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Research Article

EVALUATION OF ANTIMICROBIAL METABOLITES FROM MARINE MICROALGAE *TETRASELMIS SUECICA* USING GAS CHROMATOGRAPHY – MASS SPECTROMETRY (GC – MS) ANALYSIS

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ABSTRACT

Objective: Marine natural products have been considered as one of the most promising sources of antimicrobial compounds in recent years. Marine microalgae *Tetraselmis suecica* (Kylin) was selected for the present antimicrobial investigation.

Methods: The effects of pH, temperature and salinity were tested for the growth of microalgae. The antibacterial effect of different solvent extracts of marine microalgae *Tetraselmis suecica* against selected human pathogens such as *Vibrio cholerae, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Salmonella sp, Proteus sp., Streptococcus pyogens, Staphylococcus aureus, Bacillus megaterium* and *Bacillus subtilis* were investigated. The GC-MS analysis was done with standard specification to identify the active principle of bioactive compound.

Results: The highest cell density was observed when the medium adjusted with 40ppt of salinity in pH of 9.0 at 25^{0}C during 9th day of incubation period. Methanol + chloroform (1:1) crude extract of *Tetraselmis suecica* was confirmed considerable activity against gram negative bacteria than gram positive human pathogen. GC-MS analysis revealed the presence of unique chemical compounds like 1-ethyl butyl 3-hexyl hydroperoxide (MW: 100) and methyl heptanate (MW: 186) respectively for the crude extract of *Tetraselmis suecica*. Different fatty acids such as Methyl carprate, Methyl stearate, Decoic acid, Palmitic acid, Nonoic acid and Caprylic acid with antimicrobial activity and pharmaceutical importance were identified.

Conclusion: These findings demonstrate that the Methanol + chloroform (1:1) extract of *Tetraselmis suecica* displayed appreciable antimicrobial activity and thus have great potential solvent to extract bioactive compounds from the natural sources for current clinical and pharmaceutical importance.

Keywords: Microalgae, *Tetraselmis suecica,* Antimicrobials, GC-MS analysis.

INTRODUCTION

Antibiotic resistance in bacteria is one of the major emerging health care related problems in the world. High mortality in human population and aquaculture organisms is because of bacterial infection [1]. Recurrent exposure of bacteria to the antibiotics has developed resistant plasmid thereby producing plasmid mediated extracellular enzyme to inactivate the antibiotics. According to World Health Report of Infectious Diseases [2], overcoming antibiotic resistance is the major issue for the next millennium. To prevent antibiotic resistance new compounds which are not based on existing synthetic antimicrobial compounds have to be used. Biologically active compounds from natural resources have always been of great interest to scientists working on different diseases [3]. In the present investigation we have tried to explore the screening of novel antimicrobials from marine microalgae in order to prevent antibiotic resistance in pathogenic bacteria. Some of the chemotherapeutic agents cause certain side effects to human beings. These limitations demand for improved pharmacokinetics properties, while necessitating continued research for new antimicrobial compounds for the development of drugs from unexplored area to treat already existing diseases [4]. Marine environment contain pharmacologically important diverse group of natural products [5, 6] and enzymes [7].

Pharmaceutical industries are giving importance to the compounds derived from marine organisms [8]. Among marine organisms, marine algae are valuable natural sources effective against infectious agents. Worldwide extensive efforts have been made for the detection of bioactive compounds derived from natural resources, in order to develop safe, non-toxic and efficient antimicrobial agents for current medical practice in pharmacology. Algae of marine and terrestrial origins have been the best choice among natural resources within aquaculture and agriculture fields [9]. There are over 50,000 different species of microalgae of which only a few have been characterized [10]. This group of microorganisms is extremely diverse and represents a major untapped resource of valuable bioactive compounds [11]. Screening of bioactive metabolites of algal crude extracts is enforced in clinical practice, where antibacterial, anti-plasmodia and cytotoxicity [12], antifungal [13] and antiviral [14] activity have been accessed to these metabolites. Microalgae are rich in bioactive natural products so it has been studied as potential biocidal and pharmaceutical agents [15]. Due to the crucial role played by microalgae, it was deemed worthwhile to examine growth parameters, physiological attributes and antimicrobial activity for possible biotechnological applications.

The antimicrobial activity of algae extracts is generally assayed using various organic solvents [16]. An organic solvent always provides a higher efficiency in extracting compounds for antimicrobial activity [17]. Screening of organic solvent extracts from marine algae and other marine organism is a common approach to identify compounds of biomedical importance. Hence, an attempt has been made to evaluate the efficiency of various organic solvents, antimicrobial activity and identify the chemical components and structure by GC-MS analysis of crude algal extracts from the marine microalgae against the most common selected human pathogenic bacteria.

MATERIALS AND METHODS

Marine microalgae culture collection

Marine microalgae *Tetraselmis suecica* (Kylin) Butcher **(**Kingdom: Viridiplantae; Phylum: Chlorophyta; Class: Prasinophyceae; Order: Chlorodendrales; Family: Chlorodendraceae; Genus: Tetraselmis; Species: *Tetraselmis suecica***)** is presented in Fig.1, collected from Centre for Marine Fisheries Research Institute (CMFRI) Tuticorin, Tamilnadu, India with the help of sterile screw cap tube and maintained in our laboratory was chosen for the present study.

Stock culture maintenance

Hundred milliliter of filtered seawater was taken into 250ml of conical flask and amended a required nutrient of Miquell's medium (solution-A: Potassium nitrate: 20.2 g; distilled water: 100ml; solution-B: Sodium orthophosphate: 4g; Calcium chloride: 2g; Ferric chloride: 2g; Hydrochloric acid: 2ml; distilled water: 100ml).

Solution A (0.55 ml) and solution B (0.5 ml) were added to one liter of filtered sterilized seawater and mixed thoroughly to enrich the water. Then the culture flask was autoclaved. After sterilization 10% of actively growing phase inoculums were transferred into culture flask aseptically. The inoculated flask was incubated at 28±2°C beneath the tube light (1000 lux) for 8 days. When the maximum exponential phase was reached, the light was reduced for further growth.

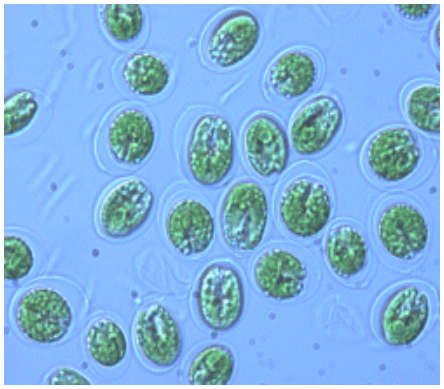

Fig. 1: Showing marine microalgae *Tetraselmis suecica*

Chemicals

All chemicals and media components procured from Hi media Laboratories Private Limited, (Mumbai, India) were used to carry out the present study.

Growth optimization of culture condition

The Miquell's medium (100ml) was prepared in 250 ml of Erlenmeyer flask. The different growth parameter including pH (3, 5, 7, 9 and 11), temperature (20, 25, 30 and 35°C) and salinity (20, 30 and 40ppt) were optimized independently. The salinity was checked with the help of 300 px- Refractometer **(**300 X 225-traditional hand land**).** Then 10 ml of actively growing phase inoculums was transferred to the culture flask and kept under the tube light with 1000 lux for 14 days.

Determination of cell density

The determination of cell density was performed by the standard method [18]. Cell counts were monitored using a Neubauer improved Haemocytometer (DHC-N01). The samples were treated with formalin to kill the cells and one drop of the sample was taken using sterile Pasteur pipettes. After placing the cover slip on the haemocytometer, the pipetted samples were introduced on the counting grid of the haemocytometer and left for a few minutes. The cells were counted under compound microscope (ADELTA OPTEC – DN10) and the total cell count was calculated using the following formula.

$$\text{Total cell count} = \frac{\text{Number of cells counted X Number of square in a group}}{\text{Number of square counted}}$$

Algal extracts preparation using different solvents

The algal cells were centrifuged (REMI-R24) at 200 rpm for 10 minutes. The pellet was collected and air dried to get a fine powder. Dried algal cells (10g) were extracted in 100ml of different organic solvents such as Acetone, n-butanol, Isopropanol, Acetone + n-butanol (1:1), Acetone + Isopropanol (1:1), Acetone + Chloroform (1:1), Butanol + Isopropanol (1:1), Chloroform + Methanol (1:1) separately under stirring condition (50rpm) for 7 days at room temperature. The solution was filtered through Whatman No.1 filter paper. Then the filtrate was dried using desiccators (Vacuum Dry - seal Desiccator 12") at 40°C for 24h. The dried powder was dissolved with respective solvents to give 50mg/ml extract. The crude extract was kept in airtight bottle and stored in a refrigerator for further antimicrobial and GC-MS studies.

Antibacterial assay

Antibacterial activity was determined against the selected human pathogens using paper disk assay (PDA) method [19]. Whatman No.1 filter paper disk of 6mm diameter was cut and sterilized by autoclaving. The sterile disk was impregnated with different solvent extracts (50µl/per disk). Control disk also maintained for each extract by impregnate respective organic solvent alone. Muller Hinton Agar (MHA) plates were prepared and overnight broth culture (1.2×10^8 cfu/ml) of test pathogens were inoculated uniformly using sterile cotton swab. The impregnated disks were placed on the plates using sterile forceps suitably spaced at equal distance. Triplicates were maintained for each test pathogen. The plates were incubated at 37°C for 24h. The zone of inhibition was measured and expressed in mm in diameter.

Test pathogens

The human gram negative pathogens such as *Vibrio cholerae, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Salmonella sp, Proteus sp.,* and gram positive pathogens namely *Streptococcus pyogens, Staphylococcus aureus, Bacillus megaterium* and *Bacillus subtilis,* collected from Kanniyakumari medical college and hospital (KMCH), Kanniyakumari District, Tamilnadu, India and maintained in our laboratory was chosen for the present antibacterial susceptibility study.

Chemical analysis of algal extracts using gas chromatography-mass spectrometry (GC-MS analysis)

The gas chromatography combined with mass spectrometry detection technique is a qualitative and quantitative analysis of the crude extracts with high sensitivity even with smaller amount of components. Consequently, identification of the chemical moiety of crude extracts of *Tetraselmis suecica* which showed precious antibacterial activities against the selected human bacterial pathogens was analyzed. The GC-MS analysis was done with standard specification by dissolving 10mg of crude extracts in one milliliter of ethyl acetate. The liquid sample of 0.1 µl was injected into 0.25 mm x 25 m column of GC-MS model (GC 17A, Japan) 5% phenyl poly siloxane as stationary phase and helium (3 ml/min) as carrier gas with the flow rate of 0.4 m/min. The temperature gradient program was adopted for the evaporation of organic solvent to identify the chemical constituent. The initial temperature was 70°C and gradually accelerated to 250°C at a rate of 10°C per minute. The sample was injected at 250°C after 18 minutes. The maximum peaks representing mass to charge ratio characteristics of the antimicrobial fractions were compared with those in the mass spectrum library of the corresponding organic compounds [20]. The concentration of such compound was calculated by the following formula:

Compound concentration percentage = [P1/P2] x 100

Where, P1 is the peak area of the compound and P2 is whole peak areas in the fractionated extracts.

Data analysis

The data were statistically analyzed through TWO way ANOVA using MINITAB software and means for different parameters were separated by applying least significant difference (LSD) test at 0.05 % level of probability to know their significance status [21].

RESULTS

Culture conditions

Marine microalgae have the rich source of bioactive compounds to defense the harmful organisms. The media optimization is the important aspect to be considered in the development of fermentation technology. Large scale production of algal metabolites usually involves a wide range of search for optimization of culture conditions. This was achieved through a systematic study by varying the various culture conditions to the microalgae. Optimum culture conditions relative to temperatures, pH and salinities levels were distinct for marine microalgae *Tetraselmis suecica.* The growth at different temperature is shown in Fig. 2. Maximum growth was recorded at 25°C and minimum growth at 35°C. The growth of microalgae at different pH is depicted in Fig. 3. Maximum growth of algae was observed at the pH of 9.0 and minimum growth was recorded at the pH of 5.0 on

9th day of incubation. The growth rate of microalgae was studied at different saline concentration such as 20, 30 and 40 ppt. Growth rate of salinity is presented in Fig. 4. Maximum growth was observed at 40 ppt. and minimum growth was recorded at 20 ppt.

These results confirmed that the microalgae *Tetraselmis suecica* belongs to halophytes. Progressive increase in the cell concentration of microalgae was observed from first day to 8th day with the maximum value on 9th day.

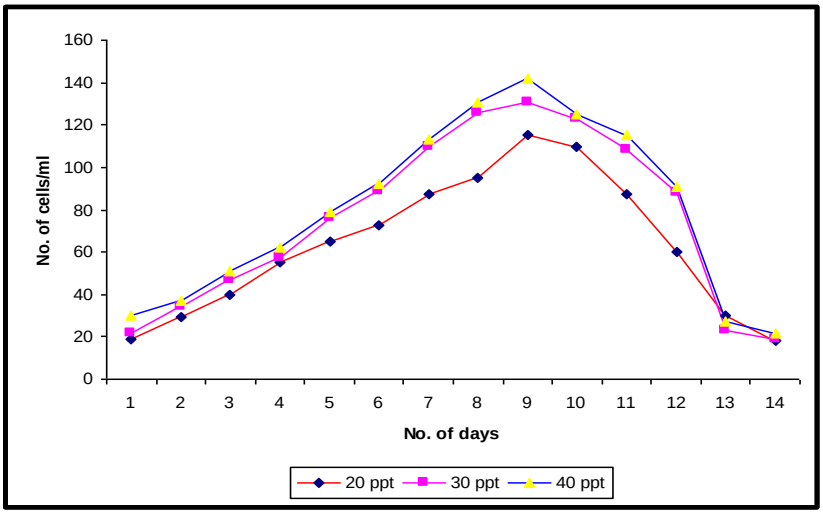

Fig. 2: Growth characterization of *Tetraselmis suecica* at various salinity.

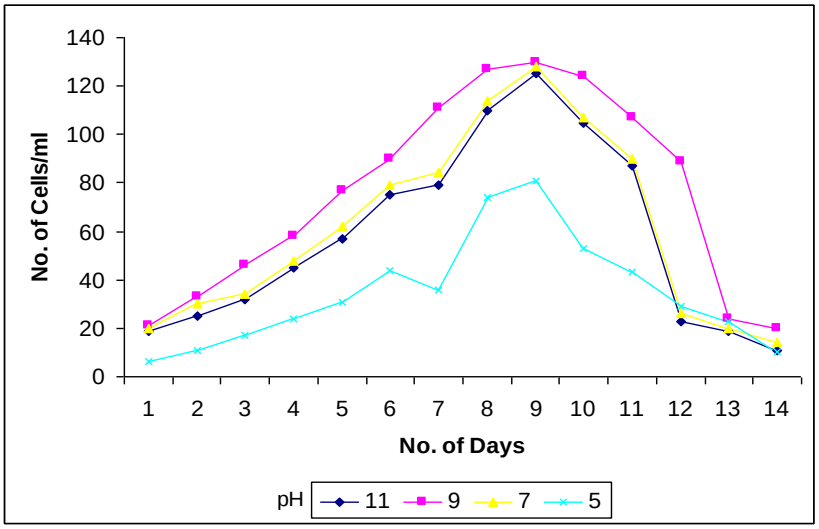

Fig. 3: Growth characterization of *Tetraselmis suecica* at various pH.

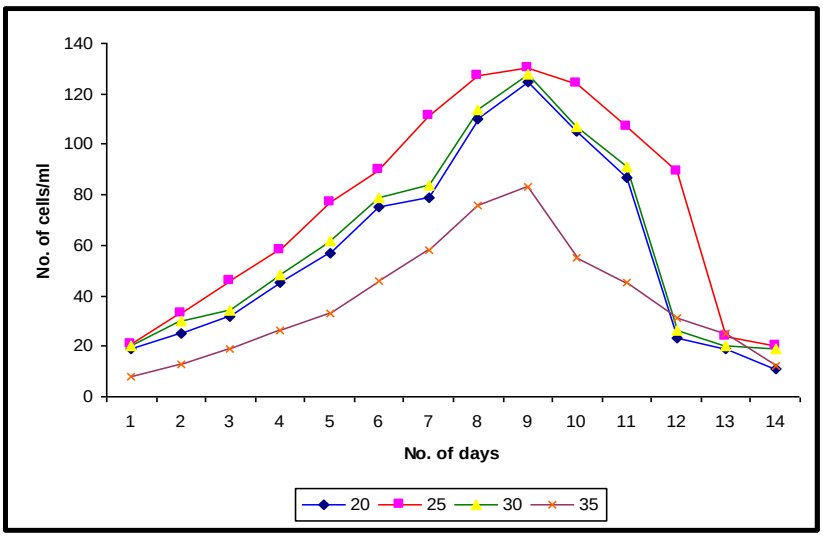

Fig. 4: Growth characterization of *Tetraselmis suecica* at various temperatures (°C)

Antibacterial assay

Algal extracts were prepared using different organic solvents for antimicrobial assay by disk diffusion method. Antibacterial activity of crude extract is portrayed in Table 1. Among the solvents used, chloroform + methanol (1:1) crude extract of *Tetraselmis suecica* exhibited maximum zone of inhibition (13.8) against *Proteus* sp is showed in Fig. 5. However Butanol + Isopropanol (1:1) solvent extract showed minimum zone of inhibition (2.7) against *Salmonella sp.* The highest inhibition zone was observed in chloroform+ methanol (1:1) extract of *Tetraselmis suecica* against gram negative bacteria *Proteus* sp (13.8) and gram positive bacteria *Streptococcus pyogens* (11.8) and Isopropanol extract against *Bacillus megaterium* (11.8) respectively. Two-way ANOVA was performed on the data of antibacterial activity of bioactive substance extracted from *Tetraselmis suecica* using different organic solvents and their combinations against selected human pathogens. Variation due to bacteria P-value was > 0.05 is statistically non-significant. Variation due to solvent based extracts P-value was < 0.01 is statistically significant is depicted in Table 2.

Table 1: Antimicrobial activity of bioactive substance extracted from *Tetraselmis suecica*

Solvent used	Zone of inhibition (mm)										
	Control	*Vibrio cholerae*	*Klebsiella pneumoniae*	*Escherichia coli*	*Staphylococcus aureus*	*Bacillus megaterium*	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Salmonella sp.*	*Proteus sp.*	*Streptococcus pyogens*
Acetone	-	9.6 ± 1.14	10.6 ± 1.14	9 ± 0.70	9.4 ± 1.14	10.6 ± 0.54	9.8 ± 1.30	8.2 ± 0.83	8.6 ± 1.51	10 ± 0.70	10.8 ± 1.09
n-butanol	-	11.2 ± 1.48	12.2 ± 1.30	9.8 ± 0.44	8.6 ± 0.54	9.8 ± 0.83	10.8 ± 2.04	10.4 ± 0.89	9.2 ± 0.44	11.2 ± 0.83	9.6 ± 0.54
Isopropanol	-	5.2 ± 0.38	4.8 ± 0.26	3.9 ± 0.41	10.6 ± 0.54	11.8 ± 0.83	2.6 ± 0.61	9.6 ± 0.54	9.2 ± 0.83	9.2 ± 0.44	11.4 ± 1.14
Acetone + n-butanol (1:1)	-	10.6 ± 0.89	10.6 ± 1.14	9.6 ± 0.89	9.4 ± 0.54	11 ± 1	11 ± 0.70	9.2 ± 2.16	8.2 ± 0.83	9.6 ± 2.07	11 ± 0.70
Acetone + Isopropanol (1:1)	-	3.8 ± 0.52	3.1 ± 0.29	9 ± 0.70	3.1 ± 0.32	4.3 ± 0.92	9 ± 0.70	2.6 ± 0.99	3.1 ± 0.72	3.3 ± 0.15	9.4 ± 1.51
Acetone + Chloroform (1:1)	-	10.2 ± 1.30	9.4 ± 1.14	9.4 ± 1.14	9 ± 0.70	9.6 ± 0.89	10.6 ± 0.54	8.8 ± 1.30	9.4 ± 1.51	12 ± 1.87	12.8 ± 2.28
Butanol + Isopropanol (1:1)	-	10.4 ± 0.54	4.2 ± 0.63	3.3 ± 0.36	11.2 ± 1.92	9.4 ± 1.34	2.9 ± 0.72	2.8 ± 0.29	2.7 ± 0.95	2.6 ± 0.33	10.4 ± 1.51
Chloroform + Methanol (1:1)	-	11.6 ± 1.14	3.8 ± 0.61	4.1 ± 0.59	10.6 ± 1.14	10.6 ± 1.14	8.2 ± 1.30	10.4 ± 1.51	13 ± 1.22	13.8 ± 1.64	11.8 ± 2.16

"-"= No activity; each value is the mean ± SD of three individual estimates

Table 2: Two-way ANOVA for the data on antibacterial activity of bioactive substance extracted from *Tetraselmis suecica* using different organic solvents and their combinations against selected human pathogens

Source of Variation	SS	df	MS	F	P-value
Total variance	819.628	79			
Variation due to bacteria	96.0905	9	10.6767	1.73439	> 0.05*
Variation due to solvent based extracts	335.716	7	47.9594	7.79081	< 0.01**
Error variance	387.821	63	6.1559		

* Statistically non-significant; ** statistically significant

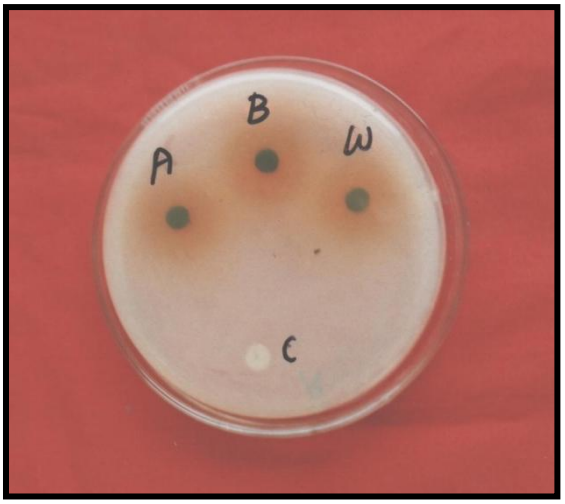

Fig. 5: Photo showing antimicrobial activity of *Tetraselmis suecica* extracts against *Proteus* sp. A - acetone + n-butanol (1:1);B- isopropanol; W- chloroform + methanol(1:1)extracts; C-control

GC – MS analysis

Marine microalgae have the ability to produce a variety of natural products, which are not produced by the terrestrial counterpart. Identification of marine natural product chemistry is a new area of research to develop a newer compound in the field of biomedical and pharmaceutical industries. This research provides a good starting point for the investigation of unique compound from marine microalgae for the treatment of human diseases. The present study was undertaken to identify the antimicrobial compound from the solvent extract of *Tetraselmis suecica* microalgae using GC-MS analysis is illustrated in Fig. 6a. The mass spectra of the compounds were investigated with those similar in the PubChem database and some of our chemical components are reported to have a known biomedical value in the pharmacological fields (data not shown).

The chief constituent of the crude extract of *Tetraselmis suecica* has unique chemical compounds like 1-ethyl butyl 3-hexyl hydroperoxide (MW: 100) is presented in Fig. 6c and methyl heptanate (MW: 186) is presented in Fig. 6e. These metabolites afford a way for future research to pinpoint the chemical constituents that possess the antimicrobial activity.

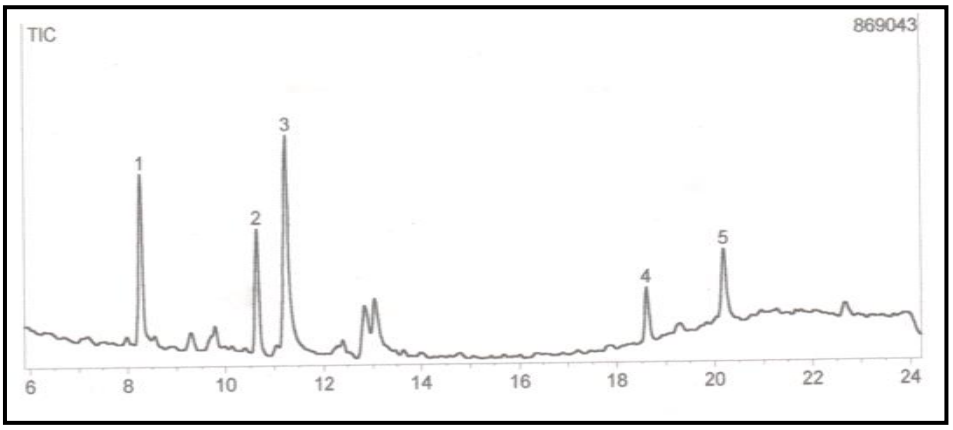

Fig. 6a: GC-MS chromatogram of antimicrobial metabolites produced by *Tetraselmis suecica*

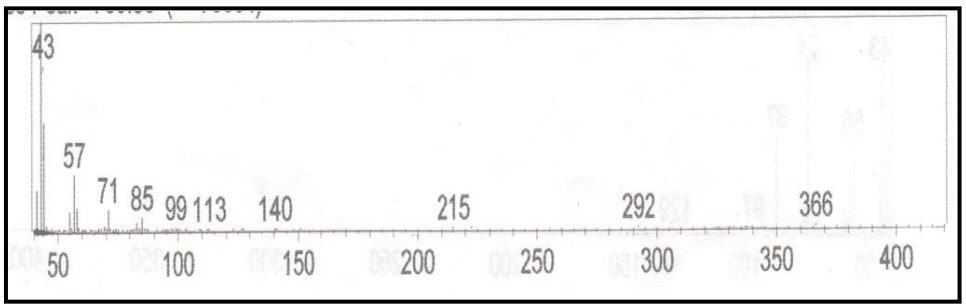

Fig. 6b: Peak separation at the retention time of 7.97; base peak 39.80

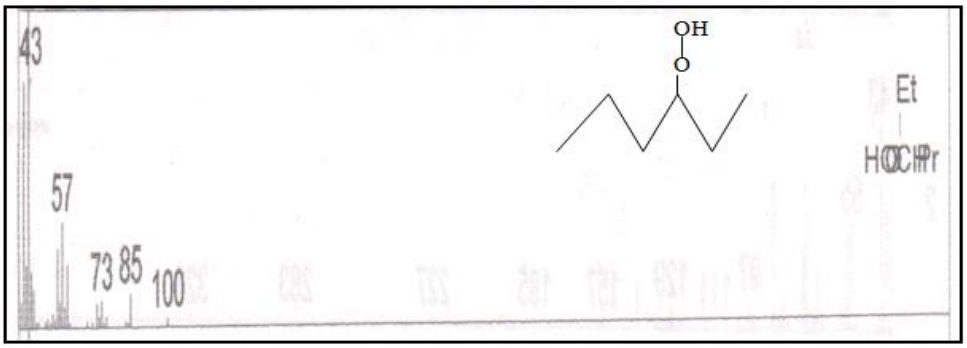

Fig. 6c: 1-ethyl butyl 3-hexyl hydroperoxide (MW: 100)

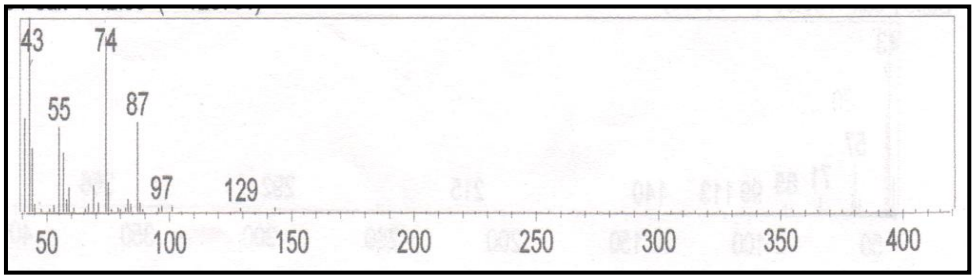

Fig. 6d: Peak separation at the retention time of 10.633; base peak 42.85

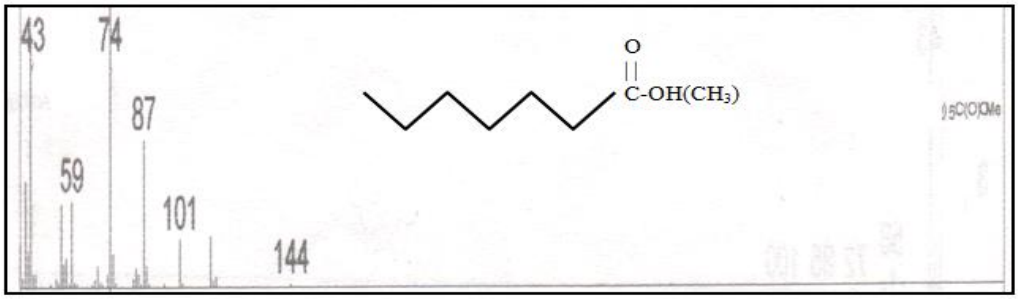

Fig. 6e: Methyl heptanoate (MW:186)

DISCUSSION

Marine environment contain novel and unique structures of the secondary metabolites produced by diverse group of organisms. The researchers have forced to view the marine environment to search novel metabolites for different perspective of biomedical and pharmaceutical industries. Marine microorganisms including bacteria, actinomycetes, fungi and microalgae have received increasing attention during past decade due to constant isolation of previously discovered compounds. Based on the literature survey only a few reports are available concerning the culture condition especially marine microalgae *Tetraselmis suecica*. Maximum growth rate and was observed at 20~30°C for *Tetraselmis chui* [22] and cell densities for *Tetraselmis sp* were obtained at the salinity range 20–35% and at the temperature of 19–21°C than other temperatures tested however pH is not significantly affecting cell density[23]. But in the present studies on *Tetraselmis suecica* were significantly higher at the pH of 9.0 on 9th day incubation this might be due to species variation. Highest growth was observed between a pH of 9 to 9.5 at 7th day incubation for marine microalgae *Chlorella* sp [24]. The earlier research supports our present culture optimization study on *Tetraselmis suecica*.

Antimicrobial activity depends on both algal species and the solvents used for their extraction [25]. In the present investigation different organic solvent extracts of *Tetraselmis suecica* were tested against selected human pathogens. Highest inhibition zone was observed in methanol + chloroform (1:1) extract of *Tetraselmis suecica* against gram negative bacteria *Proteus* sp and gram positive bacteria *Streptococcus pyogens* and Isopropanol extract against *Bacillus megaterium*. The culture supernatants and extracts derived from spray dried preparation of *Tetraselmis suecica* were observed to inhibit both gram positive and gram negative fish pathogens by *in vitro* methods [26]. The present study on crude extract of *Tetraselmis suecica* exhibited inhibition against both gram negative and gram positive selected human pathogens by *in vitro* methods. The crude extract of marine microalgae *Tetraselmis chui*, effectively inhibit the growth of tested *Vibrio* sp. [27]. Makridis et al. [28] also observed that marine microalgae *Tetraselmis chuii* and *Chlorella minutissima* possess antimicrobial activity selectively directed against gram-negative bacteria. Singh and Chaudhary [29] observed that methanolic extract of microalgae *Pithophora oedogonia* revealed strong inhibitory effect in the case of gram positive bacteria. The methanol extract showed more potent antimicrobial activity than dichloromethane petroleum ether; ethyl acetate extracts of *Spirulina platensis* [30]. Mhadhebi et al.[31] reported that the chloroformic and the ethyl acetate extract obtained from marine algae *Cystoseira crinita* and *Cystoseira sedoides* showed a higher antifungal activity against four *Candida* strains. Karabay-Yavasoglu et al. [32] endorsed the present investigation that methanolic and chloroform extracts of marine algae *Jania rubens* had significant antimicrobial activity against gram negative and gram positive bacteria.

GC-MS analysis of crude extract of *Tetraselmis suecica* demonstrated interesting compounds with significant antimicrobial activity. In the present investigation different fatty acids such as Methyl carprate, Methyl stearate, Decoic acid, Palmitic acid, Nonoic acid and Caprylic acid with antimicrobial activity and pharmaceutical importance were identified. Crude extract analysis of the described species using gas chromatography-mass spectrometry (GC-MS) had revealed several important organic volatile compounds as fatty acids and derivatives of methyl ester. It is possible to carry out the screening for bioactive compounds in the microalgae *Synechocystis* sp. [33]. The microalgae produced active extracts in terms of both antioxidant and antimicrobial activity. In the earlier research different fatty acids and volatile compounds with antimicrobial activity were identified, such as phytol, fucosterol, neophytadiene or palmitic, palmitoleic and oleic acids were obtained organic solvent extracts of *Synechocystis* sp. were chemically characterized by GC-MS analysis. Fractionated matrices of *Tetraselmis suecica* crude extract, contained 1-ethyl butyl 3-hexyl hydroperoxide and methyl heptanate which is known to demonstrate valuable therapeutic uses including anti-inflammatory**,** antipsychotics, antiseptic, antineoplastic, anti-allergic, antipyretic and analgesic effects. Interestingly, some of our resultant spectra compounds exhibited important biomedical features. Based on the results obtained, methanol + chloroform (1:1) were selected as the most appropriate solvent to extract bioactive compounds from the natural sources for current clinical and pharmaceutical importance.

CONCLUSION

A further study on these compounds can be finalizing the relation between these metabolites and the antimicrobial properties of *Tetraselmis suecica* microalgae. The GC-MS results indicate the possibilities of extraction, purification and identification of such substances as well as support and encourage the idea for using such active marine microalgae as biocide for controlling some infectious human pathogens. Further utilization of naturally occurring bioactive compounds from marine microalgae could obtain a wide variety of alternatives of manufactured therapeutics. Obviously the present finding opens a new avenue in biomedical and pharmaceutical industries.

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