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Evaluation of *in vitro* antioxidant activities and antiproliferative activity of green microalgae, *Desmococcus olivaceous* and *Chlorococcum humicola*

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Abstract

The potential antioxidant and antiproliferative activities of acetone and methanolic extracts of green micro algae, *Desmococcus olivaceous* and *Chlorococcum humicola*, were evaluated *in vitro*. Total phenolic and flavonoid content were determined in both the extracts. Free radical (DPPH) scavenging potential was higher in Desmococcus olivaceous than cholorococcum humicola. Reducing power (H_2O_2) scavenging assay, Ferric reducing antioxidant power assay (FRAP), lipid peroxidation inhibition efficiency (TBAR's assay) of both extracts were also evaluated. Methanolic extract of *D .olivaceous* and *C.humicola* showed higher reducing power and promising activity in preventing lipid peroxidation that might prevent oxidative damage to biomolecules. DNA fragmentation analysis was done by agarose gel electrophoresis. The results obtained suggest that methanolic extract of *D.olivaceous* and acetone extract of *C.humicola* were able to protect DNA from oxidative damage. Tested extracts showed strong selective cell proliferation inhibition on Hep-2 cell line, especially the methanolic extract of *D. olivaceous* with IC_{50} value of 1.56µg / ml and the acetone extract of *C.humicola* with IC_{50} value of 0.625µg / ml. Cell growth inhibition was mainly due to apoptosis proved by fragmentation analysis. The results obtained suggest that the methanolic extracts of *D. olivaceous* and acetone extracts of *C. humicola* may be promising alternative to synthetic substances as natural compound with high anti-proliferative activity.

Keywords: Phenolics,Flavanoids Free radicals, LPO, DPPH, HRSA, TBARS, MDA,FRAP, MTT, IC50

Abbreviations

LPO,Lipid peroxidation; DPPH, 1,1-diphenyl 1-2 picrylhydrazyl; HRSA, Hydrogenperoxide radical scavenging assay;TBARS, thiobarbituricacid reactive assay;MDA, malon dialdehyde; FRAP, ferric reducing antioxidant power assay; MTT,3-[4,5-dimethylthiazole-2y] 2,5- diphenyltetrazoliumbromide; IC_{50}, Inhibitory concentration.

Introduction

Reactive Oxygen species (ROS) such as superoxide anion, hydroxyl radicals and hydrogen peroxide which are generated by normal physiological process and various exogenous factors initiate peroxidation of membrane lipids as well as a wide range of other biological molecule through a process that is believed to be implicated in the etiology of several disease conditions including Coronary heart disease, Stroke,

Rheumatoid arthritis, Diabetes and Cancer. Antioxidants play an important role in inhibiting and scavenging radicals thus providing protection to humans against infections and degenerative diseases. The two most commonly used synthetic antioxidants, butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) have begun to be restricted because of their toxicity and DNA damage induction. Therefore, natural antioxidants from plant and algal extracts have attracted increasing interest due to the safety. Recent researches have been interested in finding novel antioxidants to combat and / or prevent ROS mediated diseases.

Green microalgae are widely used in the life science as the source of compounds with diverse structural forms and biological activities. Marine algae have been historically and exceptionally rich source of pharmacologically active metabolites with antineoplastic,, antimicrobial and antiviral effects.(Faulkner, 2000 ; Tziveleka *et. al*., 2003.) Green micro algae like *Scenedesmus* and *Chlorella* contain rich source of active metabolites with anticarcinogenic effects.(Farouk *et. al.,* 2002.) Antitumor promoting glyceroglycolipids from the green alga chlorella (Morimoto *et. al.,* 1995). More marine algae have been suspected of having strong antiproliferative. and antioxidants properties, including *Fucus vesiculosis* and brown alga, *Ecklonia cava* (Yasantha *et. al.,* 2000)

The aim of the present study was to evaluate antioxidant properties and anti proliferative activity of methanolic and acetone extracts of green microalgae, *D. olivaceous* and *C. humicola* by measuring scavenging activity against free radicals, reducing capacity and protection of biological molecules from ROS induced damage. These micro algae are extensively employed in the phycoremediation of various types of industrial effluents by Vivekananda Institute of Algal Technology (VIAT), Chennai (Sivasubramanian *et al.,* 2010). The algal extracts were evaluated for their suppressive effect on tumor cell growth by using MTT assay and DNA fragmentation analysis.

Materials and Methods:

Culturing and growth of algal organisms

Fresh water green microalgae *Desmococcus olivaceous* and *Chlorococcum humicola* were obtained from the culture collection of Vivekananda Institute of Algal technology (VIAT) Chennai. Algal Biomass was obtained by growing algal cultures in 20L of water and 0.25g / L of NPK fertilizer was added with a facility to pump the culture with aeration pump. The algae was grown for 10 days and harvested.

Preparation of Algal extract:

0.5g of dried algal material was extracted in 20ml of acetone and methanol kept in an orbital shaker for overnight. The obtained extracts were filtered with Whatman no.1 filter paper and the filtrate was collected. The solvents were removed under reduced pressure at 50°C to yield a concentrated extract (12% and 11%) respectively.

Quantitative analysis of antioxidative compounds

Determination of Total phenolic compounds

Total phenolic content was determined with Folin & Ciocalteau reagent according to the method described by Singleton *et. al.*, (1999) using gallic acid as standard.

Determination of Total Flavonoids:

Total Flavonoids content was determined by the method described by Zhishen *et. al.,* (1999)

Antioxidant activities assays

DPPH Radical Scavenging Assay

Free radical scavenging ability of the extracts was tested by DPPH radical scavenging assay as described by Blois, (1958). Different concentration of sample 50, 100 &150µl (0.25,0.5 &0.75 mg) of the extracts were taken in the test tubes. 3.0ml of 0.1mM DPPH in ethanol was added to each tube and incubated in dark at room temperature for 30minutes. The absorbance was read at 517nm using UV-visible spectrophotometer. BHT (butylated hydroxy toluene) was used in standard calibration. The % inhibition (I%) was calculated using the formula :

$$I\% = \frac{[\text{Abs (control) - Abs (sample)}]}{\text{Abs (control}} \times 100$$

Hydrogen peroxide(H2o2) scavenging assay (HRSA)

H_2O_2 scavenging activity was determined according to Ruch etal., (1988). A solution of H_2O_2(10mM) was prepared in

phosphate buffer (pH7.4). Reaction mixture containing 2.5ml of H_2O_2 solution and 0.1, 0.3, 0.5ml of varying concentration of algal extracts (0.5, 1.5 &2.5 mg) was made up of to 3ml with phosphate buffer. The absorbance was measured at 0min and after 60 min at 240nm. Ascorbic acid was used as the control. Total H_2O_2 scavenging activity was expressed in %.

HRSA(%) = [Abs(control) – Abs (sample) / Abs (control)] x 100

Thiobarbituric Acid Reactive Assay (TBARS)

The assay was performed as described by Halliwell Gutteridge (1999) ,in which the extent of LPO was estimated from the concentration of Malondialdehyde,, a thiobarbituric acid reacting substance which is produced due to lipid peroxidation.

50µg, 10 µg, 150 µg, 200 µ g and 250 µg of the algal extract were taken in a test tubes and were evaporated to dryness at 80°C .1 ml of 0.15M potassium chloride was added to the tubes and followed by 0.5ml of goat liver homogenate (10% W/V in Phosphate buffered saline; calcium magnesium free). Peroxidation was initiated by the addition of 100 µl of 2mM ferric chloride. After incubating the tubes for 30min at 37°C, the peroxidation reaction was stopped by adding 2ml of ice-cold HCL (0.25N) containing 15% TCA & 0.38% TBA. The tubes were kept at 80°C for 1 hr, cooled and centrifuged at 7500rpm. The absorbance of the supernatant, containing TBA-MDA complex was read at 532nm. The anti-lipid peroxidation activity (ALP%) was calculated using the formula :

$$\text{ALP\%} = \frac{[\text{Abs (control)} - \text{Abs (sample)}]}{\text{Abs (control)}} \times 100$$

Ferric Reducing antioxidant Power Assay

Ferric Reducing antioxidant Power Assay was determined by the method described by Omidreza *et. al* ., (2000).

MTT Assay

In this study, cancer cell growth inhibition activity was measured by using MTT assay (Mossman,1983; Carmichael *et.al.*, 1987). Hep-2 cell lines were maintained in minimal essential media(MEM) containing 10% heat inactivated fetal calf serum (FCS) supplemented with penicillin (100 µg/ml)

and streptomycin (100 µg/ml) at 37°c under 5% co2 in air. 48hrs monolayer culture of Hep2 cells at a concentration of 1 lakh / ml /well seeded in 24 well titre plate.

The monolayer of cells was washed twice with MEM without FCS to remove the dead cells and excess FCS. To the washed cell sheet, add 1ml of medium (without FCS) containing defined concentration of the drug in respective wells. Each dilution of the drug ranges from 1:1 to 1:64 and they were added to the respective wells of the 24 well titre plates. To the cell control wells add 1 ml MEM without FCS. Plates were incubated at 37oc in 5% co2 environment and observed for cytotoxicity using inverted microscope. In each well add 200ul of MTT concentration (5 mg/well) cells were then incubated for cytotoxicity. Add 1ml of DMSO in each well and mix, leave for 45 seconds. If any viable cells present formazan crystals after adding solubilizing reagent (DMSO) shows the purple colour formation. The suspension is transferred into the cuvette of spectro photometer and an OD values was read at 595mm by taking DMSO as a blank. Graph is plotted by taking concentration of the drug on X axis and relative cell viability on Y axis.

Cell Viability (%) = Mean OD/Control OD x 100

All determinations were carried out in triplicates. The IC_{50}, the antiproliferative activity of the tested enzymatic fractions was determined in terms of the amount (µg/ml) of the extract necessary for inhibiting 50% of the cell growth.

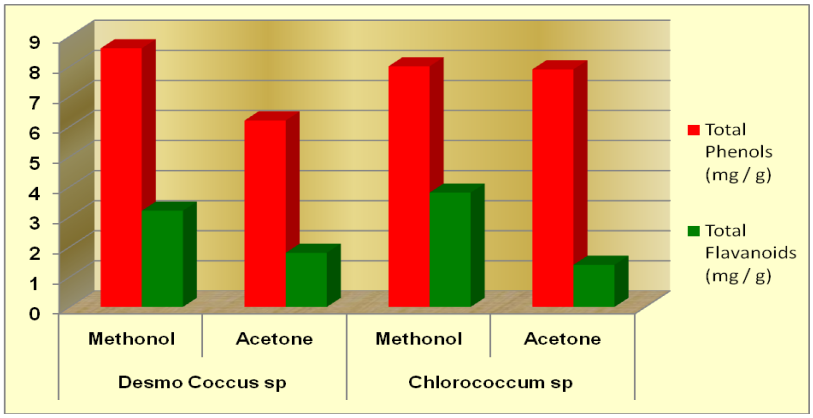
DNA fragmentation Analysis

A semiquantitative method for measuring apoptosis was described by Bortner, *et. al* (1995).

Results and Discussion

Total phenolic and flavonoid contents in the methanolic and acetone extracts were expressed as mg/g and were presented in **Fig 1.** The methanolic extract had higher phenolic and flavonoid content than acetone extract of *D. olivaceous*, and . *C.humicola* . This may be due to the differences in the polarity of the two solvents used and there by the different phenolic components differentially eluted.

FIG. 1. TOTAL PHENOLIC AND FLAVONOID CONTENTS OF METHANOLIC AND ACETONE EXTRACTS OF *DESMOCOCCUS OLIVACEOUS* AND CHLOROCOCCUM *HUMICOLA*



DPPH radical scavenging assay

Both methanolic and acetone extracts of *D. olivaceous* and *C . humicola* showed a significant dose dependent reduction of DPPH radicals. The scavenging action was higher in *Desmococcus* (95.8%) in comparison with the *Chlorococcum* (93%). This assay revealed that the extracts might prevent reactive radical species from damaging biomolecules such as Lipoprotein, DNA, aminoacids, sugar, proteins and PUFA in biological and food systems**.(Table 1)**

TABLE.1. DPPH RADICAL SCAVENGING ACTIVITY OF METHANOL AND ACETONE EXTRACTS OF *DESMOCOCCUS OLIVACEOUS* AND *CHLOROCOCCUM HUMICOLA*

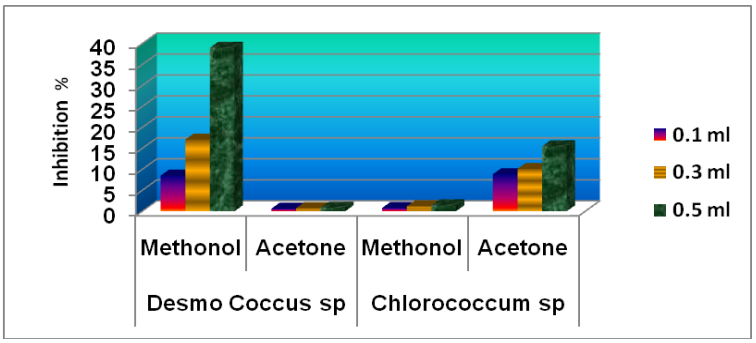
Concentraction Of Extracts (µg)	**Inhibition %**			
	Desmococcus olivaceous		***Chlorococcum humicola***	
	Methanol	**Acetone**	**Methanol**	**Acetone**
250	92.6 ± 0.5	92 ± 1.6	91 ± 1.4	87.3 ± 1.5
500	94 ± 1.5	94 ± 1.6	92 ± 1.6	89 ± 1
750	95.8 ± 0.7	95.8 ± 0.5	94 ± 1.8	93 ± 1.5

The values were expressed as inhibition %; Data's are mean of triplicate determination mean ± S.D (n=3)

Hydrogen peroxide scavenging assay (HRSA)

In this study, methanolic extract of *D. olivaceous* at 2.5mg/0.5ml exhibited 39% scavenging activity (Fig 2) and the acetone extracts of *C. humicola* exhibited, 15% scavenging activity. Acetone extract of *Desmococcus* and methanolic extract of *Chlorococcum* showed relatively low H_2O_2 scavenging activities. However, the activity increases with the sample concentration.(Fig 2)

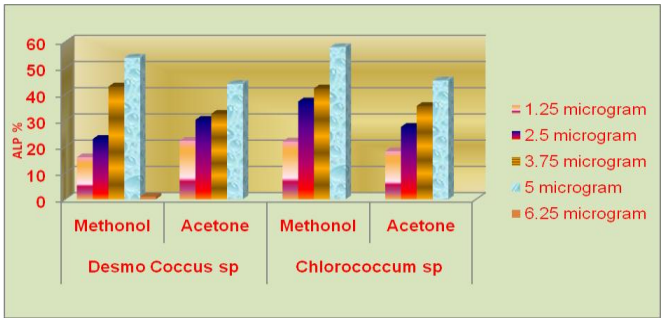
FIG 2 HYDROGEN PEROXIDE SCAVENGING ASSAY OF METHANOLIC AND ACETONE EXTRACTS OF *DESMOCCOCUS OLIVACEOUS* AND *CHLOROCOCCUM HUMICOLA*



TBARS assay

Both extracts were capable of preventing the formation of MDA in a dose dependent manner. The methanolic extracts of *D. olivaceous* & *C. humicola* was observed to be significantly better inhibitor of Lipid peroxidation compared to acetone extract. (Fig III) shows the ALP % potential of the extracts of *Desmococcus* and *Chlorococcum*.

FIG 3 ANTI LIPID PEROXIDATION ACTIVITY OF METHANOLIC AND ACETONE EXTRACTS OF DESMOCOCCUS OLIVACEOUS & CHLOROCOCCUM HUMICOLA

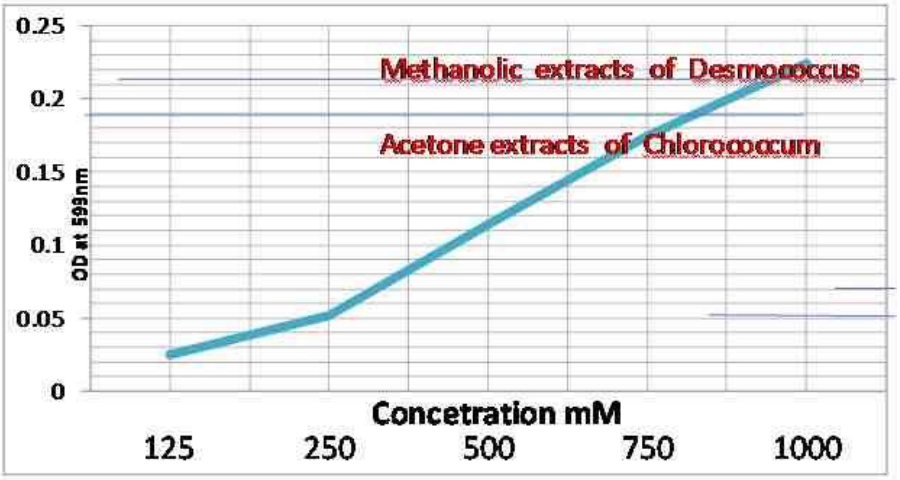


FRAP ASSAY

Methanolic extracts of *Desmococcus* and acetone extracts of *Chlorococcum* showed maximum ferric reducing antioxidant power.(Table 2 &Fig 4)

TABLE 2 & FIG 4. FERRIC REDUCING ANTIOXIDANT POWER ASSAY (FRAP)

Sl. No.	Contents	BHT control (0.16mg/ml)	*Desmococcus* sp		*Chlorococcum* sp	
			Methanol	Acetone	Methanol	Acetone
1	Absorbance at 593 nm	0.063	0.211	0.101	0.035	0.183



MTT assay

Of the extracts tested, the methanolic extract of *D. olivaceous* had the highest cell growth inhibition activity with an average IC50 value of 0.156mg/ml. In addition, the acetone extract of *Chlorococcum* also showed good cell growth inhibition activity on HEP-2 cell line (0.625mg/ml IC50). These extracts induced high antiproliferative activity in a dose dependent manner.. (Fig 5 a & b)

FIG 5 a INVITRO ANTI PROLIFERATIVE ACTIVITY OF METHANOL EXTRACTS OF *DESMOCOCCUS OLIVACEOUS*

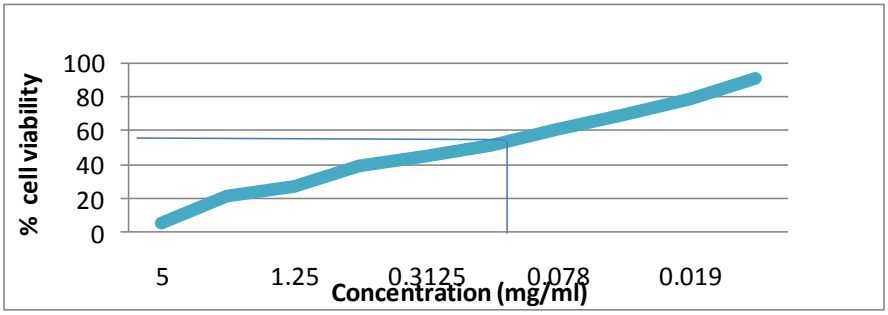
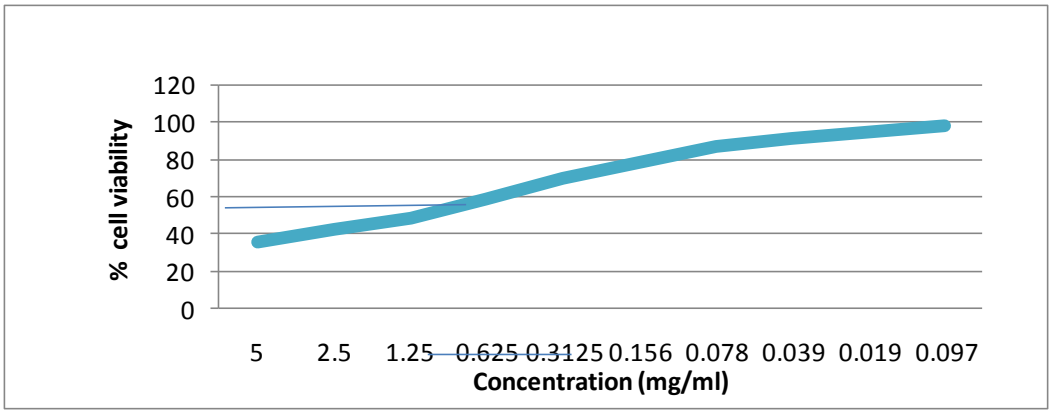


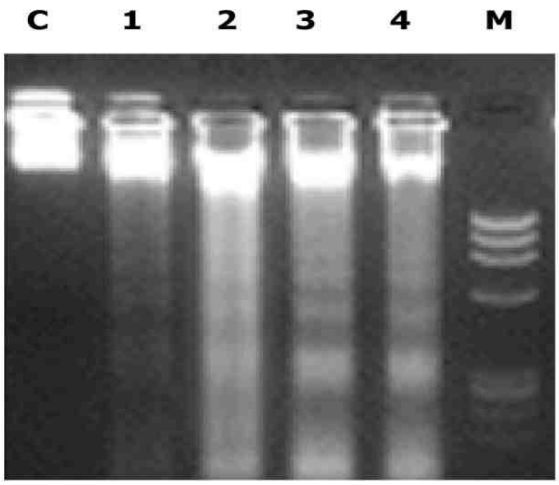
FIG 5 B INVITRO ANTI PROLIFERATIVE ACTIVITY OF ACETONE EXTRACTS OF *CHLOROCOCCUM HUMICOLA*



DNA Fragmentation analysis

In control lane, DNA is not fragmented. In lane 1 ,2 ,3 & 4 acetone extracts of *Chlorococcum* and methanolic extracts of *Desmococcus* treated with Hep-2 cell line, DNA was fragmented. Hence, 3 bands ranging from 180 to 220bp was obtained**.** **(**Fig 6)

FIG 6 DNA FRAGMENTATION BY AGAROSE GEL ELECTRO PHORESIS



The Figure 6 shows the electrophoretic pattern of DNA (c) Control and presence (1 ,2 ,3 ,4) of the acetone extracts of ***Chlorococcum*** **and methanolic extracts of** ***Desmococcus*****, (M) marker-1000bp ladder.**

Conclusion

Methanolic & acetone extracts of both *Desmococcus olivaceous* and *Chlrococcum humicola* found to possess antioxidant activity including reducing power, free radical scavenging activity (DPPH, H_2O_2) and lipid peroxidation inhibition potential. The methanolic extracts displayed greater potential in all antioxidant assays. Methanolic extract of *D. olivaceous* and acetone extract of *C. humicola* had the antiproliferative acticity on Hep2 cell line, these extracts demonstrated higher DNA damage. Cell growth inhibition was mainly due to Apoptosis, proved by DNA fragmentation analysis. Present findings encourage for further studies for isolation and identifying of active components in green algae and also in vivo studies for their mechanism of action.

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