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MOLECULAR CHARACTERIZATION AND SCREENING OF BIO-ACTIVE COMPOUNDS FROM AQUEOUS EXTRACTS OF *CHLORELLA VULGARIS*.

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ABSTRACT

The discovery of unique classes of natural products from Algae represent an important source of novel anticancerous secondary metabolites. From past two decades, more than 300 nitrogen containing secondary metabolites were identified as therapeutic agents from plants and microorganisms and some are in clinical trials I and II. Chlorella could serve as a major source of bioactive compounds with potential efficacy against Squamous cell carcinoma and Hepatocellular carcinoma. Hence there is a need to exploit other remedies with possibly less known adverse effects and from readily accessible sources like marine medicinal photo-planktons. In the present study, we have concentrated on the preliminary screening, quantitative determination and the qualitative separation of phytochemical compounds from *Chlorella sp*. The HPLC analysis of the extracts revealed that there were two unidentified components while comparing with methanol. The retention time of two compounds was 3.12 and 3.837 mins. The current study focused on aqueous studies of the *Chlorella sp.* to identify potential compounds. Further studies should be carried out with identification and testing antiproliferative activity of *Chlorella Vulgaris* with respect to oral cancer.

KEYWORDS: Hepatocellular carcinoma, cancer, phytochemical analysis, HPLC, *Chlorella vualgaris.*

INTRODUCTION

The discovery of unique classes of natural products from Algae represent an important source of novel anticancerous secondary metabolites. From past two decades, more than 300 nitrogen containing secondary metabolites were identified as therapeutic agents from plants and microorganisms. Cancer is a term used to describe characterized by the uncontrolled proliferation and spread of abnormal cells (Hayflick, 1997). MarineAn increasing number of marine algal compoounds are found to target tubulin or actin filaments in eukaryotic cells, making them an alternative source of natural products as anticanncer agents (Jordan and wilson 1998).

Prominent molecules such as the antimicrotubule agets, curacin A and dolastatin-10, have beenin preclincal and or clinical trials as potential anticancer drugs (Gerwick et al 2001). Apartoxin A is another potent cytotoxic compound worthy of further biological investigation as anticancer agent due to it mechanism of action in attenuating the FGF (fibroblast growth factor) signaling pathway.

Chlorella is widely used in artificial nutrition chain phytoplanktone - zooplanktone - fish (Elert and Woffrom, 2001; Wacker et al., 2002). Chlorella could serve as a major source of bioactive compounds with potential efficacy against Squamous cell carcinoma and Hepatocellular carcinoma. Hence there is the need to exploit other remedies with possibly less known adverse effects and from readily accessible sources like marine medicinal photo-planktons. In the present study, we have concentrated on the preliminary screening, quantitative determination, and the qualitative separation of phytochemical compounds from Chlorella sp.

MATERIALS AND METHODS

Sample Collection and Culture

Water sample with visible microalgae population were collected from sea water, Mangalore. Algae were filtered from water sample using whatman filter paper and inoculated in to 200ml of Bold's Basal Medium as given in standard protocol.

The algae was grown in 500ml flasks on Orbital Shaker Incubator shaking at 180 rpmand incubated for 7 days under natural light and lighting (40Watt Tubes). Tube containing culture sample was centrifuged at 3000rpm for 15 minutes. After centrifugation, supernatant was discarded and cells were suspended in fresh water again centrifuged for purification.

Microalgal cultures were initially separated based on microscopic observation of morphological characteristics of the colonies.

Molecular Characterization

Genomic DNA extraction: Genomic DNA was isolated by using the Insta Gene TM Matrix Genomic DNA isolation kit (Catalog # 732-6030). DNA fragments are amplified using 1$\mu\ell$ of template DNA in 20$\mu\ell$ of total PCR reaction mixture using ITS1/ITS4 primers (50 pmol) and 35 amplification cycles with denaturation: 94℃ for 45 sec, annealing: 55℃ for 60 sec, chain elongation: 72℃ for 60 sec. Removedun incorporated PCR primersandd NTPs from PCR productsbyusing Montage PCR Clean up kit (Millipore).

Sequencing

The PCR product was sequenced using the ITS1/ITS4primers.Sequencingreactionswere performed using a ABI PRISM® Big Dye TM Terminator Cycle Sequencing Kits with Ampli Taq® DNA polymerase (FS enzyme) (Applied Biosystems). Single-passsequencing was performe doneachtem plateusing below 18sr RNAIT Suniversal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were re suspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Bio systems).

Cell count and Biomass estimation.

The algal cell density was measured daily by counting the cells using a haemocytometer and respective Optical density (OD) measurement was taken at 680 nm (Lee,K And Lee,C,2001) to monitor cell growth by UV/Visible spectrophotometer.

Preparation of extract

10g of micro-algal pellet was dissolved in 50 ml of methanol. Mixture was kept at 50ºC for 4 hours and dried filtrate was measured after the filtration. 500mg of extract was solubilized in 10 ml Methanol. Then the different solvent starting from Hexane, Ethyl acetate and Chloroform was used for the further extraction using separating funnel. Each extracts were diluted to 10mg /ml and 2 ul of sample was used for TLC analysis.

DPPH radical scavenging activity

The free radical scavenging activity of *C. vualgaris* extract and fractions was evaluated using thew stable DPPH method with some modification in Rajakumar et al. (1994). one ml of

0.1mM DPPH solution in methanol was added to 1.0ml of standard/extract solution at different concentrations. The mixture was incubated for 15mins and the absorbance recorded at 510 m using semi-autoanalyzer. The inhibition concentration at 50% (IC50) value of each extract was calculated from the following formula: % Antioxidant activity = [(A0-A1)/A0 × 100]. Where A0 is the absorbance of negative control (methanol), and A1 is the absorbance of test sample with DPPH. Quercetin was used as standard control.

HPLC analysis

HPLC analysis was done using LC -20AT Shimadzu system. HPLC chromatograms were prepared by injecting 5 µl of the filtered extracts onto a (C 18, 5 µm and internal diameter 4.6cm and length of the column is 250mm) in a chromatograph equipped with a differential ultraviolet detector absorbing at 254 nm for detecting compounds. Mobile phase used was acteonitrile (60%), pH is 3.6 by adding acetic acid, flow rate was 0.6 ml/min. Quantification was done by comparison peak heights of sample and Standard.

Statistical analysis

All data were presented as mean ± S.D. and the graphpad prism 5.0 software was used to calculate the IC50 values.

RESULTS AND DISCUSSION

Sample obtained from study area were cultured in Bolds basal medium and allowed to grow by exposing to the sunlight. Characteristics and Morphological feature of the isolate have demonstrated its close similarity with genus C*hlorella vulgaris.* Cells are green colour, unicellular, spherical in shape and cup shaped nucleus as it shows in the Figures. The algal cell density was measured daily by counting the cells using a haemocytometer and graph was plotted using absorbance of cell suspension in y axis against cell count in x axis. The data was extrapolated from the standard calibration curve (Y= 66.83x-7.639 and R^2 = 0.983) (Fig .1**)**.

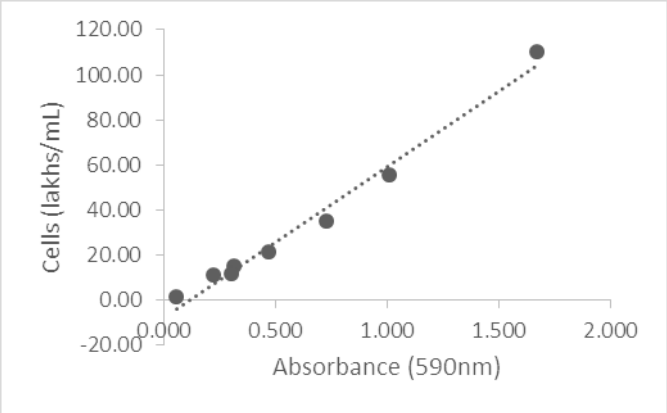

Fig 1: Standard Graph of Absorbance V/s Cell Count.

The extract showed dose dependent scavenging activity of DPPH assay. The DPPH assay result was revealed with an IC50 of 38.24µg/ml (Table 1). The activity showed more than seven fold free radicals than standard Quercitin (IC50 of 5.545µg/ml) (Figure 2).

Table 1: Free radical scavenging activity of Chlorella Vualgaris extract as determined by DPPH assay.

Plants Name	Concentration (µg/ml)	Absorbance Trail 1	Absorbance Trail 2	Absorbance Trail 3	Absorbance Average	% Inhibition	IC_{50}
Control	0.0	0.578	0.602	0.564	0.581	0.0	
Standard (Quercetin)	0.3	0.545	0.576	0.529	0.550	5.4	5.545µg/ml
	0.6	0.501	0.548	0.513	0.521	10.4	
	1.3	0.443	0.469	0.475	0.462	20.5	
	2.5	0.315	0.351	0.359	0.342	41.2	
	5.0	0.207	0.209	0.231	0.216	62.9	
	10.0	0.098	0.069	0.096	0.088	84.9	
	0.0	0.578	0.602	0.564	0.581	0.0	
	3.1	0.524	0.579	0.564	0.556	4.4	38.24µg/ml
	6.3	0.542	0.516	0.549	0.536	7.9	
Chlorella Vualgaris	12.5	0.521	0.474	0.512	0.502	13.6	
	25.0	0.433	0.442	0.468	0.448	23.0	
	50.0	0.389	0.397	0.331	0.372	36.0	
	100.0	0.201	0.233	0.175	0.203	65.1	

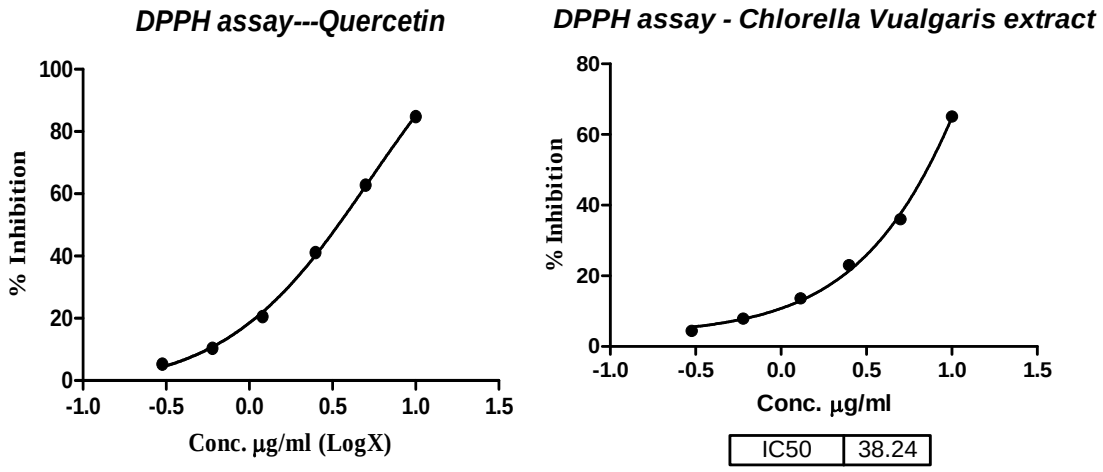

Figure 2: Antioxidant activity of Chlorella Vualgaris extract as determined by DPPH assay. Quercetin as positive standard and ginger as experimental.

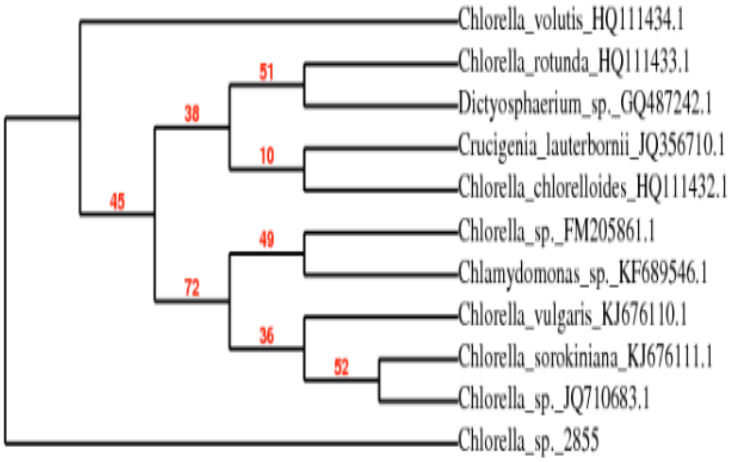

Fig 2: Phylogenetic tree showing *Chlorella Vulgaris.*

Table 1: showing HPLC data of aqueous extract of *Chlorella Vulgaris.*

Sl. No	Sample	Retention Times (min) Major Peaks	% Area	Chromatogram reference
1	Sample	3.837	51.4	Fig. 1
2		3.12	37.5	Fig. 2
	Solvent blank	2.210	100	Fig. 2

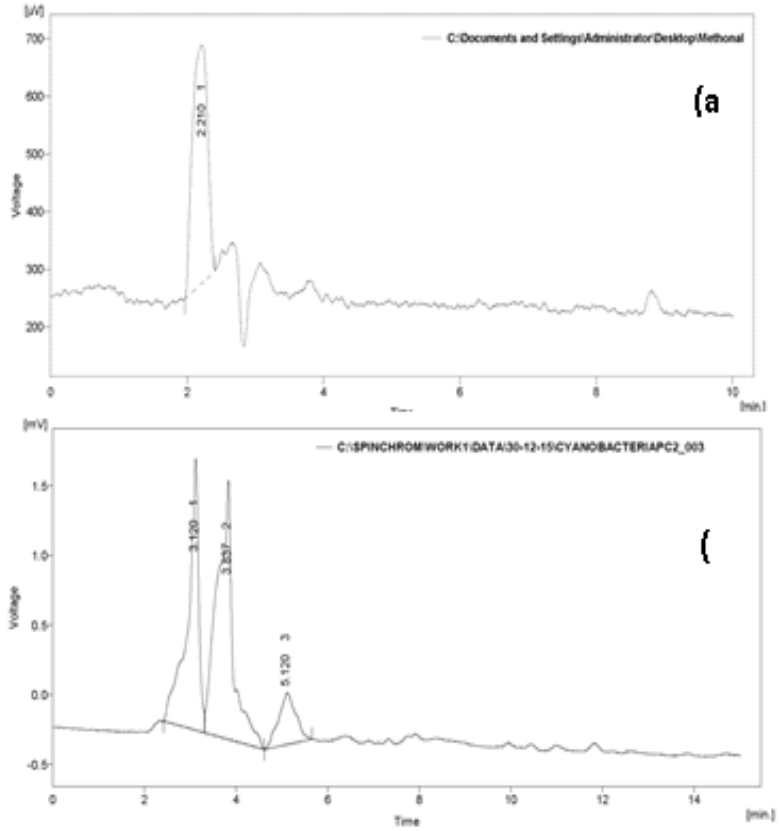

Fig 3: Graph showing HPLC data of aqueous extract of *Chlorella Vulgaris.*

The molecular characterisation of the isolate -2855 has been matched to *Chlorella Vulgaris* as showed in phylogeny tree (Fig .2). The HPLC analysis of the extracts revealed that there were two unidentified components while comparing with methanol(solvent) (fig). The retention time of two compounds was 3.12 and 3.837 mins (Fig .3 and table 1).

CONCLUSION

The obtained result in this investigation go parallel with the results of Zhukova *et. al*, 1995, who screened different species of Chlorella, recorded the secondary compounds and metabolites with an accumulation of biomolecular protection against anticancerous therapeutics. These findings were determined by using DPPH assay of Chlorella extract using quercetin as standard respectively. IC_{50} of 38.24µg/mL was found for methanolic extract of Chlorella in case of DPPH assay in comparison to quercetin (IC_{50} 5.545). The current study focused on aqueous studies of the *Chlorella sp.* to identify potential compounds. Further studies should be carried out with identification and testing antiproliferative activity of *Chlorella Vulgaris* with respect to oral cancer.

Conflict of Interest

The authors have no conflict of interest to declare.

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