PHYTOCHEMICALS, ANTIMICROBIAL AND ANTIOXIDANT SCREENING FROM FIVE DIFFERENT MARINE MICROALGAE

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ABSTRACT

Solvent extracts of five different microalgal strains including *Tetraselmis* sp., *Dunaliella* sp., *Chlorella* sp., *Synechocystis* sp., and *Oscillatoria* sp. were screened for seven phytochemical activities. Antimicrobial activity of solvent extracts was tested against seven bacterial and three fungal strains. The antioxidant potential of solvent extracts was analyzed using the DPPH scavenging assay. Flavonoids were predominantly found in all the solvent extracts. Microalgal strains showed better antifungal than antibacterial activity. Acetone extract of *Dunaliella* sp. showed highest percentage DPPH scavenging activity. The extracts showing highest antimicrobial and DPPH scavenging activity were subjected to GC-HRMS. The results showed the presence of Octadecanoic acid-4-hydroxy-methyl ester, benzoic acid, hexadecanoic acid and Tetradecanoic acid, confirming the bioactive compounds in the algal extracts.

KEY WORDS: *Microalgae, Antimicrobial activity, Phytochemical, antioxidant, algal extract*

1. INTRODUCTION

Microalgae are the major producers of organic matter, more than sixty trace elements including minerals, proteins and many bioactive substances in the ocean (Asthana, 2009). Algae are used in various food, cosmetic, nutraceutical, medical, pharmaceuticals and agricultural industries. Algae produce phytochemicals as their protective measure. These compounds could be used for protecting humans from many diseases. Many biologically and pharmacologically active substances have been isolated from algae which are difficult to be synthesized chemically (Kaushik, 2008). Algae-derived compounds have a very wide range of potential applications as animal feed and nutrition products. Some algae are considered as rich sources of natural antioxidants. Many microalgal and cyanobacterial extracts were found to have antibacterial or antifungal activities (Jaritz, 2011).Most of phytochemical compounds are accumulated in the microalgal biomass. Inhibitory activities of the extracts against the growth of microorganisms are common indicators for screening antimicrobial (Bouhlal, 2011), antiviral (Kim, 2011), antifungal (Felicio, 2010), anti-allergic (Na, 2005), anticoagulant (Dayong, 2008), anticancer (Kim, 2011), antifouling (Bhadury, 2004), antioxidant activities (Devi, 2011), cytotoxic and antitumor substances. Many substances having antioxidant property were derived from microalgae and are used to protect cells from oxidative damage during various diseases and ageing processes. The oxidative damage caused by reactive oxygen species on lipids, proteins and nucleic acids may trigger various chronic diseases, such as coronary heart disease, atherosclerosis, cancer and ageing (Finkel, 2008). These antioxidants are used as food additives in packaged foods to protect them from oxidative degradation. Application of antioxidant compounds like carotenoids, terpenoids, polysaccharides, peptides, proteins, vitamins, acrylic acid, terpenes, chlorophyllides, phenols, heterocyclic compounds, steroids, amino acids, phlorotannins, phenolic compounds, halogenated ketones, alkanes and cyclic polysulphides in food and cosmetics protects the life- style related diseases (Guedes, 2011). The discovery and development of antibiotics are among the most powerful and successful achievements in modern Science and Technology for the control of infectious diseases (Chanda, 2010). Antimicrobial effects of the algal extracts could be attributed to their phenolic components. Accordingly, pharmaceutical industries are giving importance to the compounds derived from traditional sources such as plants and non-traditional sources like marine organisms (Salem, 2011). Hence, the present study was focused on screening of phytochemicals from algal extracts and testing their antibacterial, antifungal and antioxidant potential as well as characterizing the specific compounds in the extracts using GC-HRMS.

2. MATERIAL AND METHODS

2.1. Strains collection, sub-culturing and growth conditions: Microalgal strains such as *Tetraselmis* sp., *Dunaliella* sp., *Chlorella* sp., *Synechocystis* sp., and *Oscillatoria* sp. were obtained from Central Marine Fisheries Research Institute (CMFRI) -Thoothukudi, Tamil Nadu, India. Seven different bacterial strains such as *Bacillus* sp., *Escherichia coli, Staphylococcus aureus, Salmonella* sp., *Pseudomonas aeruginosa, Klebsiella* sp. and *Enterococcus* sp. and fungal species such as *Rhizopus* sp., *Anidubans* sp. and *Fusarium* sp. were obtained from KAP Visvanathan Medical College, Tiruchirappalli, Tamil Nadu. Sea water was taken from Elliot beach, Chennai. All the microalgal strains were sub-cultured in sea water enrichment medium (sea water, 0.1 g/L $NaNO_3$ and 0.02 g/L $Na_2HPO_4.2H_2O$) maintained at 22 ^oC with occasional shaking for a period of 30 days. Nutrient and potato dextrose broth were used as media for sub-culturing bacterial and fungal strains respectively.

2.2. Biomass harvesting: The algal biomass was harvested by centrifugation at 5,000 rpm for 7 min at 15 °C and the cell pellet was dried at room temperature using a vacuum desiccator.

2.3. Extract preparation: Dried microalgal biomass was ground well using a sterile mortar and pestle and extracts were prepared using four different solvents *viz*., acetone, methanol, ethanol and chloroform. The crude extracts were stored at 4 °C for further use.

2.4. Phytochemical screening: Acetone, methanol, ethanol and chloroform extracts prepared from all the five different microalgae were used to screen various phytochemicals such as tannins, flavonoids, terpenoids, steroids, saponins, glycosides and alkaloids as described by Sanjeet et al.,(2010).

2.4.1. Test for Tannins: Algal extracts of 700 µl were mixed with 50 ml of distilled water and 1 % of ferric chloride was added drop by drop. Formation of a dark green solution indicated the presence of tannins.

2.4.2. Test for Flavonoids: Algal extract of 500 µl was dissolved in 1ml of 10 % NaOH and a few drops of concentrated HCl were added. The presence of flavonoids was indicated by the disappearance of yellow color.

2.4.3. Test for Terpenoids: To 1 ml of algal extract was taken and 400 µl of chloroform was added and mixed. A few drops of concentrated sulfuric acid were added. A reddish brown interface was formed and this indicated the presence of terpenoids.

2.4.4. Test for Steroids: To 100 µl of algal extracts was taken and 400 µl of acetic anhydride and a few drops of concentrated sulfuric acid were added. The formation of a brown ring indicated the presence of steroids.

2.4.5. Test for Saponins: To 1 ml of algal extracts, few drops of 1 % ferric chloride were added. Frothing or appearance of a creamy mass of small bubbles showed the presence of saponins.

***2.4.6. Test for Glycosides*:** To 1 ml of algal extracts, 1 ml of pyridine and a few drops of freshly prepared sodium nitroprusside solution were added. Appearance of pink to red color indicated the presence of glycosides.

2.4.7. Test for Alkaloids: To the algal extract, few drops of Wagner's reagent (solution of iodine in potassium iodide) were added. Appearance of reddish brown precipitate indicated the presence of alkaloids.

2.5. Antimicrobial Activity

Antimicrobial activity of acetone, methanol, ethanol and chloroform extracts of five different marine microalgal strains was evaluated by agar well diffusion assay against bacterial strains such as, *E. coli*, *Bacillus* sp., *Staphylococcus* sp., *Salmonella* sp., *Pseudomonas* sp., *Klebsiella* sp. and *Enterococcus* sp. Fungal species *viz.*, *Rhizopus* sp., *Anidubans* sp. and *Fusarium* sp. were also used for the assay. Each bacterial culture was swabbed on Muller Hinton agar. Five wells of 4 mm diameter were cut into agar plates and 50 µL of microalgal extracts and positive control (Streptomycin) were added to each well. The inoculated plates were incubated for 24 h at 37 ºC for bacteria and incubated for 3 days at 30 ºC for fungi. The growth inhibition zone was measured in millimeters. Tests were carried out in triplicates (Abedin and Taha, 2008; Prakash, 2011).

2.6. Antioxidant Activity

The free radical scavenging activity of acetone, methanol, ethanol and chloroform extracts of microalgal strains using stable free radical, DPPH (1,1-diphenyl-2-picrylhydrazyl) was determined spectrophotometrically. DPPH (0.004 %) was prepared in methanol and 1 ml of 0.004 % DPPH-methanol solution was mixed with 2 ml of each solvent algal extract and kept in dark for 30 min. The optical density of the oxidation reaction was measured at 517 nm using spectrophotometer with solvent and DPPH as blank (Temraz and El-Tantawy, 2008; Rajaram and Kumar, 2011). The percent inhibition was calculated using the formula,

$$\text{Percentage (\%)inhibition of DPPH activity} = \left\{\left(\frac{\text{A} - \text{B}}{\text{A}}\right) \times 100\right\}$$

Where, A = absorbance of the control (DPPH) and B = absorbance of test sample.

2.7. GC-HRMS analysis

To identify the composition of the extracts which showed best antimicrobial activity was subjected to gas chromatography- High Resolution Mass Spectroscopy (GC-HRMS, AccuTOFGcv, Jeol make, USA). GC-HRMS was performed on a 0.25 mm (id) x 30 m fused silica column lined with a 0.25 µm film of polyethylene glycol. Samples (0.1 µl) were injected in split mode of ratio 100. The column head pressure of the carrier gas was helium at 3 Kpa at the initial oven temperature and its flow rate 1.0 ml/min. The initial temperature of 100 ºC, ramp was 10 ºC/min, to 250 ºC with a hold for 5 min at 250 ºC, interface temperature was 280 ºC. The GC-HRMS was connected TIC, MS, Library data search tool (Selvan et al., 2013).

3. RESULTS AND DISCUSSION

3.1. Phytochemical screening using different solvents: The five marine algal strains were extracted with four different solvents. The phytochemicals present in the algal strains were identified as flavonoids, terpenoids, alkaloids, steroids and saponins (Table 1). *Chlorella* sp., *Synechocystis* sp., and *Oscillatoria* sp. extracts of all four solvents contain flavonoids. Flavonoids were the most predominant phytochemical found in all the solvent extracts. It is predominantly seen in acetone and ethanol extracts. Acetone, methanol and ethanol extracts of *Chlorella* sp., *Synechocystis* sp. and *Oscillatoria* sp. gave better result for flavonoids than Chloroform extracts. Alkaloids and saponins were also present in almost all the extracts except acetone extracts of *Synechocystis* sp. and *Oscillatoria* sp. A good DPPH scavenging activity could be achieved with algal extracts having phenol and flavonoid contents (Dash and Padhi, 2012). Ethyl acetate extract of *Chlorococcum humicola* have the bioactive phytochemicals such as alkaloids, flavonoids, fattyacids and saponins which showed the 80 % microbial growth inhibition (Bhagavathy, 2011). Phytocomponents like flavonoids, reducing sugar, carotenoids, tannins, alkaloids anthraquinones and quinineswere present in *Sun chlorella* extracts but Glycosides was absent (Geetha, 2010).

Table 1. Screening of phytochemicals of different solvent extracts of microalgae

Algal species	Extracts	Tannins	Flavonoids	Terpenoids	Alkaloids	Glycosides	Steroids	Saponins
Tetraselmis sp.	Acetone	-	+	+	+	-	+	+
	Methanol	-	+	-	+	-	-	+
	Ethanol	-	++	-	+	-	-	+
	Chloroform	-	+	-	+	-	-	-
Dunaliella sp.	Acetone	-	+	-	+	-	+	+
	Methanol	-	+	-	+	-	-	+
	Ethanol	-	++	-	+	-	-	+
	Chloroform	-	+	-	+	-	-	-
Chlorella sp.	Acetone	-	+++	-	+	-	-	-
	Methanol	-	+++	-	+	-	-	+
	Ethanol	-	+++	-	+	-	-	+
	Chloroform	-	++	-	+	-	-	-
Synechocystis sp.	Acetone	-	++	-	-	-	+	-
	Methanol	-	++	-	+	-	-	+
	Ethanol	-	++	-	+	-	-	+
	Chloroform	-	+	-	+	-	-	-
Oscillatoria sp.	Acetone	-	+++	-	-	-	-	-
	Methanol	-	+++	-	+	-	-	++
	Ethanol	-	+++	-	+	-	-	+
	Chloroform	-	++	-	+	-	-	+

High concentration (+++), Moderate concentration (++), Low concentration (+) and absent (-)

3.8.2. Antibacterial activity using different solvents (agar well diffusion method)

Seven different bacterial strains such as *E. coli*, *Bacillus* sp., *Staphylococcus* sp., *Salmonella* sp., *Pseudomonas* sp., *Klebsiella* sp. and *Enterococcus* sp. were used to examine the antibacterial activity of the solvent extracts of the microalgal strains (Fig. 2). Acetone and ethanol extracts of *Tetraselmis* sp. showed maximum zone of inhibition against *Pseudomonas* sp. (6.17 mm) and *Enterococcus* sp. (4.05 mm). Most of the solvent extracts of *Synechocystis* sp. exhibit highest zone of inhibition against *E. coli*, *Bacillus* sp, *Staphylococcus* sp. and *Salmonella* sp. The pathogenic bacterial strains were resistant to some of the solvent extracts whereas, *Synechocystis* sp., and *Oscillatoria* sp. showed highest inhibitory activity than other microalgal solvent extracts. *Tetraselmis* sp. showed maximum zone of inhibition against *E. coli* and *Pseudomonas* sp. for all solvent extracts and acetone extracts against *Enterococcus* sp. Acetone and methanol extracts of *Synechocystis* sp. showed maximum zone of inhibition against *E. coli*, *Bacillus* sp, *Staphylococcus* sp. and *Salmonella* sp. Similarly extracts of *Oscillatoria* sp., *Phormidium* sp. and *Lyngbya majuscula* were found to have activity against human pathogenic bacteria such as *Streptococcus* mutants, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Klebsiella pneumonia* (Sethubathi and Prabu, 2010). Methanol extract of *Oscillatoria sancta* have better zone of inhibition against *P. mirabilis*, *P.vulgaris* and *S. aureus* (Prakash, 2011) which shows its antibacterial capability. *S.aureus* was particularly sensible to palmitoleic and oleic acids, organic solvent extracts of *Synechocystis* sp. were identified to have superior in antimicrobial activity, when it was chemically characterized by GC-MS analysis, it revealed the presence of various fatty acids and volatile compounds such as phytol, fucosterol, neophytadiene, palmitic, palmitoleic and oleic acids (Plaza, 2010).

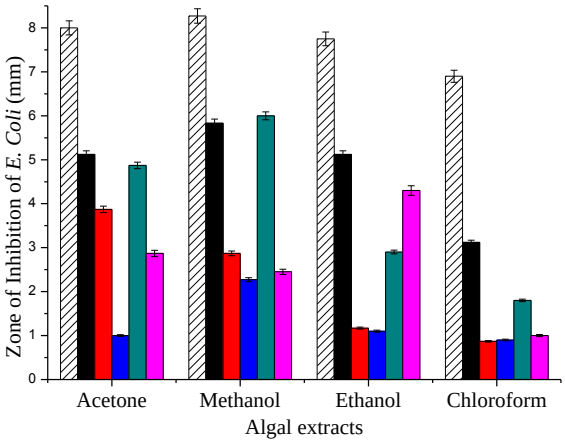


Fig. 1A

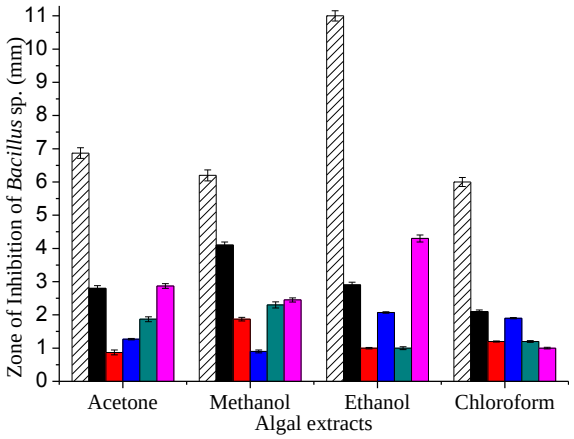


Fig. 1B

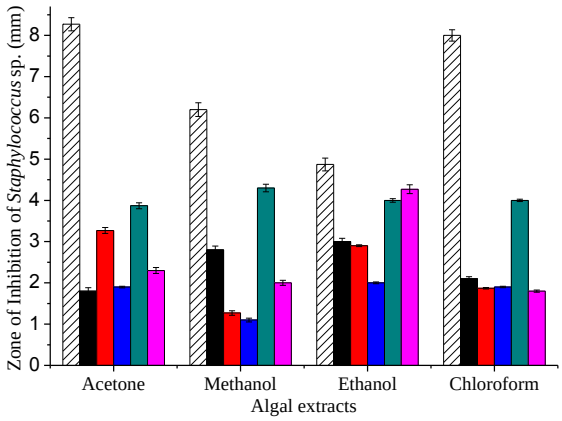


Fig. 1C

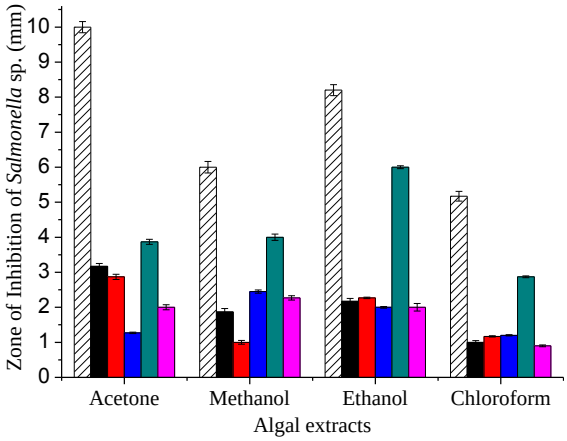
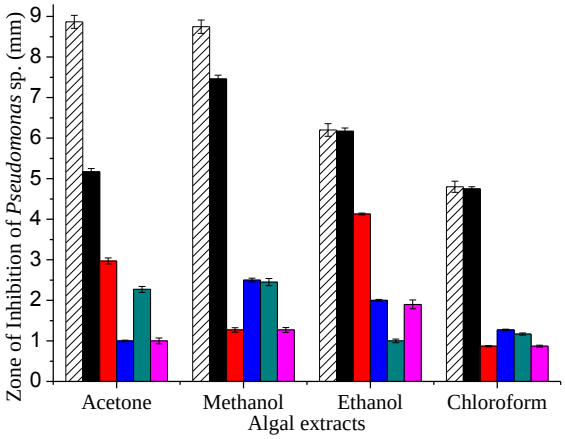


Fig. 1D



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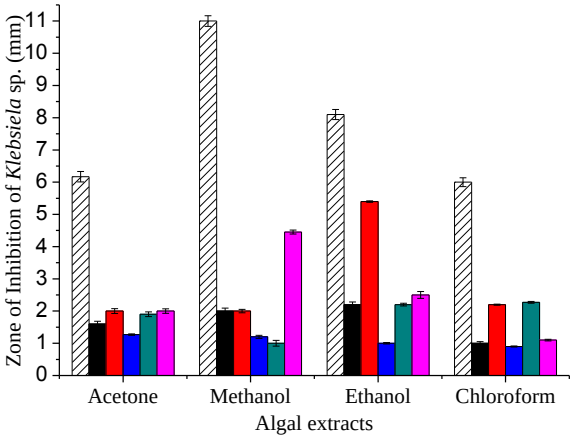

Fig. 1F

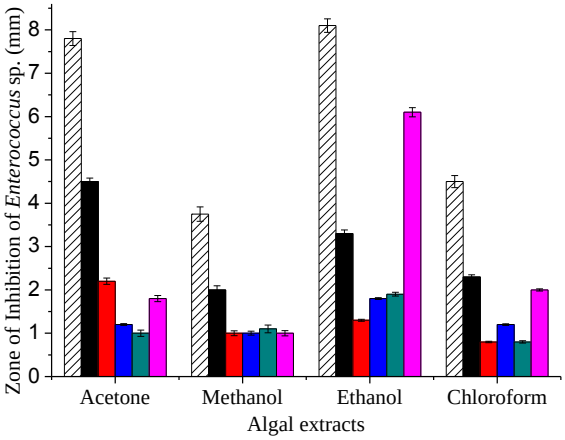

Fig. 1G

Fig.1 Antibacterial activity of algal extracts
Fig. 1A. Activity against *E. coli*
Fig. 1B.Activity against *Bacillus* sp.
Fig. 1C. Activity against *Staphylococcus* sp.
Fig. 1D.Activity against *Salmonella* sp.
Fig. 1E. Activity against *Pseudomonas* sp.
Fig. 1F.Activity against *Klebsiella* sp.
Fig. 1G.Activity against *Enterococcus* sp.

Control (Streptomycin)
Tetraselmis sp.
Dunaliella sp.
Chlorella sp.
Synechocystis sp.
*Oscillatoria*sp.

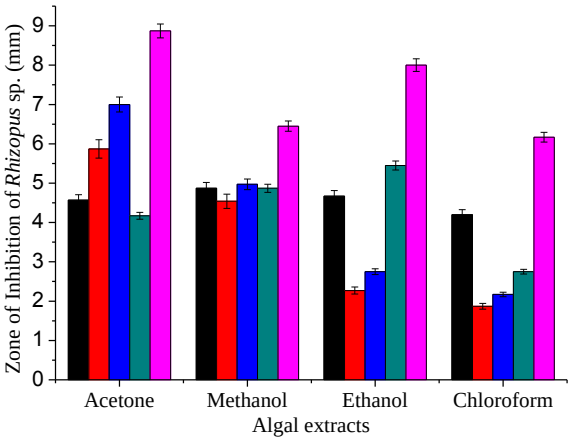

Fig. 2A

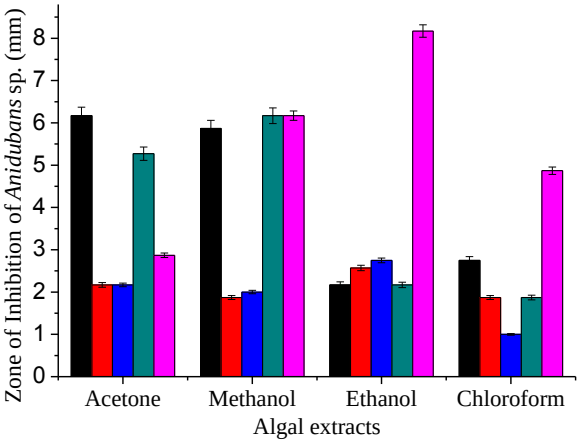

Fig. 2B

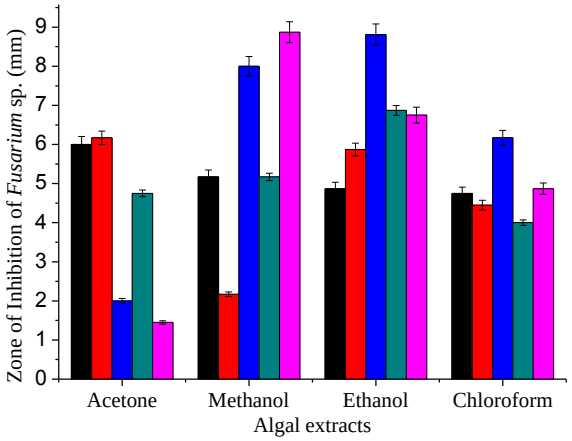

Fig. 2C.

Fig. 2 Antifungal activity of algal extracts
Fig. 2A.Activity against *Rhizopus* sp.
Fig. 2B.Activity against *Anidubans* sp.
Fig. 2C.Activity against *Fusarium* sp.

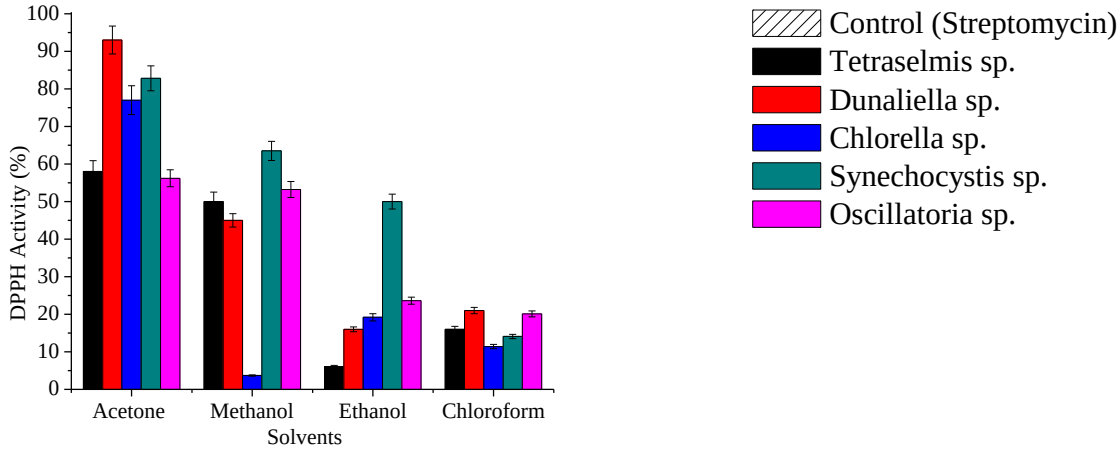

Fig. 3. DPPH activity of solvent extracts of microalgal strains

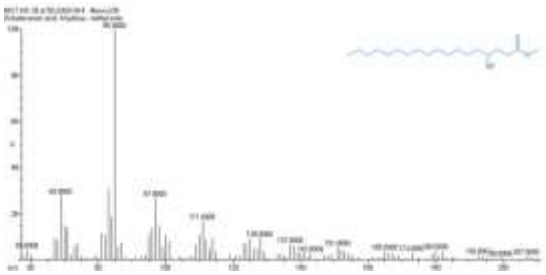

Fig. 4A

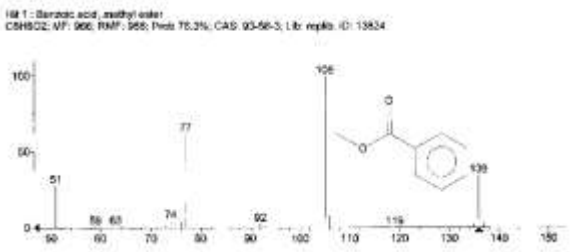

Fig. 4B

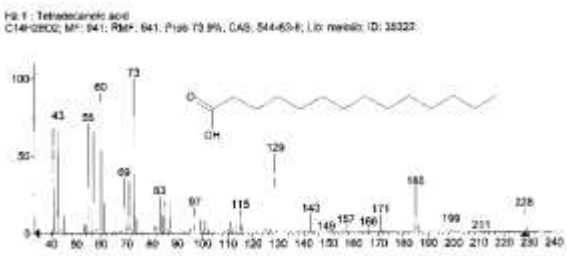

Fig. 4C

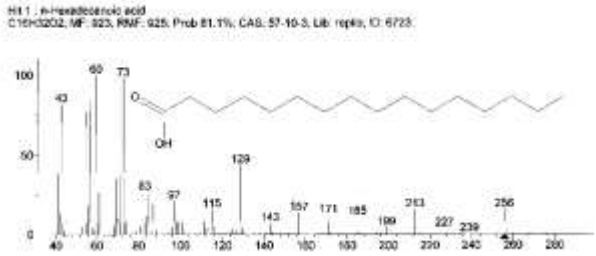

Fig. 4D

Fig 4. Characterization of components of active antimicrobial extracts by GC-HRMS analysis
Fig. 4A. NSIT library analysis depicts the presence of Octadecanoic acid-4-hydroxy-methyl ester,
Fig. 4B. NSIT library analysis represents the presence of benzoic acid,
Fig. 4C. NSIT library analysis shows the presence of hexadecanoic acid
Fig. 4D. NSIT library analysis describes the presence of Tetradecanoic acid.

3.8.3. Antifungal activity using different solvents: Fungi such as, *Rhizopus* sp., *Anidubans* sp. and *Fusarium* sp. were used to examine the antifungal activity of all the four different solvent extracts of the microalgae. Acetone extract of *Dunaliella* sp. showed resistance against *Rhizopus* sp. and *Fusarium* sp. whereas acetone extract of *Chlorella* sp. showed maximum zone of inhibition against *Rhizopus* sp. But, *Oscillatoria* sp. and *Synechocystis* sp. solvent extracts showed resistance against almost all the three fungal strains tested (Fig. 2). Almost all solvent extracts of *Tetraselmis* sp. showed better antifungal activity against all the three fungal strains. Acetone, methanol and ethanol extract of *Dunaliella* sp. showed resistance against *Rhizopus* sp. and *Fusarium* sp. whereas ethanol extract of *Chlorella* sp. showed maximum activity against *Fusarium* sp. But, acetone and methanol extracts of *Chlorella* sp. showed better activity against *Rhizopus* sp. Methanol extract of *Synechocystis* sp. showed maximum zone of inhibition against all the three fungal strains but acetone extract showed maximum activity against *Anidubans* sp. and *Fusarium* sp. All solvent extracts of *Oscillatoria* sp. showed maximum activity against all the fungal strains than *Synechocystis* sp. *Oscillatoria* sp. and *Synechocystis* sp. of all solvent extracts had maximum resistance against all the three fungal species than other microalgae. The long-chain unsaturated fatty acids (C16, C20) like palmitoleic, oleic, linoleic and linolenic acids attribute the antifungal activity. *Synechocystis* sp. extracts were rich in palmitoleic acid which tends to active against all these fungal strains. Thus the amount of palmitoleic and oleic acids in the extracts are responsible for activity against the fungal strains (Plaza, 2010).

3.8.4. Antioxidant activity using the DPPH scavenging assay: The DPPH scavenging assay was used to study the antioxidant potential of each solvent extract. *Dunaliella* sp., *Chlorella* sp. and *Synechocystis* sp. showed maximum activity in acetone extracts, followed by methanol extracts of *Synechocystis* sp., *Oscillatoria* sp., *Tetraselmis* sp. and *Dunaliella* sp. (Fig. 3). DPPH is a better reagent for investigating the free radical scavenging activities of compounds. Acetone extracts of all microalgal strains showed maximum inhibition of DPPH, especially *Dunaliella* sp. All four solvent extracts of *Synechocystis* sp. and *Oscillatoria* sp. showed better inhibition against DPPH activity. A number of reports are available on the evaluation of antioxidant activity in microalgae *Dunaliella* (Herrero, 2006). These studies concluded that several microalgal genera contain potent antioxidants. Flavonoids are large group of naturally occurring compounds having effective biological activities including antioxidant and tumoristatic potential (Li, 2007).

3.8.5. Characterization of active antimicrobial extract through GC-HRMS analysis: The GC-HRMS analysis has confirmed the presence of Octadecanoic acid-4-hydroxy-methyl ester (Fig. 4a), benzoic acid (Fig. 4b), hexadecanoic acid (Fig. 4c) and Tetradecanoic acid (Fig. 4d) in algal extracts. Antibacterial and antifungal activities of the algal extracts were reportedly because of the presence of Lauric, palmitic (hexadecanoic acid), linolenic, linoleic, oleic, stearic (Octadecanoic acid) and myristic acids (Tetradecanoic acid) (Agoramoorthy, 2007). Octadecanoic acid from neam extract tested on three bacterial strains *viz*., *S. aureus, E. coli* and *Salmonella* sp. and showed better inhibition activity against *S. aureus* than *E. coli* and *Salmonella* sp. (Zhoung-hui, 2010). Zakaria, 2011 studied the hydrocarbons inhibitory effect on *P. aeruginosa*. The fatty acids such as oleic acids and hexadecane exhibited the antimicrobial and antioxidant activity. These acids act like an anionic surfactant to exhibit the antibacterial and antifungal activity at low pH. GC-MS NIST library analysis of *Sun chlorella* extracts revealed the occurrence

of 1, 2-Benzenedicarboxylic acid, diisooctyl ester, 9, 12, 15 - Octadecatrienoic acid, n-Hexadecanoic acid, Octadecanoic acid, 9, 12-Octadecadienoic acid, n-Hexanoic acid, Decanoic acid, Oleic acid etc (Geetha, 2010). *D.salina* extract holds different chemical constituents such as 3, 3, 5-Trimethyl heptanes, 2, 3, 4-Trimethyl heptanes, 3, 3, 4-Trimethyl heptanes, 2, 4, 4-Trimethyl heptanes, Isotetradecane, Tetradecyl iodine, n-pentadecane, Tridecane, n-Octasane, n-Dotriacontane, n-Nonocosane, n-Heptacosane, and n-Pentadecane (Bai and Krishnakumar, 2013).

4. CONCLUSION

Five different microalgal species showed rich sources of valuable medicinal compounds and their extracts exhibited a significant range of phytochemicals especially, flavonoids, alkaloids and saponins. These microalgal strains were also found to possess antioxidant activities whereas, the antifungal activity was better than antibacterial activity. Acetone extracts gave better results when compared to others. The characterization of the active compounds from microalgal extracts revealed the presence of Octadecanoic acid-4-hydroxy-methyl ester, benzoic acid, hexadecanoic acid and Tetradecanoic acid.

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