escherichia coli*). Los extractos que mostraron actividad antibacteriana fueron purificados mediante cromatografía de capa fina. La actividad antibacteriana de las fracciones purificadas fue evaluada nuevamente y confirmada contra las mismas bacterias. En las fracciones purificadas, la identificación de los grupos funcionales fue realizada mediante análisis espectroscópico infrarrojo. Basado en los análisis cromatográficos y de espectroscopia de masa se demostró que el principal compuesto bioactivo fue 2,4-bis (1,1-dimetiletil)-fenol. Por lo tanto, el compuesto bioactivo aislado de microalgas marinas del Océano Austral puede ser una nueva fuente alternativa de antibacterianos en el futuro.

Palabras clave: actividad antibacteriana, compuestos fenólicos, microalga, Océano Sur.

INTRODUCTION

Now a days a major threat to public health is emergence of drug resistance. In fact, chronic break out of multidrug resistance is of more concern than any other health issues (Tanwar *et al.* 2014, Ventola 2015, Ferri *et al.* 2017, Tayler *et al.* 2019). Since the last 90 years of antibiotic discovery, major throwback is observed in the present time where literally no drug can be applied to certain diseases causing decrease in life expectancy. Thus, finding alternative to antibiotics is a major challenge nowadays (Allen *et al.* 2014; Czaplewski *et al.* 2016). Whereas terrestrial plants or microbes have been studied a lot for discovery of bioactive compounds, relatively less are explored from extreme environments like ocean. Ocean offers infinite biodiversity potential most of which are still uncharacterized and may eventually develop into novel therapeutics (Newman & Cragg 2012, Gupta *et al.* 2015, Banerjee *et al.* 2019). Southern Ocean (Indian sector) harbors affluent microbial diversity that are promising source of unique bioactive compounds (Banerjee *et al.* 2019, Sengupta *et al.* 2019). Hence, they are of great scientific interest and make Southern Ocean as incipient biomedical resource (Chin *et al.* 2006, Harvey 2008, Banerjee *et al.* 2019).

Marine microalgae survive at extreme environments including high pressure, salinity, or temperature (Paerl *et al.* 2000). To adapt to these extreme conditions, microalgae produce variety of secondary metabolites with effective biological potentials like anticancerous, antibacterial, antiprotozoal, anticoagulant, antituberculosis, antimalarial, anti-inflammatory or antifungal activity (Montaser & Luesch 2011, Gerwick & Moore 2012, Gupta *et al.* 2014). Apart from these, marine microalgae are also referred as potential source of biofuels or in cosmeceuticals (Quinn *et al.* 2008, Bhatnagar & Kim 2010, Mayer *et al.* 2010, Penesyan *et al.* 2010, Gomma *et al.* 2015, Mourelle *et al.* 2017, Maeda *et al.* 2018). Especially in recent times, different species of *Chlorella* has become an important source for biodiesel production (Cheirsilp *et al.* 2012, Kirrolia *et al.* 2014, Zhang *et al.* 2014, Mathimani *et al.* 2017, Mathimani *et al.* 2018, Chi *et al.* 2019), wastewater treatment and heavy metal removal (Kumar & Goyal 2010, Das *et al.* 2018, Amin & Chetpattananondh 2019). Our present study involves disc diffusion method for identification of the antibacterial compounds from Southern Ocean (Indian sector) origin marine microalgae *Chlorella* sp. PR-1 against some pathogenic and non-pathogenic bacteria. The extraction of the bioactive compounds has been done according to the standard methods followed by its identification using typical thin layer chromatography (TLC), infrared spectroscopy (FTIR) and gas chromatographic technique coupled with mass spectrometry (GC-MS).

MATERIALS AND METHODS

Isolation of *Chlorella* sp. Pr1 and extract preparation

Marine microalgae *Chlorella* sp. PR-1 (MN850879) was reported to be isolated from water sample (44°S; 48°E) collected from Southern Ocean (Indian sector) expedition 2011 (Gupta *et al.* 2014). Map of the study site prepared using Ocean Data View software is represented in Fig. 1. The hydrographic characteristics of the sampling station is noted in Table 1.

Sterilized natural seawater enriched with F/2 media was used to culture *Chlorella* sp. PR-1 (Guillard 1975). It was incubated at 56 µmol m^{-2} s^{-1} luminous irradiance, light/dark conditions of 16:8 h and gentle agitation at 20 °C. The batch culture was harvested at late log phase after 8 days of incubation by centrifugation at 3000 rpm for 5 min and further oven drying at 45 °C for 48 h. All the solvent used for extraction, isolation and purification were of HPLC grade.

Dried algal biomass of 50 mg was soaked in 50 ml methanol (99.9%) and was kept for two days continuous shaking at room temperature, which was then filtered in whatman filter paper. The methanol extract was further subjected for liquid-liquid separation according to modified Kupchan & Tsou (1973) protocol. After the separation, five different extracts *viz*. n-hexane, carbon tetrachloride (CCl_4), chloroform, ethyl acetate, and methanol fractions were collected and used for the antibacterial assay.

Antibacterial studies

Antibacterial activity of all the *Chlorella* sp. PR-1 filtrates was performed by Kirby Bauer disc diffusion method (Bauer *et al.* 1966) against gram-negative *Pseudomonas aeruginosa* (NCIM 2036), *Salmonella typhimurium* (NCIM 2501) and *Escherichia coli* (NCIM 2832) along with gram-positive *Staphylococcus aureus* (NCIM 2122), *Bacillus licheniformis* (NCIM 2042) and *Bacillus subtilis* (NCIM 2193). Sterile cotton swabs were used to spread freshly grown test bacteria on Mueller Hinton (MH) agar (Himedia®) plates and were left for some times to allow complete absorption of the inoculum. The disc loaded with 15 µl of each extracts was placed on each plates and then kept for incubation at 37 °C for 24 h. Clear zone was observed around the disc, which denoted the presence of antibacterial compounds in the extracts. Zone of inhibitions were measured in millimetres (mm).

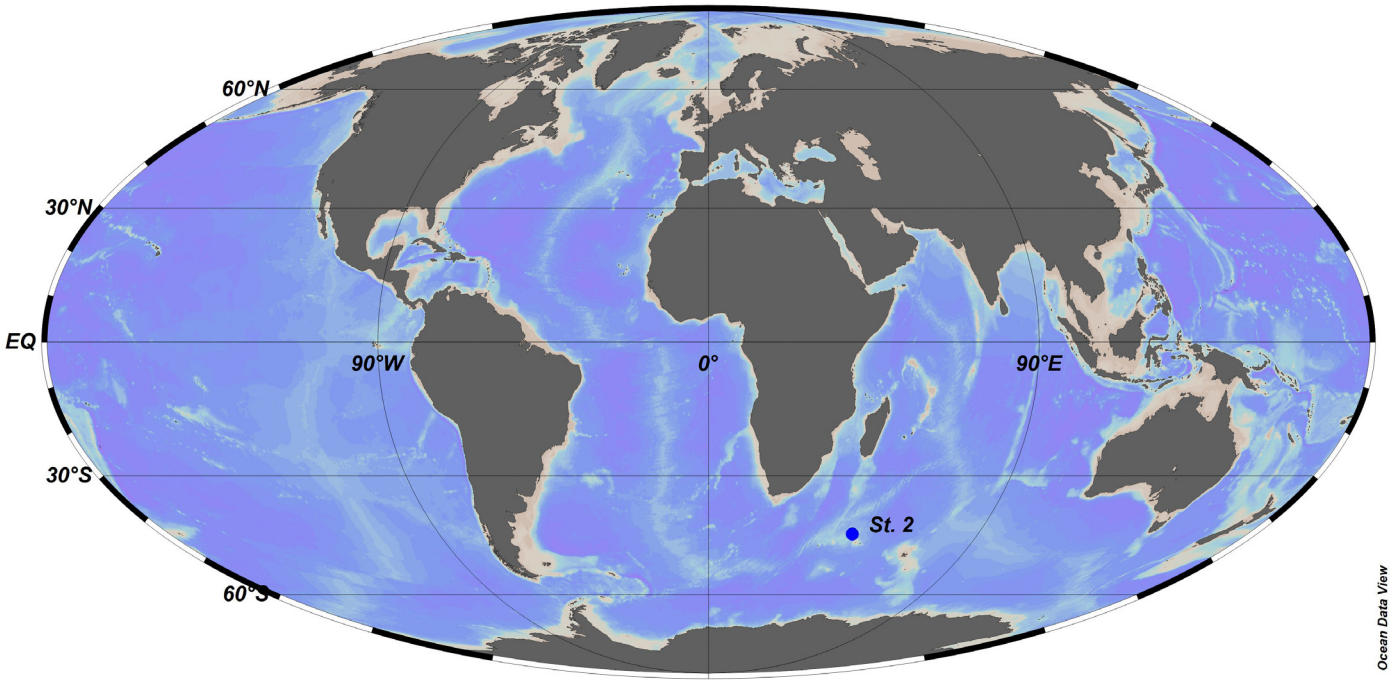

Figure 1. Map of the study zone representing the point of water sample collection at station 2 of the Southern Ocean (Sector India). The map was generated using Ocean Data View software (Schlitzer 2010). / Mapa de la zona de estudio que indica el punto de la recolección de muestras de agua en la estación 2 del Océano Sur (Sector Indico). Mapa generado mediante software Ocean Data View (Schlitzer 2010).

Table 1. Hydrographic Characteristics of the sampling station in the Southern Ocean (Indian sector). / Características hidrográficas de la estación de muestreo en el Océano Sur (Sector Indico).

Station name	Temperature (°C)	Latitude (°S)	Longitude (°E)	Depth of sampling (m)	Depth (m.b.s.l*)	Sea surface temperature/ SST (°C)	Wind speed (Knotts)	Wind direction
Station 2	12	44	48	90	5225	20.5	2.5	112°

*meters below sea level

Extraction and identification of bioactive compound

CCl_4 fraction of *Chlorella* sp. PR-1 showing the best antibacterial activity was further assayed for detail evaluation on the presence of antibacterial compound on TLC. The active fraction was loaded on silica plate (Silica gel 60; Merck, India) with ethyl acetate/ hexane (3:2) solution. Thick single band with antibacterial action was identified under UV-transillumination at 366 nm. The elution was subjected for GC-MS (Agilent Technologies, USA) having HP 5MS + 10m Duraguard capillary column (30m×0.25mm×0.25µm) attached to MSD Triple axis detector, operating in electron impact mode at 70 eV. Helium was used as carrier gas at constant flow rate of 1 ml/min, ion source temperature 230 °C, quadrupole temperature 150 °C, auxiliary temperature 280 °C and sample injection volume 1 µl. As the GC-MS data revealed total 23 compounds, CCl_4 extract was further subjected for column chromatography to fractionate all 23 compounds. The column was packed with silica (Silica gel 60-120; Merck, India). EtOAc : Hexane solvent was used in a gradient manner with increasing polarity. All the column chromatography fractions were collected and antibacterial assay was again performed against *S. typhimurium* that showed the best bioactivity. Fraction showing positive activity was further subjected to FTIR analysis (IR-Prestige 21, Shimadzu Corporation, Japan) between 400-4000 cm^{-1} by the KBr pellet technique, followed by high pressure liquid chromatography (HPLC) with Symmetry C18 5µm 4.6×150mm column (Waters, Ireland). The mobile phase consisted of water with 0.1% acetic acid (solvent A) and acetonitrile (solvent B). The different gradients of solvent B were 0-4 min gradient with 4% solvent B for 4-6 min, 17% for 6-10 min and 30% solvent B and 10-15 min 4% solvent B. The flow rate was 1 ml/min, injection volume 20 µl and the monitoring wavelength of 280 nm. After that, the fraction was subjected to GC-MS (GCMS-QP2010 Plus, USA) with Rtx-5Ms (30m×0.25mm ID×0.25µm df) column attached through MSD Triple axis detector. The operating characteristics was as earlier described with 1 µl injection volume. Identification of the compound was based on the comparison of their retention time with the compound database of WILEY8.LIB library.

RESULTS

Antibacterial studies

All the fractions of the marine isolate *Chlorella* sp. PR-1 were tested against both the sets of gram positive and gram negative bacteria *S. typhimurium*, *S. aureus*, *E. coli*, *P. aeruginosa*, *B. subtilis* and *B. licheniformis*. Among these, CCl_4 fraction of *Chlorella* sp. PR-1 demonstrated positive biocidal activity against *S. typhimurium* with 10±0.5 mm zone of inhibition; whereas rest of the hexane, ethyl acetate and methanol fractions did not exhibited any significant activities against all the bacteria (Fig. 2).

Structural identification of the bioactive compound

CCl_4 fraction of *Chlorella* sp. PR-1 showing antibacterial activity against *S. typhimurium* was subjected to TLC and the specific band was observed at R_f value of 0.42. GC-MS analysis revealed 23 different natural compounds present in the extract. These compounds were mainly comprised of hexachloroethane (18.42%), followed by isophthalic acid and heptyl 2-methyl prop-2-en-1-yl ester (16.25%). Rest

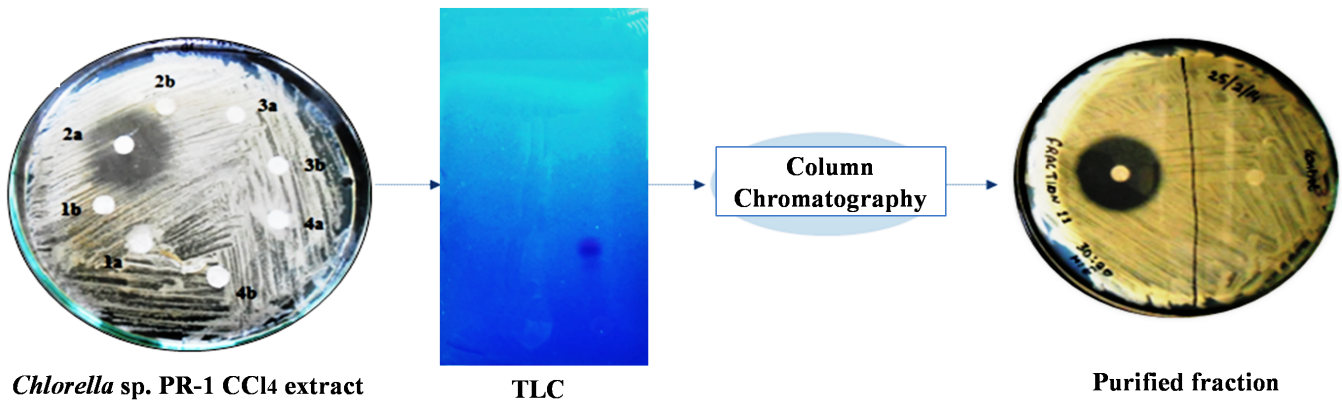

Figure 2. Antibacterial activity of *Chlorella* sp. PR-1 against *Salmonella typhimurium* confirmed using TLC and fraction purified through column chromatography. Purified fraction confirms the inhibition to *S. typhimurium*. / Actividad antibacteriana de *Chlorella* sp. PR-1 contra *Salmonella typhimurium* confirmada usando TLC y fracción purificada mediante columna cromatográfica. Fracción purificada confirma la inhibición de *S. typhimurium*.

of the compounds were less than 10% which constituted Phenol 2,2-methylenebis [6-(1,1-dimethylethyl)-4-methyl-] (9.25%), [2,4-bis (1,1-dimethylethyl)- phenol] (8.65%), etc. Through column chromatography, it was observed that 11th fraction eluted with ethyl acetate: hexane (30:20) exhibited antibacterial activity against *S. typhimurium*. HPLC analysis of carbon tetrachloride fraction shows single peak with 2.510 retention time. Area and height of the peak were 188768 and 34941 respectively (Fig. 3). For identification of the functional groups, FTIR spectroscopy was performed at different wavelength. The bands 2924.09 and 2826.36 were assigned to C-H stretching vibration, while 1743.65 was of cyclopentanone. The IR absorptions at 1550.77 and 1377.17 were also indicative of benzene ring and tertiary alcohol respectively. GC chromatogram showed 3 peaks; out of which one peak (Rt=17.954) belonged to 2,4-bis (1,1-dimethylethyl)-phenol. GC-MS of the crude extract showing 23 peaks, also contained one 2,4-bis (1,1-dimethylethyl)- phenol peak on it (Fig. 4). So, 2,4-bis (1,1-dimethylethyl)- phenol was the actual compound responsible for biocidal property. The structure of the compound was identified based on the comparison of its retention time with the compound database present in NIST library. The rest two peaks belonging to 1-Tetradecene and Pentadecane were probably due to the column impurities that came along the compound.

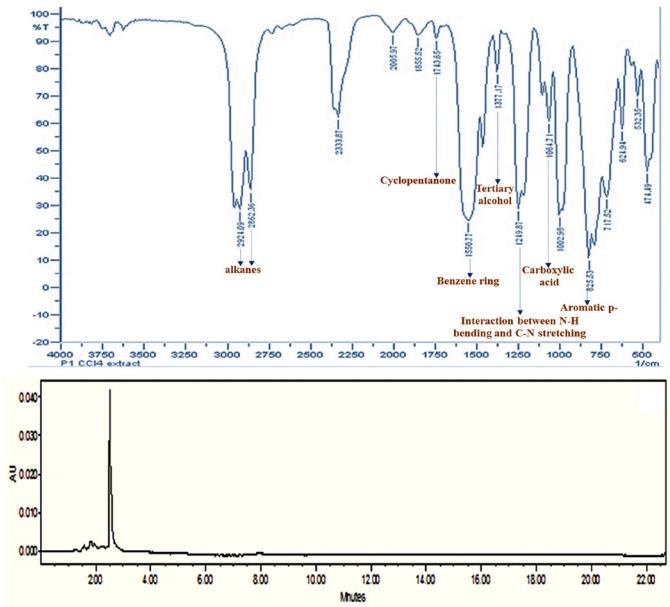

Figure 3. Structural identification of antibacterial compounds produced by *Chlorella* sp. PR-1 through FTIR and GC analysis. / Identificación estructural del compuesto antibacteriano producido por *Chlorella* sp. PR-1 mediante análisis de FTIR-GC.

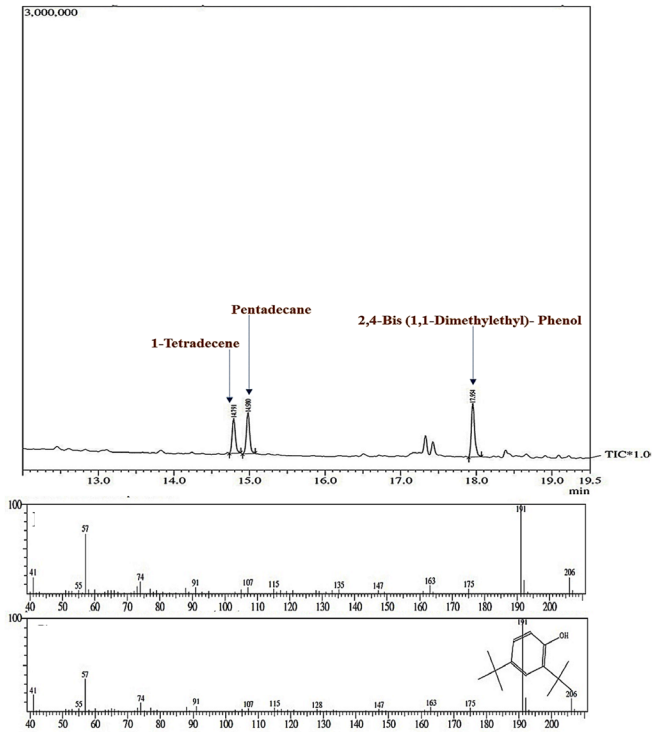

Figure 4. GC-MS analysis showing 2,4-bis (1,1-dimethylethyl)-phenol as the primary bioactive compound for antibacterial activity against *S. typhimurium*. / Análisis de GC-MS que muestra el 2,4-bis (1,1-dimetiletil)-fenol como compuesto bioactivo primario para la actividad antibacteriana contra *S. typhimurium*.

DISCUSSION

Microalgal biomass is widely studied as a promising source to several industry-based applications; like biofuel production, bioremediation, carotenoids, polyunsaturated fatty acids, phycobiliproteins and more (Syed *et al.* 2015; Liang *et al.* 2019, De Souza *et al.* 2018). The ability of microalgae to grow in otherwise inhospitable conditions, as well as their ability to metabolic adaptation under stress conditions, makes them attractive as raw material for large-scale cultivation and industrial applications (Katiyar *et al.* 2017). Marine environments supports diverse group of microorganisms and among them microalgae are identified to be one of the key members because of the biotechnological interests (Falaise

et al. 2016). In the present study, microalgae *Chlorella* sp. PR1 was isolated as a part of Southern Ocean (Indian sector) expedition 2011. Because of relatively fewer reports on biology of Southern Ocean microorganisms, this habitat is an example of one of the least explored ecosystem on earth with immense future possibilities (Banerjee *et al.* 2019). In this study, CCl_4 fraction of the marine microalgae *Chlorella* sp. PR1 displayed promising antibacterial property against the pathogen *S. typhimurium*. In 1944, the pioneer work of Pratt demonstrated the antimicrobial activity of green alga *Chlorella* against several gram negative and gram positive bacteria (Pratt *et al.* 1944). A similar study was also reported by Asthana *et al.* (2009) where 0.22 µg methanol extract of Antarctic cyanobacterium *Nostoc* CCC537 inhibited *S. typhimurium* with 10 mm zone of inhibition. After that, several bioactive compounds were isolated from microalgae that are available in market. In another report, methanol and chloroform (1:1) extract of marine algae *Dunaliella salina* have appreciable antimicrobial activity against a group of both gram positive and gram negative pathogenic bacteria (Krishnakumar *et al.* 2013). The carotenoid pigment and chlorophyll of green algae *Chlorococcum humicola* showed the activity of inhibiting the growth of virulent bacteria and pathogenic fungi (Bhagavathy *et al.* 2011). Bioactive fatty acids from *Haematococcus pluvialisa* and *Scenedesmus obliquus* were also reported to have antibacterial property against pathogenic bacteria (Rodríguez-Meizoso *et al.* 2010; Santoyo *et al.* 2009; Guedes *et al.* 2011). Compared to all these reports, in the present study 15 µg CCl_4 extract of *Chlorella* sp. PR-1 inhibited the growth of *S. typhimurium* with 10±0.5 mm zone of inhibition. The bioactive antibacterial compound responsible for the inhibition of *S. typhimurium* was further characterized through TLC, column chromatography, FTIR, HPLC and GC-MS. The FTIR spectrum indicated the presence of C-H stretching vibration, cyclopentanone, benzene ring and tertiary alcohols that are commonly found to have antimicrobial properties against both gram positive and gram negative bacteria (Cowan 1999, Barnos *et al.* 2007, Kostic *et al.* 2012, Wang *et al.* 2012, Zhang *et al.* 2012, You *et al.* 2014). In our preliminary study report, it has been characterized that 2,4-Bis (1,1-dimethylethyl)-phenol isolated from marine microalgae *Chlorella* sp. PR-1 is the biocide that showed promising antibacterial action to *S. typhimurium*. The likely mechanism by which the growth of *S. typhimurium* was inhibited is phenol-induced pyroptosis and apoptosis involving flexible deployment of caspases (Kinsella & Stallings 2020). S. *typhimurium* is susceptible to OH group of the phenols that act as a proton exchanger reducing the pH gradient across the cell membrane and thus leading to cell death. Phenolic compounds also interact with some crucial enzymes responsible for the production of the precursors of bacterial cell membrane or enzymes involved in fatty acid biosynthesis (Gyawali & Ibrahim 2014). Marine microalgal compounds is also newly revealed to inhibit enterobacteria, *S. typhimurium* (Machado *et al.* 2020). Phenols constitute one the largest group of secondary metabolites isolated from microalgae. *In vitro* antibacterial activity of the microalgae against *Bacillus* spp., *Salmonella* spp. and *from Staphylococcus aureus were* linked with phenolic compounds (Pina-Perez *et al.* 2017). Further, nuclear magnetic resonance (NMR) analysis may elucidate the detail structural features of the bioactive compound that may be used as alternative antimicrobials in future. Thus, the present study discloses a novel, marine microalgae-origin biocide, which may open new trends in biopharmaceutical industries.

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