

## EVALUATION OF BIOACTIVE METABOLITES FROM HALOPHILIC MICROALGAE *DUNALIELLA SALINA* BY GC – MS ANALYSIS

S. KRISHNAKUMAR<sup>1\*</sup> V. DOOSLIN MERCY BAI<sup>2</sup> AND R. ALEXIS RAJAN<sup>3</sup>

<sup>1</sup>Department of Biomedical Engineering, Sathyabama University, Chennai – 600119, Tamil Nadu, <sup>2</sup>Department of Biomedical Engineering, Rajiv Gandhi College of Engineering and Technology, Puducherry - 607402, <sup>3</sup>Center for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam 629501, Tamil Nadu, India. Email: drskrishnakumarphd@gmail.com

Received: 12 July 2013, Revised and Accepted: 22 Aug 2013

### ABSTRACT

**Objective:** In the last decade the screening of secondary metabolites and pharmacologically active compounds from marine microalgae has increased. In the present investigation *Dunaliella salina* has been chosen for the antibacterial metabolites studies.

**Methods:** Marine microalgae *Dunaliella salina* (green algae) was selected for the present secondary metabolites investigation. The effects of pH, temperature and salinity were tested for the growth of microalgae. The antibacterial effect of different solvent extracts of *Dunaliella salina* against selected human pathogens such as *Vibrio cholerae*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella sp*, *Proteus sp*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Bacillus megaterium* and *Bacillus subtilis* were examined.

**Results:** The uppermost cell growth was observed when the medium adjusted with pH of 9.0 in 40ppt of salinity at 25°C during 9<sup>th</sup> day of incubation. Among the solvents used, chloroform + methanol (1:1) crude extract of *Dunaliella salina* exhibited maximum zone of inhibition (10.4 mm) against *Vibrio cholerae*. Methanol + chloroform (1:1) crude extract confirmed considerable activity against gram negative bacteria than gram positive pathogen. GC-MS analysis revealed that, the presence of unique chemical compounds like 3, 3, 5-Trimethylheptane (M.W. 142.2) and n-Hexadecane (M.W. 226.2) respectively for the crude extract of *Dunaliella salina*.

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

**Keywords:** Microalgae, *Dunaliella salina*, Secondary metabolites, GC-MS analysis.

### INTRODUCTION

More than 70% of the Earth surface is covered with water, in which the most dominant group of living organisms is algae. Microalgae play a key role in the productivity of oceans. Marine organisms produce pharmacologically important diverse group of natural products [1, 2] that include algae, which produce novel and unexplored sources of potentially useful bioactive compounds that might represent useful leads in the development of new pharmaceutical agents [3]. Biologically active compounds from natural resources have always been of great interest to scientists working on different diseases [4]. Algae have been used in traditional medicine for a long time and also some algae have bacteriostatic, bactericidal, antifungal, antiviral and antitumor activity [5]. Microalgae are rich source of structurally novel and biologically active metabolites. So it has been studied as potential bioactive compounds of interests in the pharmaceutical industry [6, 7]. This group is extremely diverse and it constitutes a rich source of bioactive ingredients, such as vitamins [8], pigments, fatty acids, sterols and polysaccharides [9, 10]. Recurrent use of chemotherapeutic drugs and delay in adequate therapy has developed resistance of pathogens and cause some undesirable side effects and potentially increased mortality [11]. These limitations demand improved pharmacokinetics properties, while demand continued to researchers for new antimicrobial compounds from unexplored habitat for the development of novel drugs for already existing pathogens [12]. Hence the present study has paid attention on the potential applications of marine microalgae *Dunaliella salina* particularly for the treatment of human pathogenic microorganisms, which can be used as the alternative source for the commonly used dormant chemotherapeutic agents.

*Dunaliella salina* is a motile unicellular halotolerant green alga belonging to the class Chlorophyta and family Polyblepharidaceae most frequently found naturally in habitats like salt marshes [13]. *Dunaliella* sp. produce a biomolecule of β-carotene which is used in the food [14], cosmetic, pharmaceutical industries as a coloring agent, antioxidant [15], anti-tumor agent [16], and heart disease preventive [17].

A wide range of pharmacologically active substance has been observed with different organic solvent extracts of microalgae. Several of the bioactive compounds found their application in human diseases and others as structural models for the development of new drugs. The antimicrobial activity of microalgae extracts is generally assayed using various organic solvents [18]. An organic solvent always provides a higher efficiency in extracting compounds for antimicrobial activity as compared to aqueous extract [19, 20]. Screening of organic solvent extracts from microalgae and other marine organism is a common approach to identify compounds of biomedical importance. In this context, an effort has been initiated to evaluate the efficiency of various organic solvents, antimicrobial activity and identify the chemical constituents and structure by GC-MS analysis of crude marine microalgae extracts against the most common human pathogenic bacteria.

### MATERIALS AND METHODS

#### Microalgae culture collection

Marine microalgae *Dunaliella salina* (Kingdom: Plantae; Phylum: Chlorophyta; Class: Chlorophyceae; Order: Volvocales; Family: Dunaliellaceae; Genus: *Dunaliella*; Species: *D. salina*) was collected from Centre for Marine Fisheries Research Institute (CMFRI) Tuticorin, Tamilnadu, India in a sterile screw cap tube which was kept in a ice chest box and brought to our laboratory. The microalgae were sub-cultured and maintained as a pure culture was chosen for the present investigation.

#### Stock culture maintenance

Filtered sea water (100 ml) was taken into 250 ml of conical flask and a required nutrient of Miuell'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: 2 ml; distilled water: 100 ml) was dissolved. Solution A (0.55 ml) and solution B (0.5 ml) were added to one liter of filtered sterilized seawater and mixed meticulously to enrich the water and autoclaved. After sterilization 10% of actively growing

mid phase inoculum was transferred into culture flask aseptically. The inoculated flask was incubated at  $28\pm2^{\circ}\text{C}$  underneath the fluorescent light of 1000 lux for 8 days. When the maximum exponential growth phase was reached, the light was reduced for further growth.

### Chemicals

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

### Growth optimization of marine microalgae

The Miquell's medium (100 ml) 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  $35^{\circ}\text{C}$ ) and salinity (20, 30 and 40ppt) were optimized independently. The salinity was authenticated with the help of 300 PX- Refractometer (300 X 225-traditional hand land). Then 10 ml of actively growing log phase inoculum was transferred to the culture flask aseptically and reserved under the fluorescent light of 1000 lux for 14 days.

### Determination of cell density

The determination of cell density was performed by the method given by James and Al-Khars [21]. Cell counts were examined using a Neubauer improved Haemocytometer (DHC-N01). The microalgae were treated with formalin to kill the cells and one drop of the culture was taken with the help sterile Pasteur pipette. After placing the cover slip on the haemocytometer, the pipetted culture samples were poured on the counting grid of the haemocytometer and left for a few minutes. The cells were counted with the aid of compound microscope (ADELTA OPTEC – DN10) under the magnification of 40X and the total cell count was calculated by the following formula.

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

### Microalgae extract preparation using different organic solvents

The microalgae cells were centrifuged (REMI-R24) at 200 rpm for 10 minutes. The pellet was collected and air dried under room temperature to get a fine powder. Dried microalgae cells of 10g were extracted in 100ml of different organic solvents specifically 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 continuous stirring of 50rpm for 7 days at room temperature. The solution was filtered through Whatman No.1 filter paper. Then the filtrate was dried using desiccator (Vacuum Dry - seal Desiccator 12") at  $40^{\circ}\text{C}$  for 24h. The dried powder was suspended with respective solvents to give 50mg/ml of crude extract. The crude extract was kept in sealed container and stored in a refrigerator for further antimicrobial and GC-MS studies.

### Human 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 pyogenes*, *Staphylococcus aureus*, *Bacillus megaterium* and *Bacillus subtilis* collected from Kanyakumari Medical College and Hospital (KMCH), Kanyakumari District, Tamilnadu, India and maintained in our laboratory was chosen for the present antibacterial susceptibility study.

### Antibacterial assay

Antibacterial activity was determined against the chosen human pathogens using paper disk assay (PDA) method described by El Masry et al. [22]. Whatman No.1 filter paper disk of 6mm diameter was incised and sterilized by autoclaving. The sterile disk was saturated with different solvent extracts (50 $\mu\text{l}$ /per disk). Control disk was also sustained for each extract by impregnating respective organic solvent alone. Muller Hinton Agar (MHA) plates were prepared and overnight broth culture ( $1.2\times10^8$  cfu/ml) of test pathogens were inoculated uniformly using sterile cotton swab. The impregnated disks were placed on the plates using sterile forceps

properly spaced at equal distance. Triplicates were maintained for each test pathogen. The plates were incubated at  $37^{\circ}\text{C}$  for 24h. The zone of inhibition was measured and expressed in mm in diameter.

### GC-MS analysis of microalgae extract

The gas chromatography combined with mass spectrometry detection technique is a qualitative and quantitative analysis of the crude extracts with high sensitivity even with trace amount of constituents. Identification of the chemical moiety of crude extracts of *Dunaliella salina* which showed valuable antibacterial activities against the selected human pathogens was analyzed. The GC-MS analysis was done by standard specification by dissolving 10mg of crude extracts in one milliliter of ethyl acetate. The aliquot of 0.1  $\mu\text{l}$  was injected automatically into 0.25 mm x 25 m column of GC-MS model (GC 17A, Japan) 5% phenyl poly siloxane as stationary phase. Helium was used as a carrier gas at 17.69 psi pressure with the flow of 3ml/min at the flow rate of 0.4m/min. The temperature gradient program was implemented for the evaporation of organic solvent to identify the chemical constituent. The initial temperature was  $70^{\circ}\text{C}$  and gradually accelerated to  $250^{\circ}\text{C}$  at a rate of  $10^{\circ}\text{C}$  per minute. The sample was injected after 18 minutes at  $250^{\circ}\text{C}$ . 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 [23]. The concentration of such compound was calculated by the following formula:

$$\text{Compound concentration percentage} = [\text{P1}/\text{P2}] \times 100$$

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 [24].

## RESULTS

### Microalgae Culture conditions

Marine halophilic microalgae posses the flourishing source of bioactive compounds to compete the harmful pathogens. Culture 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 altering the diverse culture conditions to the microalgae. Optimum culture conditions relative to temperatures, pH and salinities levels were adopted for marine microalgae *D. salina*. The growth characteristic of microalgae at various temperatures is shown in Fig. 1. Maximum cell growth of microalgae was recorded at  $25^{\circ}\text{C}$  and minimum growth at  $35^{\circ}\text{C}$  on 9<sup>th</sup> day of incubation. The cell growth of microalgae at different pH is depicted in Fig. 2. Maximum cell growth was observed at the pH of 9.0 and minimum growth was recorded at the pH of 5.0 on 9<sup>th</sup> day of incubation. The microalgae cell growth rate was studied at different salinities such as 20, 30 and 40 ppt concentration. Algal cell growth at various salinity is presented in Fig. 3. Minimum growth was recorded at 20 ppt and maximum cell growth was observed at 40 ppt on 9<sup>th</sup> day of incubation after that gradually declined. These results confirmed that the microalgae *Dunaliella salina* belongs to halophytes. Logarithmic increase in the cell count of microalgae was observed from first day to 8<sup>th</sup> day with the maximum value on 9<sup>th</sup> day of incubation after which there was a gradual decline in almost all the culture conditions.

### Antibacterial assay

Microalgae extracts were prepared using different organic solvents for antibacterial assay by paper disk assay method. Antibacterial activity of crude extract is represented in Table 1. Among the solvents used, chloroform + methanol (1:1) extract of *Dunaliella salina* exhibiting maximum zone of inhibition (10.4mm) against *Vibrio cholerae* is shown in Fig. 4. However Isopropanol

solvent extract showed minimum zone of inhibition (2.0mm) against *Proteus* sp. The highest inhibition zone was observed in chloroform+ methanol (1:1) extract of *Dunaliella salina* against gram negative bacteria *Vibrio cholerae* (10.4mm) and gram positive bacteria *Staphylococcus aureus* (10.0mm) and *gram* positive bacteria *Streptococcus pyogenes* (10.0mm) and Acetone + Chloroform (1:1) extract against *Streptococcus pyogenes* (10.0mm) respectively. Two-

way ANOVA was executed on the data of antibacterial activity of bioactive substance extracted from *Dunaliella salina* using different organic solvents and their combinations against selected human pathogens is depicted in Table 2. Variation due to bacterial pathogens P-value was > 0.05 is statistically non-significant. Variation due to organic solvent based extracts P-value was < 0.05 is statistically significant.

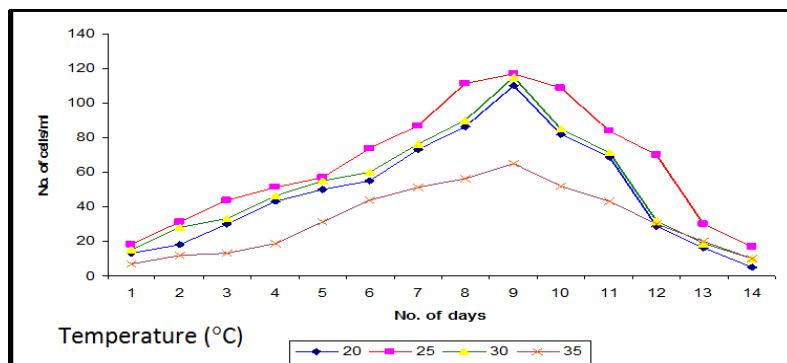


Fig. 1: Growth characterization of *D. salina* at various temperature (°C)

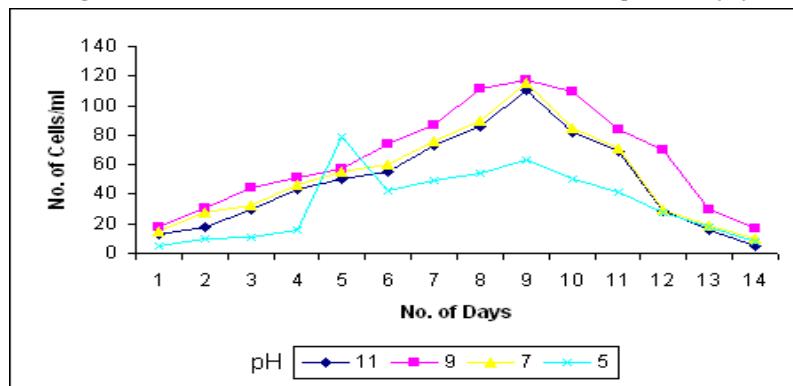


Fig. 2: Growth characterization of *D. salina* at various pH

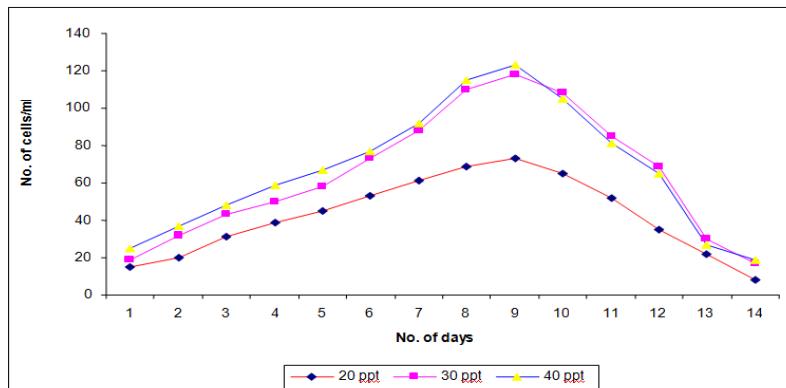


Fig. 3: Growth characterization of *D. salina* at various salinity

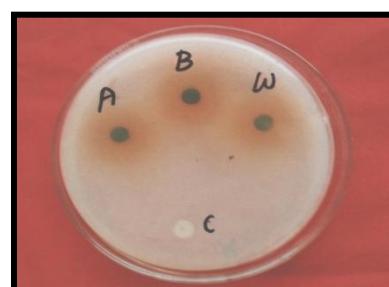


Fig. 4: Antimicrobial activity of different solvent extracts of *D. salina* against *Vibrio cholerae*

A- acetone + n-butanol (1:1); B - isopropanol; W - chloroform + methanol; C - control

**Table 1: Antimicrobial activity of bioactive substance extracted from *D. salina***

Solvent used	Zone of inhibition (mm)										
	Control	<i>Vibrio cholerae</i>	<i>Klebsiella pneumonia</i>	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>	<i>Bacillus megaterium</i>	<i>Pseudomonas aeruginosa</i>	<i>Bacillus subtilis</i>	<i>Salmonella sp.</i>	<i>Proteus sp.</i>	<i>Streptococcus pyogenes</i>
Acetone	-	3.8 + 0.65	9 ± 1.58	9 ± 0.70	4.0 + 0.72	2.9 + 0.65	2.8 + 0.45	3.5 + 0.81	9 ± 0.70	9 ± 0.70	9 ± 0.70
n-butanol	-	9.4 ± 1.14	2.9 + 0.53	8.6 ± 0.54	8.6 ± 0.54	8.2 ± 0.83	8.4 ± 1.34	7.8 ± 0.83	8.8 ± 0.83	9.4 ± 1.14	10 ± 0.70
Isopropanol	-	2.7 + 1.34	9.6 ± 1.14	3.7 + 0.83	4.4 + 0.85	8.8 ± 0.83	4.1 + 0.71	9 ± 1.07	10 ± 1.22	2.0 + 0.55	3.6 + 0.63
Acetone + n-butanol (1:1)	-	9.6 ± 0.54	4.3 + 0.72	8.4 ± 1.14	7.8 ± 0.83	8.8 ± 0.83	8.6 ± 0.54	8.8 ± 0.44	8.8 ± 0.44	8.2 ± 0.44	9.8 ± 0.83
Acetone + Isopropanol (1:1)	-	3.8 + 0.38	3.9 + 0.85	10 ± 0.70	9.6 ± 0.89	9.4 ± 1.14	4.7 + 0.85	5.5 + 1.03	5.8 + 0.33	9.6 ± 0.89	5.1 + 0.41
Acetone + Chloroform (1:1)	-	4.3 + 0.65	5.5 + 0.54	6.7 + 0.89	10 ± 0.81	9.6 ± 1.34	9.2 ± 1.48	8.4 ± 0.54	5.6 + 0.25	6.1 + 0.51	10 ± 0.70
Butanol + Isopropanol (1:1)	-	9 ± 0.70	8.4 ± 0.54	8.8 ± 0.83	10 ± 1	5.1 + 0.72	10.2 ± 0.83	9.6 ± 1.14	9.4 ± 1.14	9.2 ± 0.44	9.6 ± 1.34
Chloroform + Methanol (1:1)	-	10.4 ± 1.14	9.6 ± 0.54	9.4 ± 0.54	10 ± 0.70	9.6 ± 0.89	10 ± 1.22	8.6 ± 1.34	9.8 ± 0.83	10 ± 0.70	10 ± 0.70

"-" 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 *D. salina* using different organic solvents and their combinations against selected human pathogens**

Source of Variation	SS	df	MS	F	P-value
Total variance	484.185	79			
Variation due to bacteria	30.2636	9	3.36262	0.65984	> 0.05*
Variation due to solvent based extracts	132.864	7	18.9806	3.72449	< 0.05**
Error variance	321.057	63	5.09615		

\*Statistically non-significant; \*\* Statistically significant

#### GC - MS analysis

Marine microalgae have the ability to produce a variety of natural products due to adverse environmental habitat, which are not produced by the terrestrial counterpart. Identification of marine natural product chemistry is a current scenario of research to develop a unique compound in the field of biomedical and pharmaceutical industries. This research provides an exceptional opening for the investigation of novel compound from halophilic microalgae for the treatment of human diseases. The present investigation was undertaken to discover the antibacterial compound from the organic solvent extract of *Dunaliella salina*

using GC-MS analysis is illustrated in Fig 5a. The number of compounds (peaks) reported in the crude extract is portrayed in Table 3. 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 *Dunaliella salina* having unique chemical compounds namely 3, 3, 5-Trimethylheptane (M.W. 142.2) is presented in Fig 5c and n-Hexadecane (M.W. 226.2) is presented in Fig 5e. These secondary metabolites pay for a new avenue for future research to pinpoint the chemical constituents that possess antimicrobial activity.

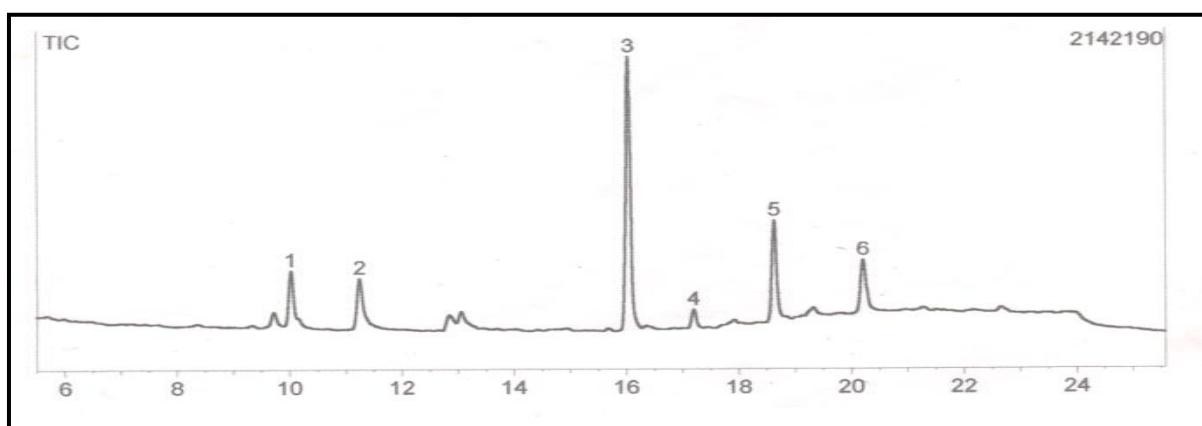


Fig. 5a: Detection of mixed secondary metabolites produced by *D. salina* using GC-MS analysis

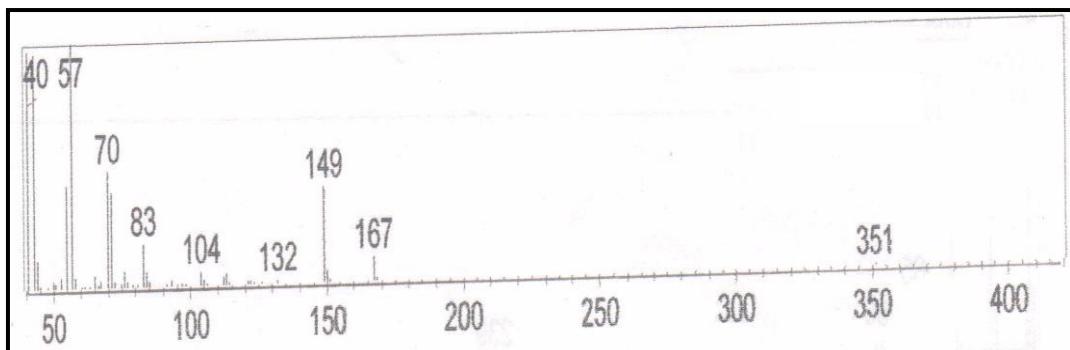


Fig. 5b: Peak separation at the retention time of 16.033; base peak 56.85

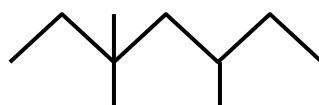
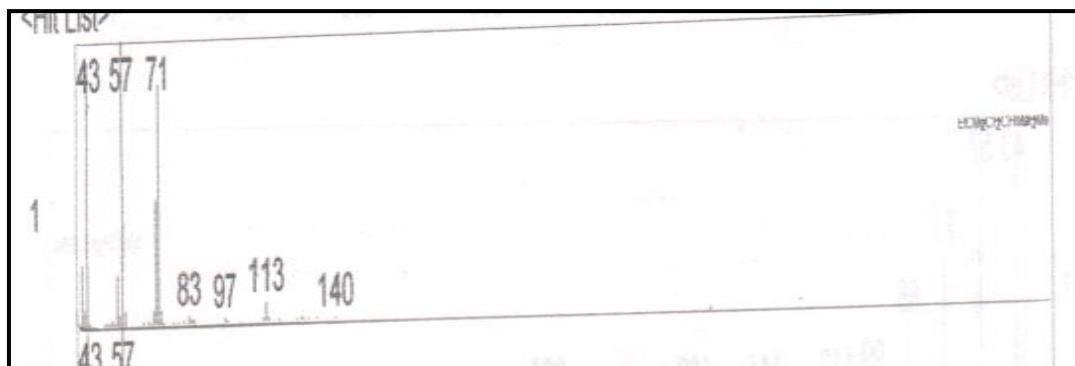


Fig. 5c: 3, 3, 5-Trimethylheptane (M.W. 142.2)

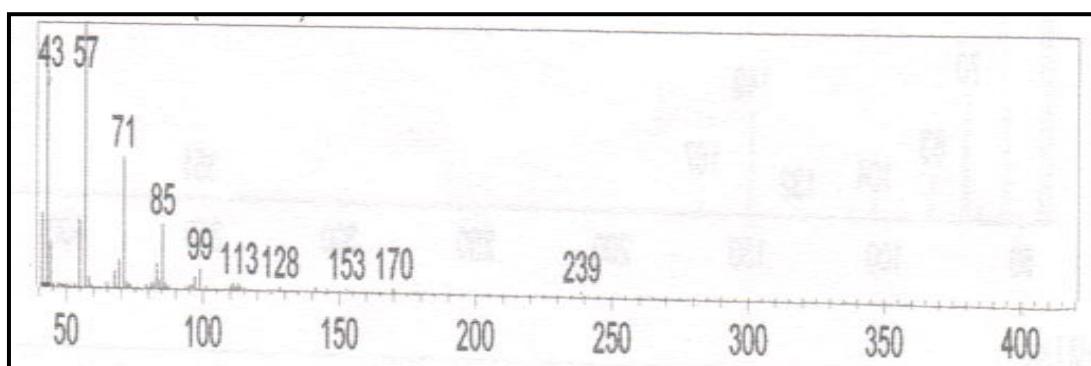


Fig. 5d: Peak separation at the retention time of 18.625; base peak 56.90

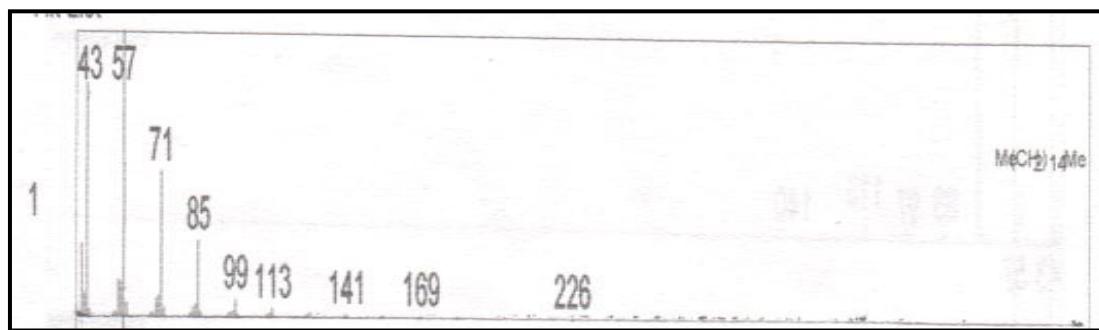


Fig. 5e: n-Hexadecane (M.W. 226.2)

**Table 3: Number of compounds (peaks) reported by GC-MS analysis for the extract of *D.salina***

PK. No.	R. Time	I. Time	F. time	Area	Height	A/H(sec)	MK % Total Name
1.	10.022	9.908	10.150	1762104	321584	5.479	8.53
2.	11.247	11.125	11.442	2163552	302440	7.154	10.48
3.	16.030	15.883	16.258	10265722	1715669	5.984	49.70
4.	17.198	17.108	17.317	615967	111829	5.508	2.98
5.	18.625	18.500	18.783	36785845	623512	5.895	17.80
6.	20.204	20.075	20.367	2170245	320063	6.781	10.51

## DISCUSSION

The production of microalgal bioactive metabolites requires large quantities of algal biomass. The optimization procedure should be done by testing the best strains and the most effective strategies under optimal conditions. Several cultivation technologies that are used for high production of microalgal biomass have been developed by researchers and commercial producers. In the present study an attempt has been made to optimize the culture condition in order to get huge quantities of microalgae biomass to meet our demand. The chemical composition of several microalgae is influenced by culture conditions like temperature, pH, salinity and other micronutrients and macronutrients. It has been well documented by the earlier researcher [25, 26, 27, 28]. Abu-Rezq et al. [29] endorsed that the growth pattern of *D. salina* (Kuwaiti and Australian) cultured at different temperatures achieving growth rates of up to  $2.90 \times 10^6$  and  $2.40 \times 10^6$  cells ml-1, respectively. The growth pattern in both samples decreased with increasing temperature. This indicates that *D. salina* prefers low temperatures ( $20^\circ\text{C}$ ) rather than high temperatures ( $32^\circ\text{C}$ ). Garcia-Gonzalez et al. [30] achieved highest production range of 2 to  $4 \times 10^6$  cells ml-1 of *D. salina* in outdoor cultures in a closed tubular system. They found that the maximum culture performance of *D. salina* at a temperature of  $25^\circ\text{C}$  with pH of  $7.5 \pm 0.5$ , controlled by means of addition of  $\text{CO}_2$  gas. Cifuentes et al. [31] studied growth pattern and carotenogenesis in different strains of *D. salina* under different experimental temperature. They reported that using a temperature of  $20 \pm 4^\circ\text{C}$  under a 12:12 (light and dark phases) photoperiod recorded maximum growth rate and carotene production. These finding corroborate with our present investigation on *D. salina* at a temperature of  $20^\circ\text{C}$  for highest biomass and its secondary metabolites production on 9<sup>th</sup> day of incubation under experimental condition. On the other hand, Singh et al. [32] suggested that fixing of water temperature at  $30^\circ\text{C}$  showed maximum cell growth induction on *D. salina* under experimental condition on limiting nutrients.

The growth pattern of microalgae culture media at different pH was investigated. The algal growth was increased with increasing pH. Microalgae *D. salina* demonstrates that highest cell growth was observed at the pH of 9.0 rather than the pH of 5.0 on 9<sup>th</sup> day of the experimental period. Zhao et al. [33] confirmed that maximum growth was observed between a pH of 9 to 9.5 at 7<sup>th</sup> day incubation for marine microalgae *Chlorella* sp. The earlier research supports our present culture optimization study on *D. salina*.

The growth rate was increased with increasing salinity (40 ppt) rather than at low salinity (20 ppt) on *D. salina* in the present investigation under experimental condition. These results agreed with earlier research obtained by Dolapsakis et al. [34]. Oren [35], who found that desired optimal growth of *D. salina* could be achieved along the seashore or close to salt lagoons and salt-producing industries with increased salt concentration during natural season. *D. salina* is able to tolerate varying NaCl concentrations, ranging from 0.2% to approximately 35% observed by Farahat et al. [36]. Hadi et al. [37] reported the growth of *D. salina* in culture media containing different NaCl concentrations. The microalgae can grow in a media containing an extremely wide range of salt concentration from 0.17 M to 4.0 M NaCl. Raja et al. [38] reported that maximum cell number of *D. salina* was recorded when the media amended with 4.0M NaCl on the 18<sup>th</sup> day of incubation. However 3.5M of NaCl favored increased accumulation of  $\beta$ -carotene [39]. In contrast, Leach et al. [40] concluded that it was possible to obtain a cell concentration of  $0.8 \times 10^6$  cells ml-1 when the culture was maintained at a salinity of 18% NaCl w/w with a pH of 8.5. Thus,

*D. salina* is a hyper-halotolerant organism found in high densities in saline lakes. It has adapted to survive in high salinity environments by accumulating glycerol to balance osmotic pressure.

In recent years, antibacterial metabolites extraction from algae has attracted the most attention among other possible sources. The antimicrobial activity of microalgae has been attributed to compounds belonging to several chemical classes - including indoles, terpenes, acetogenins, phenols, fatty acids and volatile halogenated hydrocarbons [41, 42]. Antimicrobial activity depends on both algal species and the solvents used for their extraction [43]. The antimicrobial activity of algae extracts is generally assayed using various organic solvents, such as acetone, ether and chloroform, methanol [44]. An organic solvent always provides a higher efficiency in extracting compounds for antimicrobial activity [45]. However, the antimicrobial activity detected in several pressurized extracts from *D. salina* may be explained not only by several fatty acids, but also by such compounds as cycloclinal, neophytadiene and phytol [46]. The methanol extract showed more potent antimicrobial activity than dichloromethane, petroleum ether and ethyl acetate extracts of *Spirulina platensis* [47]. Karabay-Yavasoglu et al. [48] 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. These findings correlated with our present observation of the combination of methanol with chloroform (1:1) extract showed promising antibacterial activity against gram negative and gram positive bacteria, as shown in Table 1. In contrast chloroform and ethyl acetate extract obtained from marine algae *Cystoseira crinita* and *Cystoseira sedoides* showed a higher antifungal activity Mhadhebi et al. [49].

GC-MS analysis of crude extract of *D. salina* demonstrated interesting compounds with significant antimicrobial activity. In the present investigation, 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, Cetane, Isotetradecane, Tetradecyl iodine, n-pentadecane, Tridecane, n-Octasane, n-Dotriacontane, n-Nonacosane, n-Heptacosane, n-Pentadecane 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 and its derivatives. The microalgae produce active extracts in terms of both antioxidant and antimicrobial activity. In the earlier research different fatty acids and volatile compounds such as phytol, fucosterol, neophytadiene or palmitic, palmitoleic and oleic acids, with antimicrobial activity were identified, which were obtained by the organic solvent extracts of *Synechocystis* sp. chemically characterized by GC-MS analysis [50]. Al-Wathnani [51] identified that cyanobacteria and green algae have been found potential for the production of several compounds including biomedically important organic metabolites such as 3-Methyl-2-(2-Oxopropyl) Furan, ethane1,1-diethoxy butanal, heptanal and octanal by GC-MS analysis. Dooslin Mercy Bai and Krishnakumar [52] noticed that GC-MS analysis 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. The fractionated matrices of *D. salina* extract contained leading chemical compounds namely 3, 3, 5-Trimethylheptane and n-Hexadecane which has pharmaceutical importance. Interestingly, some of our resultant chromatogram compounds exhibited significant biomedical features. In this study, based on the results

obtained, methanol + chloroform (1:1) preferred as the most suitable organic solvent to extract bioactive compounds from marine microalgae for current biomedical and pharmaceutical importance.

## CONCLUSION

The following optimum culturing conditions such as salinity of 40ppt, temperature of 20°C and a high pH of 9.0 on 9<sup>th</sup> day of incubation period were maintained to get a maximum algal biomass of *D. salina* for the possible methods for bioactive metabolites extraction. Further research is required regarding exact chemical constituent responsible for the biocidal activity and its clinical trial for human therapeutic applications. This research could open an interesting new facet of microalgal biotechnology in future. The production of new antibiotic substances and production of biofuels will make *D. salina* a main topic for many future microalgal investigations.

## ACKNOWLEDGEMENT

The authors are grateful to Sathyabama University, Department of Biomedical Engineering, Chennai, Tamil Nadu, India and Rajiv Gandhi College of Engineering and Technology, Department of Biomedical Engineering, Puducherry, India for providing all the needed facilities complete this work successfully. Our exceptional thanks to Dr.A.Kumaresan, Professor and Head, ICAR centre, Sri Parasakthi College for women, Courtallam, Tamilnadu, India for the valuable guidance and constant support.

## REFERENCES

- Ravikumar S, Krishnakumar S, Jacob Inbaneson S, Gnanadesigan M Antagonistic activity of marine actinomycetes from Arabian Sea coast. Archives of Applied Science Research. 2010; 2(6):273-280.
- Krishnakumar S, Premkumar J, Alexis Rajan R, Ravikumar S Optimization of potential antibiotic production by salt-tolerant actinomycetes *Streptomyces* sp. - MSU29 isolated from marine sponge. International J on Applied Bioengineering. 2011; 5(2):12-17.
- Iwamoto C, Yamada T, Ito Y, Minoura K, Numata A Cytotoxic cytochalasans from a *Penicillium* species separated from a marine alga. Tetrahedron. 2001; 57: 2904-2997.
- Hemraj Upmanyu N, Gupta A, Jindal A, Jalhan S Pharmacological activities of *Stephania glabra*, *Woodfordia fruticosa* and *Cissampelos pareira* - A review. International J of Pharmacy and Pharmaceutical Sciences. 2012; 4(3): 16-23.
- Justo GZ, Silva MR, Queiroz MLS Effects of green algae *Chlorella vulgaris* on the response of the host hematopoietic system to intraperitoneal Ehrlich ascites tumour transplantation in mice. Immunopharmacology. 2001; 123:199-131.
- Rangaiah SG, Lakshmi P, Manjula E Antimicrobial activity of sea weeds *Gracilaria*, *Padina* and *Sargassum* sp. on clinical and phytopathogens. Int J Chem Anal Sci. 2010; 1:114-117.
- Ely R, Supriya T, Naik CG Antimicrobial activity of marine organisms collected off the coast of Southeast India. J Exp Mar Biol Ecol. 2004; 309:121-127.
- Ghoshal D, Mach D, Agarwal M, Goyal A, Goyal A Osmoregulatory isoform of dihydroxyacetone phosphate reductase from *Dunaliella tertiolecta*: Purification and characterization. Protein Expr Purification. 2002; 24: 404-411.
- Laurienzo P Marine polysaccharides in pharmaceutical applications: an overview. Marine Drugs. 2010; 8:2435-2465.
- Pushpamali WA, Nikapitiya C, Zoysa MD, Whang I, Kim SJ, Lee J Isolation and purification of an anticoagulant from fermented red seaweed Lomentariacatenata. Carbohydrate Polymers. 2008; 73: 274-279.
- Kumar A, Roberts D, Wood KE et al. Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. Critical Care Med. 2006; 34: 1589-96.
- Al-Haj NA, Mashan NI, Shamudin MN Antibacterial activity in marine algae *Eucheuma denticulatum* against *Staphylococcus aureus* and *Streptococcus pyogenes*. Res J Biol Sci. 2009; 4(4): 519-524.
- Garcia F, Freile-Pelegrin Y, Robledo D Physiological characterization of *Dunaliella* sp. (Chlorophyta, Volvocales) from Yucatan, Mexico. Bioresour Technol. 2007; 98: 1359-1365.
- Dufosse L, Galaup P, Yaron A, Arad SM, Blanc P, Chidambaram Murthy KN, Ravishankar GA Microorganisms and microalgae as sources of pigments for food use: a scientific oddity or an industrial reality? Trends Food Sci Technol. 2005; 16: 389-409.
- Chidambaram Murthy KN, Vanitha A, Rajesha J, Mahadeva Swamy M, Sowmya PR, Ravishankar GA In vivo antioxidant activity of carotenoids from *Dunaliella salina* - a green microalga. Life Sci. 2005; 76: 1381-1390.
- Ben-Amotz A, Avron M The biotechnology of cultivating the halotolerant alga *Dunaliella*. Trends Biotechnol. 1990; 8: 121-126.
- Tornwall ME, Virtamo J, Korhonen PA, Virtanen MJ, Taylor PR, Albanes D, Huttunen JK Effect of α-tocopherol and β-carotene supplementation on coronary heart disease during the 6-year post-trial follow-up in the ATBC study. Eur Heart J. 2004; 25: 1171-1178.
- Cordeiro RA, Gomes VM, Carvalho AFU, Melo VMM Effect of Proteins from the Red Seaweed *Hypnea musciformis* (Wulfen) Lamouroux on the Growth of Human Pathogen Yeasts. Brazilian Arch Biol Technol. 2006; 49(6): 915-921.
- Masuda M, Abe T, Sato S, Suzuki T, Suzuki M Diversity of halogenated secondary metabolites in the red alga *Laurencia nipponica* (Rhodomelaceae, Ceramiales). J of Phycology. 1997; 33: 196-208.
- Lima-Filho JVM, Carvalho AFFU, Freitas SM, Melo VMM Antibacterial activity of extracts of six macroalgae from the northeastern Brazilian coast. Brazilian J of Microbiology. 2002; 33: 311-313.
- James CM, Al-Khars AM Intensive continuous culture system using tubular photo-bioreactor for producing microalgae. Aquaculture. 1990; 87(3-4): 381-393.
- El-Masry HA, Fahmy HH, Abdelwahed ASH Synthesis and Antimicrobial activity of some new benzimidazole derivatives. Molecules. 2000; 5: 1429-1438.
- Pandey A, Milind M, Naik, Dubey SK Organic metabolites produced by *Vibrio parahaemolyticus* strain An3 isolated from Goan mullet inhibit bacterial fish pathogens. Goa, India. African J of Biotechnol. 2010; 9(42): 7134-7140.
- Steel RGD, Torrie J H, Dickey DA Principles and Procedures of Statistics. 3rd Ed. McGraw Hill Book Co. Inc., New York, USA 1997.
- Richmond A Microalgal biotechnology at the turn of the millennium: a personal view. Journal of Applied Phycology. 2000; 12(3-5):441-51.
- Renaud SM, Parry DL, Luong-Van T, Kuo C, Padovan A, Sammy N Effect of light intensity on proximate biochemical and fatty acid composition of *Isochrysis* sp. and *Nannochloropsis oculata* for use in tropical aquaculture. Journal of Applied Phycology. 1991; 3:43-53.
- Renaud SM, Zhou HC, Parry DL, Thinh LV, Woo KC Effect of temperature on the growth, total lipid content and fatty acid composition of recently isolated tropical microalgae *Isochrysis* sp., *Nitzschia closterium*, *Nitzschia paleacea*, and commercial species *Isochrysis* sp. (clone T.ISO). Journal of Applied Phycology. 1995; 7: 595-602.
- Borowitzka MA, Borowitzka LJ, Kessly D Effect of salinity increase on carotenoids accumulation in the green alga *Dunaliella salina*. Journal of Applied Phycology. 1990; 2: 111-119.
- Abu-Rezq TS, Al-Hooti S, Jacob DA Optimum culture conditions required for the locally isolated *Dunaliella salina*. Journal of Algal Biomass Utln. 2010; 1(2): 12-19.
- Garcia-Gonzalez M, Moreno J, Manzano JC, Florencio FJ, Guerrero MG Production of *Dunaliella salina* biomass rich in 9-cis-β-carotene and lutein in a closed tubular photobioreactor. Journal of Applied Biotechnol. 2005; 115: 81-90.
- Cifuentes A, Gonzalez M, Conejeros M, Dellarossa V, Parra O Growth and carotenogenesis in eight strains of *Dunaliella salina* Teodoresco from Chile. Journal of Applied Phycology. 1992; 4: 111-118.

32. Singh E, Babcock R, Radway JA Photobioreactor modification for *Dunaliella salina*. Marbec Summer Undergraduate Research Fellowship, Marine Bioproducts Engineering Center, University of Hawaii at Manoa and University of California, Berkeley 2000.
33. Zhao B, Zhang Y, Xiong K, Zhang Z, Hao X, Liu T Effect of cultivation mode on microalgal growth and CO<sub>2</sub> fixation. Chemical Engineering Research and Design DOI: 2011; 10.1016.
34. Dolapsakis NP, Tafas T, Abatzopoulos TJ, Ziller S, Economou-Amilli A Abundance and growth response of microalgae at Megalon Embolon Solar Salt works in northern Greece: An aquaculture prospect. Journal of Applied Phycology. 2005; 17(1): 39-49.
35. Oren A (2005) A hundred years of *Dunaliella* research: 1905-2005. Saline Systems. Licensee BioMed Central Ltd. Available online as DOI 10:1186/1746-1448-1-2.
36. Farahat N, Rabhi M, Falleh H, Jouini J, Abdelly C, Smaoui A "Optimization of salt concentrations for a higher carotenoid production in *Dunaliella salina* (Chlorophyceae)." Phycological Society of America, DOI 2011; 10:1529-8817.
37. Hadi MR, Shariati M, Afsharzadeh S Microalgal biotechnology: carotenoid and glycerol production by *Dunaliella* sp. algae isolated from the Gave khooni salt marsh, Iran. Biotech Bioproc Eng. 2008; 13(5): 540-544.
38. Raja R, Anbalagan C, Senthil Swamy, Lakahmi D, Rengaswamy R Nutritional studies on *Dunaliella salina* (Volvocales, Chlorophyta) under laboratory conditions. Seaweed Res Utilization. 2004; 26(1&2): 127-164.
39. Fisher M, Pick U, Zamir A A salt induced 60-Kilodalton plasma membrane protein plays a potential role in the extreme halotolerance of the alga *Dunaliella*. Plant Physiol. 1994; 106: 1359-1365.
40. Leach G, Oliveira G, Morais R Production of a carotenoids-rich product by alginate entrapment and fluid-bed drying of *Dunaliella salina*. Journal of the Science of Food and Agriculture. 1998; 76: 298-302.
41. Viso AC, Pesando D, Baby C Antibacterial and antifungal properties of some marine diatoms in culture. Botanica Marina. 1987; 30:41-45.
42. Findlay JA, Patil AD Antibacterial constituents of the diatom *Navicula delognei*. Journal of Natural Products. 1984; 47: 815-818.
43. Radhika D, Veerabahu C, Priya R Antibacterial activity of some selected seaweeds from the Gulf of Mannar Coast, South India. Asian journal of pharmaceutical and clinical research. 2012; 5(4): 89-90.
44. Cordeiro RA, Gomes VM, Carvalho AFU, Melo VMM Effect of Proteins from the Red Seaweed *Hypnea musciformis* (Wulfen) Lamouroux on the Growth of Human Pathogen Yeasts. Brazilian Arch Biol Technol. 2006; 49(6): 915-921.
45. Tuney I, Cadirci BH, Unal D, Sukatar A Antimicrobial activities of the extracts of marine algae from the coast of Urla (Izmir, Turkey). Turk J Biol. 2006; 30:171-175.
46. Herrero M, Ibañez E, Cifuentes A, Reglero G, Santoyo S *Dunaliella salina* microalga pressurized liquid extracts as potential antimicrobials. Journal of Food Protection. 2006; 69: 2471-2477.
47. Karabay-Yavasoglu NU, Sukatar A, Ozdemir G, Horzum Z Antibacterial activity of volatile component and various extracts of *Spirulina platensis*. Phytother Res. 2007a; 21(2): 754-757.
48. Karabay - Yavasoglu NU, Sukatar A, Ozdemir G, Horzum Z Antimicrobial activity of volatile components and various extracts of the red algae *Jania rubens*. Phytother Res. 2007b; 21: 153 - 156.
49. Mhadhebi L, Chaibek K, Bouraoui A Evaluation of antimicrobial activity of organic fractions of six marine algae from Tunisian Mediterranean Coasts. International J of Pharmacy and Pharmaceutical Sciences. 2012; 4(1): 534-537.
50. Plaza M, Santoyo S, Jaime L, Garcia-Blairsy Reina G, Herrero M, Senorans FJ, Ibanez E Screening for bioactive compounds from algae. Journal of Pharmaceutical and Biomedical Analysis. 2010; 51:450-455.
51. Al-Wathnani H, Ismet Ara RR, Tahmaz TH, Al-Dayel, Bakir MA Bioactivity of natural compounds isolated from cyanobacteria and green algae against human pathogenic bacteria and yeast. Journal of Medicinal Plants Research. 2012; 6(18): 3425-3433.
52. Dooslin Mercy Bai V, Krishnakumar S Evaluation of antimicrobial metabolites from marine microalgae *Tetraselmis suecica* (Kylin) using gas chromatography - mass spectrometry (GC - MS) analysis. International J Pharmacy and Pharmaceutical Sciences. 2013; 5 (3):17-23.