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Invitro Assessment of Antimicrobial and Antioxidant Activity of Bioactive Compounds from Marine Algae

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A B S T R A C T

In recent years, naturally derived metabolites are main target for the treatment of various biological disorders. It is highly used in the medicinal chemistry to avoid side effects in the treatment. In this context, our research focused to derive antibacterial and antioxidant properties from the algal metabolites. Marine algal species such as *Chlorella salina, Nannochloropsis oculata, Dunaliella salina* and *Isochrysis galbana*are focused. The algal species were inoculated in Artificial and F2 natural sea water media. The growth rate was monitored dailyin the media. The maximum growth rate of the algal species was obtained at 10-12 days of incubation time. Mass multiplication was done at small scale reactor vessel (60L) to get the maximum yield of biomass. The extracted biomass was dried and used in antibacterial and antioxidant assays. Antibacterial activity done on the four different human pathogens namely *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus and Proteus vulgaris*. The highest inhibitory activity was found in *Dunaliella salina*. Further, antioxidant potential was done from the potential extract in which *Dunaliella salina* exhibited the highest activity in DPPH as well as FRAP assays. Inhibitory concentration (IC_{50}) of *Dunaliella salina* was calculated as 80µg/ml whereas *Isochrysis galbana* was observed as 100 µg/ml. Moreover, chlorophyll, carotenoids and total phenolic content was studied. Results of GC – MS that the crude extracts showed that the crude extract of *Dunaliella salina* contain Triclosan, Dodecane and α-Amyrin which are nowadays used as drug ingredients for the treatment of cancer.

Introduction

Marine algae was one of the largest producers of biomass in the marine environments (Bhadury *et al.*, 2004). Marine micro algae are focused in biomass production due to their ability to survive under environmental stresses like light intensity, dark, heat, UV exposure, nitrogen and also the metabolites production. Some of the marine micro algal species like *Chlorella salina, Isochrysis galbana, Dunaliella salina,* and *Nanno chloropsis oculata* exhibits notable bioactivities like antibacterial,anti-inflammatory, anti-algal, antifungal, analgesics, antioxidant activities which are effective in the prevention of biofouling and have other likely uses, e.g. in therapeutics and nutraceuticals (Smit, 2004).

Antioxidants are involved in the prevention of cellular damage as these molecules interact with free radicals and end up the reaction. It paves the way for medicinal focus on cancer, cardiovascular, aging, and many diseases because of their tissue protecting effects by neutralization of ROS. Antioxidants compounds can be derived from both natural and synthetic method. There are many synthetic antioxidants like propyl gallate, butylated hydroxytoluene are available commercially but are unsafe and toxic in nature (Madhavi, 1995). Many researchers are interested deriving antioxidant compounds from natural sources (Rankovic *et al.,* 2009).Therefore they are used in food supplements or food ingredients, feed additives, pharmaceutical and cosmetic industry. Algae are also used as fertilizers and medicines.

Chlorella vulgaris possesses high antioxidant capacity and thus could be a potential new source of natural antioxidants (Goiris *et al.,* 2012). It is noted that *Isochrysis galbana* has rich bioactive compounds than many other algal species (Prakash *et al.,* 2004). Several reports indicate that *Dunaliella* has anti-oxidative effects (Vanitha *et al.,* 2007). The present study was conducted to evaluate the antibacterial and antioxidant activity of the bioactive compounds from the marine algae.

Materials and methods

Sample collection and extraction of bioactive compounds

The marine algae for the present investigational study were obtained from the Central Marine Fisheries Institute (CMFRI), Cochin, Kerala. The algal species were screened based on the growth kinetics. (reference) Mass culturing of the algal species was performed and they were centrifuged at 3000 rpm for 20 minutes. The biomass obtained was extracted using ethanol by ultrasonicator and the extract was air dried to be preserved for further studies.

Carotenoids Extraction

The carotenoid was obtained by separation of cold acetone extract with petroleum ether solvent in a separating flask. The upper phase containing carotenoids was collected and stored at 20ºC.

Estimation of Chlrophyll and Carotenoid Content

In order to estimate the chlorophyll and carotenoid content centrifugation was done at 3000 rpm for 15 minutes using 5 ml of micro algal cultures. The pellet was washed with distilled water. After removal of water by centrifugation, it was suspended in acetone and vortexed well. Acetone extract was separated from the cell debris by centrifuging it in 3000rpm for 15 minutes.

Total carotenoid and chlorophyll levels were determined by UV/visible spectroscopy in 80 % acetone by using the Lichtenthaler equation. Beta carotene was measured at 480 nm.

Thin Layer Chromatography analysis of carotenoids

The qualitative amount of carotenoids in the algal sample was carried out by using Thin Layer Chromatography with mobile phase of methanol and toluene in a ratio (5:95). R_f values were calculated. By comparing the standard R_f values for the chosen mobile phase, the carotenoid sample are identified.

R_f = Distance travelled by the substance/Distance travelled by the solvent.

Protein Estimation for Algae

In order to estimate the protein content, 50ml of sample was centrifuge at 3000rpm for 1-2min. Then the cells are washed with distilled water and spin again at 3000rpm for 2min.The chlorophyll was extracted by centrifugation and discard it. The pellet was suspended in small amount of water for protein estimation using Lowry *et al*., 1951. Optical density was measured at 660nm using UV-Visible spectrometer (Hitachi U-2900).

Effect of Light Intensity on Total Carotene Production

Light intensity plays important role in controlling the carotene accumulation in algal cells. The effect of light intensity on growth and pigment content of the *Dunaliella salina* are increased compared to other marine algae. The chlorophyll content per cell decreased and β-carotene content per cell are increased on the high light intensity.

Total phenolics Determination

Different concentrations of 50, 100, 150, 250, 500 mg/l of gallic acid (20 µl of gallic acid solution at every concentration made, 1.58 ml water, 100 µl of folin reagent and 300 µl sodium carbonate, 2 ml in total) was used as standard to determine the phenolic concentration of the samples. Then the tubes were mixed and allowed to stand for 1 hour in the dark at room temperature. The absorbance was measured at 765 nm using spectrophotometer.

Determination of Antibacterial Activity

The antibacterial activity was tested against four species of bacteria namely *Escherichia coli, Proteus vulgaris, Klebsiella pneumoniae and Bacillus subtilis.* Kirby Bauer well diffusion method was administered to test the antibacterial activity and the more effective algal species was selected to test the efficiency against the methicillin-resistant *Staphylococcus auerus*.

Determination of Antioxidant Activity

FRAP assay was done according to (Thaipong *et al.,* 2006). 0.2 M phosphate buffer of pH 6.6, 1% potassium ferrocyanide, 10% of trichloroacetic acid and 0.1% ferric chloride was used to carry out the FRAP assay. L-Ascorbic acid was used as the standard.

DPPH (2,2-diphenyl-2-picrylhydrazyl) assay was performed as per (Vadlapudi *et al*., 2012). The extracts prepared at different concentrations were added to the 8ml of 0.004% (w/v) stock solution of DPPH in ethanol (95%). The scavenging activity of---- on the DPPH radical was determined by measuring the absorbance at 517nm in spectrophotometer. The DPPH radical scavenging activity was calculated using the following equation:

(Absorbance of control-Absorbance of sample/Absorbance of control)*100

Results and Discussion

Culturing and screening of algae for bioactive compunds

A total of four isolates such as *Chlorella salina, Nannochloro psisocualata, Dunaliella salina and Isochrysis galbana* were cultured in laboratory condition at 18ºC with a light intensity of 0.05 cal/cm2 min and tested under microscope.

The unialgal cultures were confirmed by microscopic observation at 100X and the results were presented in figure 4.2. The results showed that the structure of four

isolates were very unique in nature. Also *Chlorella salina* and *Nannochloropsis oculata* were green algae; *Dunaliella salina* was a halophilic green algae and *Isochrysis galbana* was observed to be Brown algae.

The growth curve of the four algal species, *Chlorella salina, Nannochloropsis oculata, Dunaliella salina* and *Isochrysis galbana* were studied based on the log phase of the samples. *Nannochloropsis oculata* was ruled out because of their fastest rate of attainment of log phase.The growth pattern was studied at specific optical densities for each algae.

In *Isochrysisg albana* the optical density was read at 540nm (Drora *et al.,* 1985). The results revealed that *I.galbana* reached the log phase by 11 to 12 days. The wavelengths for studying the growth of rest of the marine algae were done based on various literatures (Sanjoy *et al.,* 2011, Huang *et al.,* 2011)

Extraction of Marine Algae

Mass culturing of the algal species were done and they were centrifuged at 3000rpm for 20 minutes. The pellet obtained was extracted using ethanol by ultrasonicator and the extract was air dried and preserved for further studies. The extraction using sonicator was found to be better as it is a time consuming process when compared with Soxhlet extraction as per Ankit *et al.,* 2010.

Estimation of Chlorophyll and Carotenoid Content

The total carotene content was high in *Dunaliella salina* (2.32 µg/ml) compared to other algal species.Next to *Dunaliella salina, Isochrysisg albana* showed high amount of carotene content but both chlorophyll a and b content was found to be high in *Isochrysis galbana.*

Protein Estimation (Lowry *et al*)

Protein content of algal species were much useful to study the stress level of the respective algae with respect to the environmental conditions. In this 2 species *Isochrysis galbana* and *Dunaliella salina* were compared and result says that the species 2 has the highest amount of protein expression compared to species 1 which indicates that the stress level of species 2 is higher than the species 1.

Total Phenolic Determination

The amount of total phenolics varied. The obtained results do not show a similarity at all the samples. The ethanolic extracts contains more amount of the phenols compared to other algal species. The *Isochrysis galbana* contains more amount of the phenols in all the solvent extracts and the *Nannochlropsis oculata* contains low amount of the phenols compared to other species.

Light intensity plays important role in controlling the carotene accumulation in algal cells. The effect of light intensity on growth and pigment content of the *Dunaliella salina* are increased compared to other marine algae. The chlorophyll content per cell are decreased and β-carotene content per cell are increased on the high light intensity.

Antibacterial Screening

The antibacterial activity was studied using two Gram positive and Gram negative bacteria. *Chlorella salina* showed good zone of inhibition against *Escherichia coli* and *Klebsiella pneumoniae* than against Gram positive. *Dunaliella salina* exhibited activity against both Gram positive as well as Gram negative bacteria. *Staphylococcus aureus*

and *Proteus vulgaris* was inihibited to a larger extend by *Isochrysis galbana*. A zone of about 12mm and 14mm was shown by the above two microbes respectively. The positive controls used were Gentamicin, Ampicillin and Trimethoprim and negative control as ethanol used for extraction. It was reported that *Chlorella salina* and *Isochrysis galbana* have a considerable antibacterial activity than *Dunaliella salina* in a study conducted by Srinivasakumar *et al.,* (2009). In contrast to this it was observed that *Dunaliella salina* exhibited better results than the other two species. The study also revealed that ethanol extract shows good activity against both Gram negative as well as Gram positive bacteria.

Activity of *Dunaliella Salina* Against Methicillin-Resistant *Staphylococcus Aureus*

The three species of marine algae showed good antibacterial activity against two Gram negative as well as two Gram positive bacteria. Of this *Dunaliella salina* exhibited more inhibition to the growth of Staphylococcus aureus. Thus it was tested against three strains (NP-267, NP-295, NP-377) of Methicillin-Resistant *Staphylococcus aureus* (MRSA). Gentamicin was used as the positive control and the solvent used for extraction was taken as the negative control.

Zone of inhibition was observed between 15mm and 19mm for the ethanolic extract of *Dunaliella salina.* Among these three strains NP-267 was inhibited the maximum. It was reported in one of the study carried out by Ohta *et al*., in 1994 that *Dunaliella primolecta*, another species of *Dunaliella salina* showed the presence of anti-Methicillin Resistant *Staphylococcus aureus* substance under optimum culture conditions. Thus the results obtained using the ethanolic extract of *Dunaliella salina* against MRSA go in line with the finding of Ohta *et al*.

Analysis of Variance was done for the antibacterial activity of *Dunaliella salina* against three strains of MRSA. No significant difference was observed during the experiment using the triplicates. Thus it was obtained that *Dunaliella salina* was a good inhibitor to these three strains of MRSA.

Antioxidant Activity of *Dunaliella Salina* and *Isochrysis galbana*

Antioxidant activity was determined for the two species of marine algae namely *Dunaliella salina* and *Isochrysis galbana* based on their activity in the above experiments. The antioxidant property was measured using two assays. The Ferric Reducing Antioxidant Power (FRAP) assay as well as DPPH(2,2-diphenyl-2-picrylhydrazyl) assay was carried out with the ethanolic extracts of two species of which *Dunaliella salina* showed good antioxidant property.

Ferric Reducing Antioxidant Power (Frap) Assay

Antioxidant property of *Dunaliella salina* and *Isochrysis galbana* was done using Ferric Reducing Antioxidant Power (FRAP) assay. This is based on the reducing power of ferric to ferrous form (which has an intense blue color). The reaction was monitored by measuring the absorbance at 700nm. The reaction is non-specific, in that any half reaction that has redox potential lower that reduce ferric ferrous half reaction, will drive the ferrous (Fe III to Fe II) ion formation. The change in absorbance was directly related to the total reducing power of the electron donating antioxidants present in the reaction mixture. The standard used

for the FRAP assay was L-Ascorbic acid. With reference to L-Ascorbic acid, *Dunaliella salina* showed more reducing power than *Isochrysis galbana*. The standard error bar was also plotted in the graph.

Many literatures revealed the antioxidant nature of *Dunaliella salina* due to the presence of carotenoid pigment which is absent in *Isochrysis galbana*. The study carried out by Herrero *et al.,* in 2006 observed that *Dunaliella salina* is an excellent source of antioxidants. The literatures showed that extraction process using ethanol possessed more efficient for the isolation of antioxidants. Results pointed out that the extracts contained, several different minor carotenoids besides β-carotene and isomers that seemed to make a contribution to the antioxidant activity of the extracts.

DPPH (2,2-Diphenyl-2-picrylhydrazyl) assay

The antioxidant activity of *Isochrysis galbana* and *Dunaliella salina* were assessed on the basis of the free radical scavenging effect of the stable free radical DPPH as per the method of Kumar *et al.,* 2008. The DPPH method permitted to calculate the electron or hydrogen atom-donating properties of antioxidants and the rate of reaction towards the free radicals. The spectrophotometric technique uses the 2,2-diphenyl-2- picrylhydrazyl free radical (DPPH), which shows a characteristic spectrum with a maximum absorbance close to 517nm in methanol. The addition of an antioxidant compound resulted in a decrease of absorbance proportional to the concentration and antioxidant activity of the compound. The discolouration of purple colour of the DPPH was observed after the incubation. Both extracts of the algal species showed an absorbance range within the L-Ascorbic acid which served as the standard. Of the two test samples the ethanolic extract of *Dunaliella salina* showed higher antioxidant activity when compared with *Isochrysis galbana*. DPPH assay is a widely used method to determine the antioxidant property of marine species (Vadlapudi, 2012).

Gas Chromatography – Mass Spectrometry Analysis

The GC-MS analysis showed various peaks representing the compounds present in the ethanolic extract of the algal species *Dunaliella salina.* From the graph it was clear that about eight compounds were identified at 5th, 8th, 12th, 16th, 23rd, 28th, 35th and 40th minutes. Of these one of the highest peak was at 15.97 which corresponds the compound diphenly ether. The derivatives of diphenyl ether shows good antibacterial as well as good anticancer activity. Triclosan, the diphenyl ether derivative is added in the drugs prescribed for patients suffering from a Methicillin-Resistant Staphylococcus aureus (MRSA) infection as per Roger *et al*., 2007. Other peaks corresponding to α-amyrin, dodecane, tetradecane etc. also shows notable anticancer property. Dodecane is one of the other compounds present in the ethanolic extract of *Dunaliella salina* is a potent anti proliferative agent which can be used in the treatment of cancer (Filosa *et al*., 2006).

Table.1 Activity of *Dunaliella salina* against methicillin-resistant *Staphylococcus aureus*

Bacterial strains	Zone of inhibition for ethanolic extract of			
	control	*Chlorella salina*	*Isochrysis galbana*	*Dunaliella salina*
Escherichia coli	18	10.27± 0.28	10.03±0.08	11.33±0.52
Klebsiella pneumonia	17	12.07± 0.38	11.23±0.08	12.37±0.41
Staphlycoccus aureus	18	7.90± 0.45	8.1±0.11	8.73±0.12
Proteus vulgaris	15	0	2.23±0.14	6.17±0.12

Table.2 Antibacterial activity of ethanolic extracts of *Dunaliella salina*

Bacterial strains	**Extract(mm)**			**Mean± SEM**	**Positive control(mm)**
	Trail 1	**Trail 2**	**Trail 3**		
NP-267	19	19.2	19.1	19.1±0.8174	20
NP-295	18	18.1	18.2	18.1±0.8174	22
NP-377	17	17.2	17.1	17.1±0.8174	22

Figure.1 Four species of algal culture

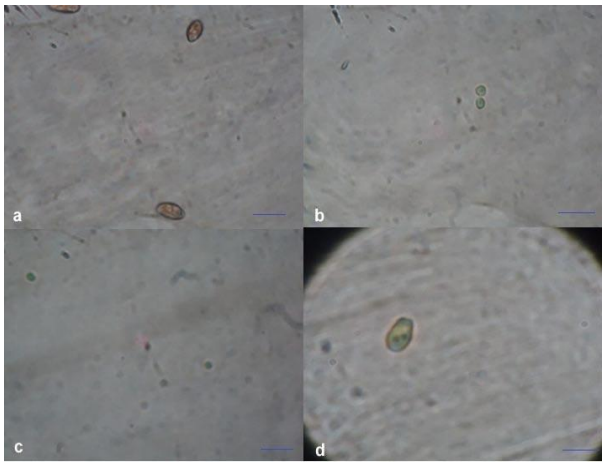

Figure.2 Microscopic images of algae

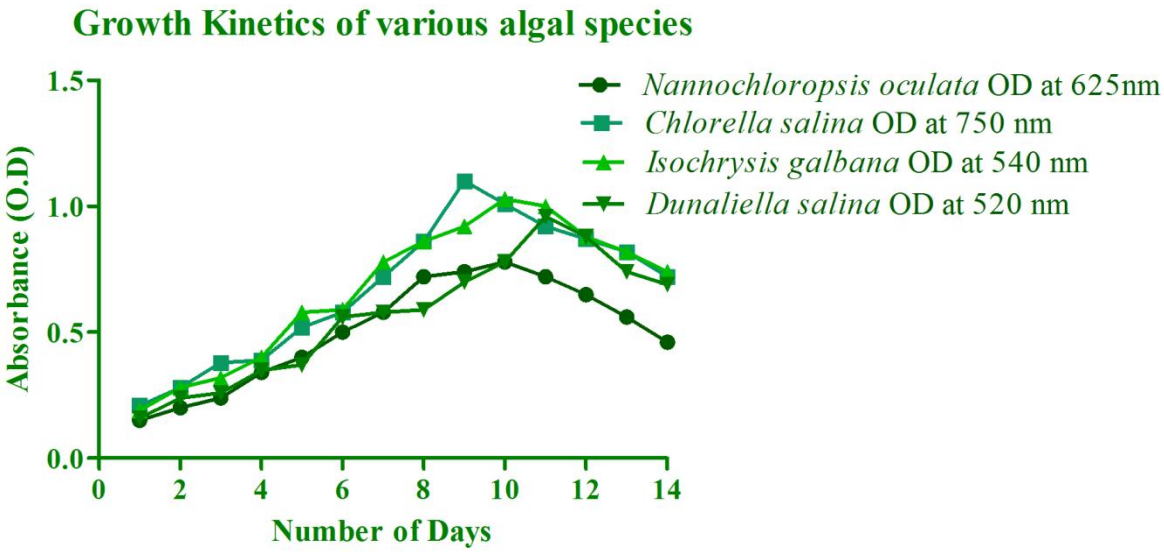

Figure.3 Growth Kinetics Of Various Algal Species.

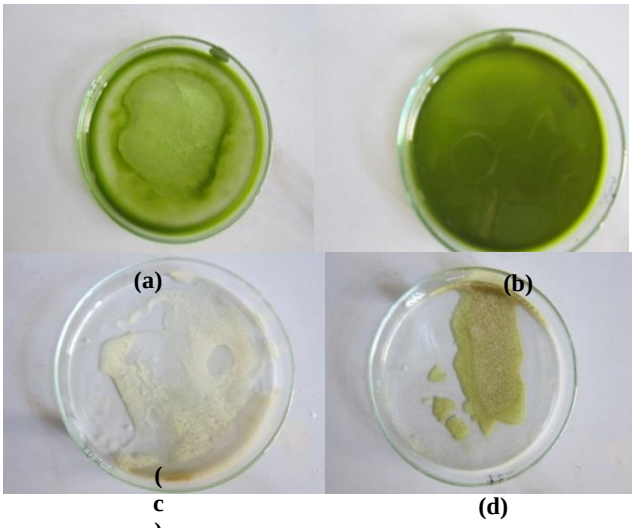

Figure.4 Four species of dried algal extracts

a. *Nannochloropsis oculata;* b.*Chlorella salina;* c. *Dunaliella salina;* d.*Isochrysis galbana*

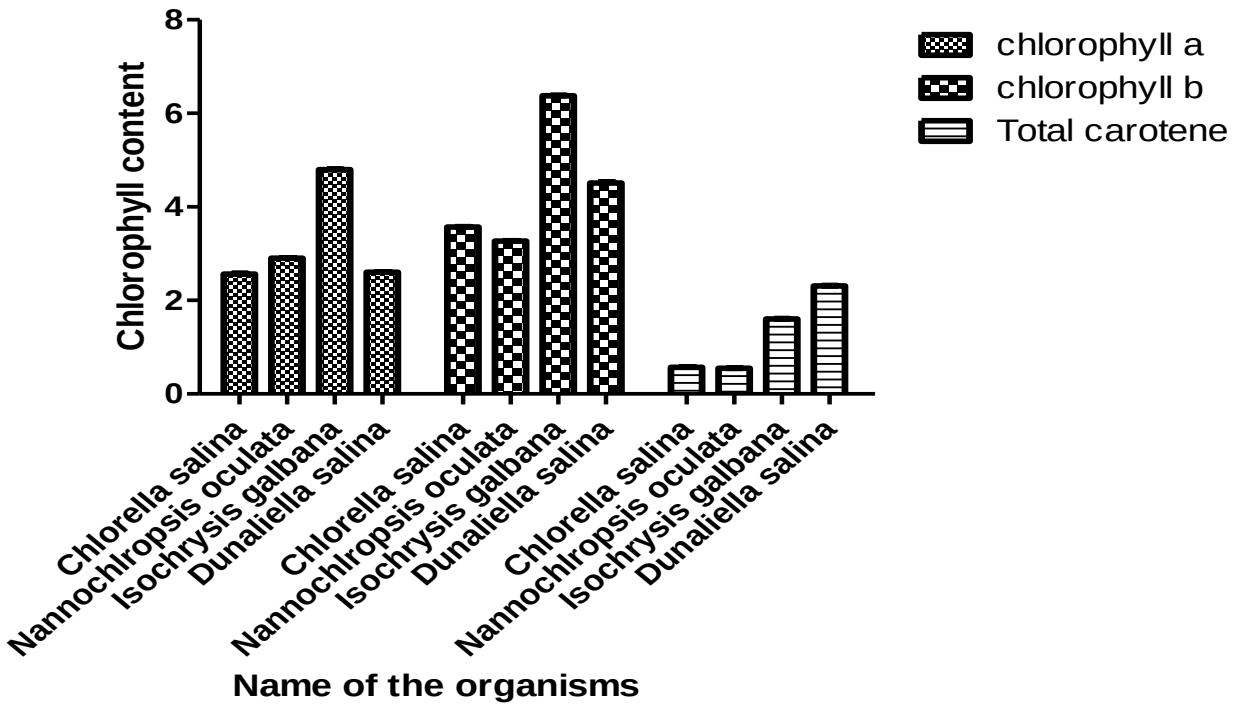

Figure.5 Estimation of chlorophyll and carotenoids in microalgal species

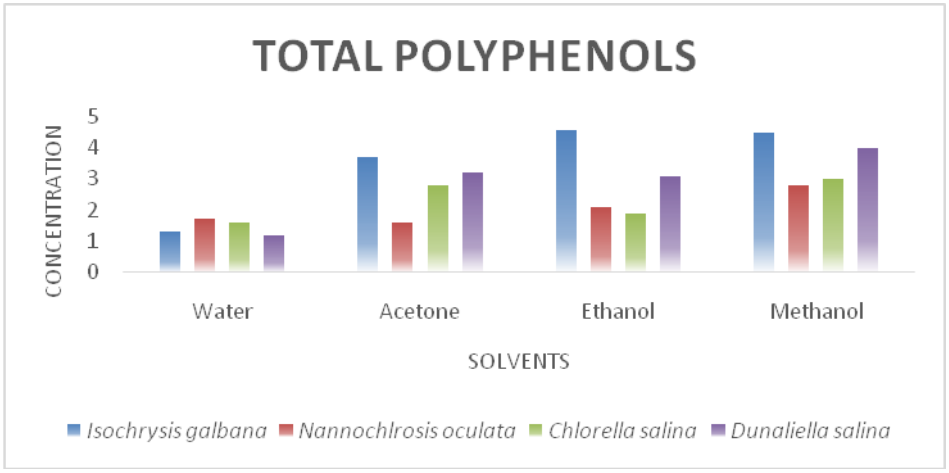

Figure.6 Comparison of Total phenolic compounds in different solvents

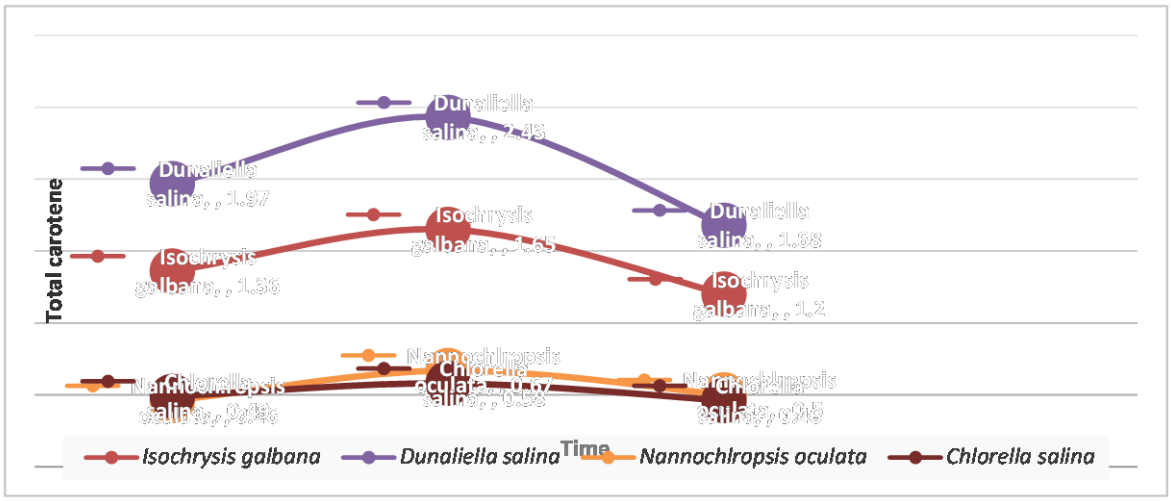

Figure.7 Effect of light intensity on carotene production

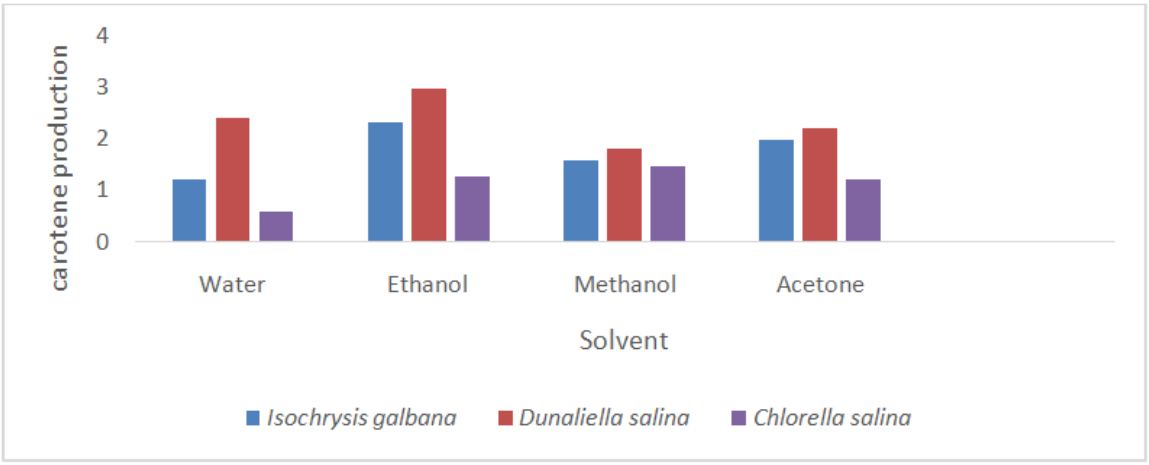

Figure.8 Effect of various solvents on the carotene production

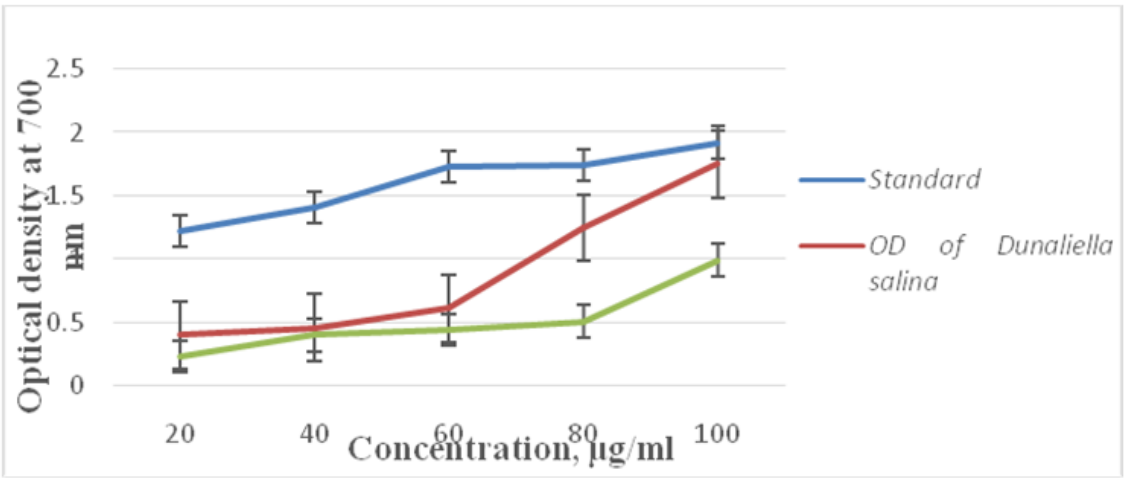

Figure.9 Antioxidant property using FRAP assay

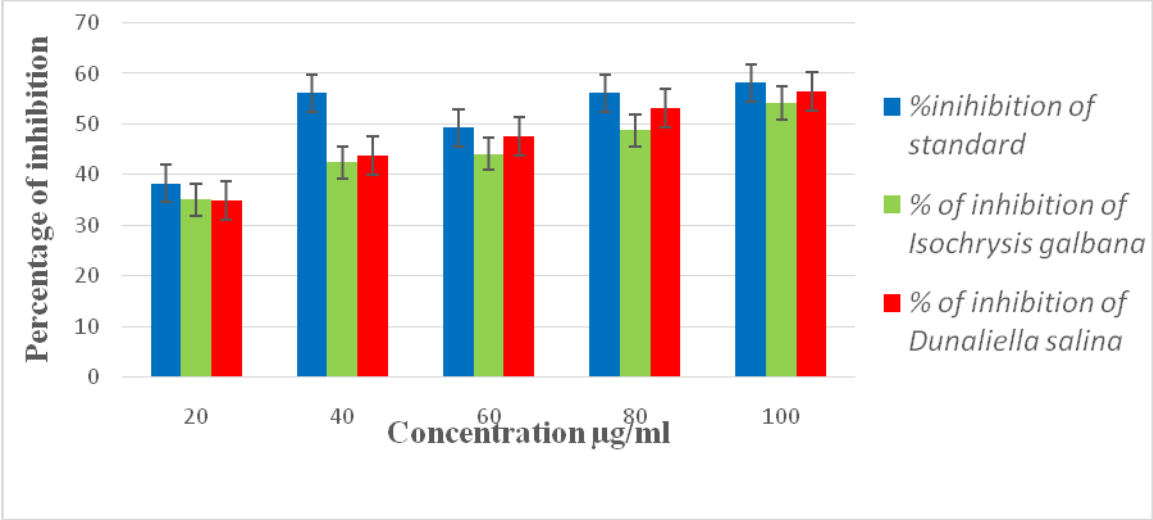

Figure.4 Antioxidant property using DPPH assay

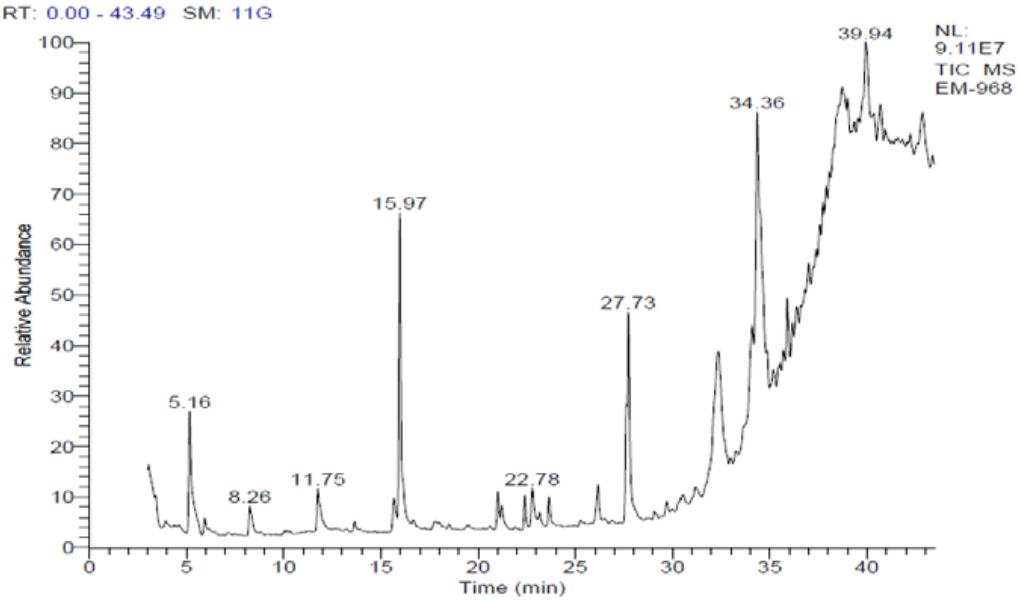

Figure.4.8 Gas Chromatography – Mass Spectrometry analysis

In conclusion, marine microalgae serve as a rich source of the bioactive compounds which can be used in the pharmaceutical field in the place of synthetic drug compounds. *Chlorella salina*, *Isochrysis galbana* and *Dunaliella salina* were extracted with ethanol using Ultrasonication. Based on the results of tests, it can be stated that the microalgal extracts have relatively strong antimicrobial and antioxidant

properties which requires furthermore studies to determine the antibacterial and antioxidant agent for therapy of various diseases. *Dunaliella salina* exhibited a remarkable activity against *Staphylococcus aureus*and it was taken to test against the Methicillin-Resistant *Staphylococcus aureus*. Quantitaive analysis for all the above tests were done using the Analysis of Variance (ANOVA).Gas Chromatography-Mass Spectrometry (GC-MS) analysis was done for *Dunaliella salina* which showed better activity in all the tests which showed the presence of volatile compounds. Triclosan is a compound which can be used to inhibit the growth of MRSA culture in the wounds. Further investigation is needed to identify the bioactive compounds in the extracts and isolate and purification of the compounds. Thus *Dunaliellasalina* is a good source of biologically active compounds and could be used as a substitute for the synthetic drug in future.

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