

# Investigation of Antioxidant Capacity and Phytochemical Composition of Sun Chlorella -An Invitro Study

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## Abstract

Sun Chlorella is a natural whole food supplement derived from a fresh water single celled green algae called *Chlorella pyrenoidosa*. The aqueous and organic extracts (hexane and ethyl acetate) obtained from Sun chlorella were screened on reactive oxygen species scavenging capacity, total antioxidant capacity (TAC) and lipid peroxidation inhibition potential. The aqueous extract compared to hexane and ethyl acetate extracts exhibited significant high levels of antioxidant potential, lipid peroxidation inhibition potential along with phenolic and flavonoid contents. In addition, the correlation coefficient between the TAC, phenolic content was found to be significantly positive for the aqueous extract ( $R^2 = 0.0995$ ) when compared to hexane ( $R^2 = 0.0162$ ), ethyl acetate ( $R^2 = 0.0395$ ) extracts. As the aqueous extract of sun chlorella showed significant antioxidant potential by positively modulating the antioxidant activity in *in vitro* (fish liver homogenate sample) study sample, it is rightly to be considered as a fish feed.

**Keywords:** Antioxidant potential; Sun Chlorella; Lipid peroxidation; Microalgae; Phytochemical analysis; Carotenoids

## Introduction

Oxidative stress occurs as a consequence of excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and it is ameliorated by endogenous antioxidant enzyme activity and exogenous dietary antioxidants [1]. Antioxidants present in the diet can delay lipid peroxidation by inhibiting the initiation or propagation phase of oxidizing chain reactions by scavenging free radical [2]. Algal biomass and algal -derived compounds have a very wide range of potential applications, from animal feed aquaculture to human nutrition and health products [3]. Some algae are considered as rich source of natural antioxidants. The extracts from selected green, brown and red algae have been reported to demonstrate antioxidant activity by a variety of invitro methodologies. The above evidence suggests a potential for protective effects of microalgae against lipid peroxidation and oxidative stress. Sun Chlorella is a natural whole food supplement derived from a superior species of fresh water single celled green algae called *Chlorella pyrenoidosa*. *Chlorella pyrenoidosa* has a wide range of potent antioxidants including chlorophyll, Chlorella Growth Factor (CGF), beta-carotene, vitamin E, vitamin C and polyphenolic compounds. Chlorella species of green algae comprise a suite of enduring dietary supplement ingredients that have been touted to have almost panacea-like properties. Despite its intense green color (due to the high content of chlorophyll), Chlorella species also harbour robust quantities of the carotenoid lutein [4]. Hence the current study involves the evaluation and comparison of the antioxidant potential of the aqueous and different organic extracts of Sun chlorella.

## Materials and Methods

### Chemicals

Rutin was obtained from Acros Organics (New Jersey, USA). 1,1-diphenyl-2-picryl hydrazyl (DPPH) was purchased from Sigma, India. Nitro blue tetrazolium (NBT), nicotinamide adenine dinucleotide phosphate reduced (NADH), phenazine methosulphate (PMS), trichloro acetic acid (TCA), thiobarbituric acid (TBA), ethylene diamine tetra acetic acid (EDTA), hydrogen peroxide ( $H_2O_2$ ), ferric chloride, potassium ferric cyanide, naphthyl ethylene diamine dihydrochloride (NEDD), sodium nitro prusside, 2-Deoxy-2-ribose and butylated

hydroxy toluene (BHT) were obtained from SRL Chemicals (Mumbai, India). Ascorbic acid and vitamin E were obtained from SD Fine Chem. Ltd. (Biosar, India).

### Sample preparation

A precisely weighed (~ 0.2 g) amount of commercially available Sun Chlorella was extracted with 2 ml of hexane for 30 minutes at 20°C temperature. The tube was centrifuged at 4500g for 10 min and the supernatant was recovered. The extraction was repeated with 2 ml of hexane and the two supernatants were combined. The residue was subsequently extracted twice with ethyl acetate (2 ml each time) for 30 min at 20°C temperature and the supernatants were combined. Then, the residues were further extracted twice with water (2ml each time) for 30 min at 80°C and the supernatants were combined. The hexane and the ethyl acetate extracts were purged to dryness using nitrogen and together with the aqueous extracts were stored at -10°C before use. The aqueous extract was directly used in antioxidant assays, while hexane and ethylacetate fractions were diluted appropriately with ethanol and immediately used in the antioxidant assays.

### In vitro study model preparation

The *Mugil cephalus* commonly called as grey mullet fish was used as the study material for the invitro study. It was sacrificed by severing their spinal cord and the liver was removed immediately. One gram of liver tissue was weighed, washed twice with ice cold Kohler's homogenizing buffer (250mM Sucrose, 5mM MOPS buffer,

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1mM EDTA, 0.1 % ethanol, 200mM PMSF in isopropanol and  $\epsilon$ -amino caproic acid pH 7.4) and minced into small sections. The homogenate was prepared by adding 5 mL of the same buffer and using approximately six strokes. The resultant was centrifuged at 2000 rpm for 10 min. The supernatant was stored at 4°C and used as the homogenate for TBARS formation inhibition studies.

#### Antioxidant potential assays

**DPPH Radical scavenging assay:** 1, 1-diphenyl -2-picrylhydrazyl (DPPH) scavenging potential of different solvent extracts of Sun Chlorella was measured based on scavenging ability of antioxidants present in it. The method based on Navarro et al., 1993 as modified by Brand-Williams [5] was employed to investigate the free radical scavenging activity of varying concentrations (2-10 mg/mL) in different solvent extracts of Sun chlorella [5]. An inhibition curve was plotted using vitamin C as standard and compared between different solvent fractions.

**Superoxide anion ( $O_2^-$ ) scavenging assay:** Nagai et al. [6] method was used for assessing the superoxide anion scavenging ability of Sun Chlorella using PMS-NBT system in varying concentration (2-10 mg / mL) of different extracts of Sun Chlorella [6]. Curcumin was used as a positive control in this assay.

**Hydroxyl radical ( $OH^-$ ) scavenging assay:** The ability of different solvent extracts of Sun Chlorella to scavenge the hydroxyl radicals generated by the Fenton reaction was measured by Halliwell et al. [7] method using 2-deoxy -2-ribose and EDTA. The reaction was performed in 100 $\mu$ L of 1.0 mM ascorbic acid and 100 $\mu$ L of varying concentrations (2-10 mg / mL) of different solvent extracts of Sun Chlorella using vitamin E as the standard.

**Nitric oxide radical (NO) scavenging assay:** Nitric oxide radical scavenging activity was determined by the use of the Griess Illosvay reaction [8] using naphthylethylene diamine dihydrochloride (NEDD) reagent in 0.5 mL of varying concentrations (2-10 mg/ mL) of different solvent extracts of Sun Chlorella using rutin as standard.

**Reducing activity:** Reducing activity was evaluated according to the method of Oyaizu [9] in one mL of varying concentrations (0.2 -1.0 mg/mL) of different solvent extracts of Sun Chlorella using aqueous solution of Vitamin C as standard [9].

#### ABTS<sup>+</sup> radical cation scavenging activity

Total antioxidant capacity of the three different extracts of Sun Chlorella was evaluated by its ability to scavenge ABTS<sup>+</sup> radical cation according to the method described by Erel [10] in 5 $\mu$ L of varying concentrations (2-10 mg/mL) of different solvent extracts of Sun Chlorella [10]. The degree of quenching of free radical generation in individual sample was quantified by comparing the assay results to a traditional standard trolox.

#### In vitro Lipid peroxidation inhibition potential assay

This assay was based on the method described by Liu [11]. In the present study lipid peroxidation inhibition potential of different solvent extracts of Sun Chlorella was identified by their ability to inhibit CuSO<sub>4</sub> induced lipid oxidation process in fish liver homogenate using malondialdehyde as the standard. Briefly, the reaction mixture composed of 0.1 mL of fish liver homogenate and 0.1 mL of 5 $\mu$ M CuSO<sub>4</sub> was incubated in the absence and presence of different concentrations (2-10mg/mL) of different solvent extracts of Sun Chlorella at 37°C for 1 hour and the inhibition of TBARS formation was determined. The reaction was stopped after the incubation

period by the addition of 20% acetic acid, 8.1% SDS and 0.8% TBA and incubated at 80°C for 20 minutes. The mixture was cooled, centrifuged at 1000 rpm for 10 minutes and the intensity of the MDA-TBA complex in the supernatant was measured using its absorbance at 532 nm. Antioxidant activity was calculated as percent inhibition of peroxidation relative to control. The concentration of extract causing a 50 % reduction in lipid oxidation IC<sub>50</sub> was then calculated.

#### Identification of phyto-components of Sun Chlorella

The phyto-components present in the aqueous extract of Sun Chlorella was identified by means of qualitative analysis: phenols were identified using Folin-ciocalteau reagent; flavonoids by reaction with magnesium and concentrated hydrochloric acid; reducing sugar by heating with benedict's reagent; alkaloids with 2N hydrochloric acid and Mayer's reagent; carotenoids by TLC method; saponins by shaking with water, tannins by treatment with lead acetate; quinines by reaction with concentrated sulphuric acid; coumarins identified with alcoholic sodium hydroxide; anthroquinones reaction with magnesium acetate.

#### Analysis of phytochemical constituents of Sun Chlorella extract

**HPTLC Analysis:** The aqueous extract of Sun Chlorella was subjected to HPTLC analysis using CAMAG TLC scanner III (Camag, Muttenz, Switzerland) [12]. An aliquot of about 10 $\mu$ L of Sun Chlorella extract was applied as a 0.5 cm band on a 2-mm thick silica gel G coated aluminum plate (Merk, Silica Gel GF254, Germany). The spot was dried and the plate was developed using the mobile phase that contained butanol, glacial acetic acid and water in the ratio 65:15:25. The developed plate was dried at room temperature and the naturally colored spot was then scanned with a CAMAG TLC Scanner III in the ascending mode at 254 nm coupled with SP 4100 integrator.

**Gas Chromatography-Mass Spectrometry (GC-MS) analysis:** 20 g of the powdered sample of Sun chlorella was taken in a beaker to which 50 ml of absolute alcohol was added and kept soaked over night. The volatiles were steam-distilled and filtered along with 2 gm sodium sulfate using Whatmann filter paper No. 41 (wetted with ethanol) to remove the sediments and traces of water in the filtrate. The filtrate was concentrated and the volume was reduced to 1 ml by bubbling nitrogen gas into the solution.

The GC-MS analysis of volatile components of Sun Chlorella were carried out on a GC Clarus 500 Perkin Elmer, equipped with an Elite-1 (100% Dimethyl Poly Siloxane 30 m x 0.25 mm ID) with 1 $\mu$ m film thickness. The conditions of the analysis were as follows: injection temperature 250°C; oven temperature maintained at 110°C for 2 min, followed by a linear programmed temperature from 110 to 200°C at a rate of 10°C for one min and from 200 to 280°C at a rate of 5°C min<sup>-1</sup> for 9 min. The flow rate of the carrier gas, helium was 1 ml min<sup>-1</sup>; 2 $\mu$ l of the sample was injected. The Turbo mass gold mass spectrometer model had electron energy of 70 eV, inlet line temperature of 200°C and source temperature of 200°C with a mass range (m/z) of 45-450 a.m.u. The identification of each compound was carried out by comparison of relative retention time and mass spectral data obtained with literature and a computerized MS data bank (NIST ver. 2.0- year 2005).

**Determination of total phenolics and flavonoids:** Total phenolic compounds were determined according to the protocol described by Chandler and Dodds [13] using Folin-Ciocalteau reagent in different solvent fractions of Sun Chlorella (2 mL) [13]. A gallic acid standard curve was obtained for the calculation of phenolic content.

The flavonoid content was determined by a colorimetric method involving aluminium chloride in different solvent extracts of Sun Chlorella using rutin as standard [14]. The flavonoid contents were expressed in terms of mg rutin equivalents/g.

## Statistical Analysis

Results were expressed as mean value  $\pm$  SD. The values were subjected to statistical analysis using normal tests of significance. The statistical significance was arrived by comparing the results of the three different types of extract using One Way ANOVA [15]. The SPSS software package version 7.5 was used to test the significance of the experiments performed and for correlation analysis. Differences were taken to be statistically significant for values of \*\* $p < 0.001$  and \* $p < 0.01$ .

## Results

### DPPH· radical scavenging activity

DPPH· is one of the chemical compound that possess a proton free radical and it shows a maximum absorption at 517 nm because of its bright purple colour. When DPPH· encounters proton radical , its purple colour fades rapidly and this scavenging action forms the basic mechanism for measuring antioxidant activity. As shown in (Table 1), the aqueous extract of Sun Chlorella showed a significant dose dependent inhibition of DPPH· radical scavenging activity ( $p < 0.01$ ) compared to organic fractions with a 50% inhibition ( $IC_{50}$ ) at a concentration of 10mg/mL. When considering the organic fractions

of Sun Chlorella, the DPPH· radical scavenging capacity was more towards the ethyl acetate fraction followed by hexane. The activities of the Sun Chlorella extracts were dose dependent characterized by increasing scavenging activity with rise in sample concentration, though the activities were lower than the standard.

### Superoxide anion ( $O_2^-$ ) scavenging activity

The superoxide anion derived from dissolved oxygen by phenazine methosulphate/NADH-coupling reaction reduces nitrobluetetrazolium. The decrease in the absorbance at 560 nm with the different solvent extracts of Sun Chlorella indicates the consumption of  $O_2^-$  anion in the reaction mixture. As represented in (Table 2), all the solvent extracts of Sun Chlorella and the standard curcumin showed the scavenging activity but the scavenging effect was significantly higher in the hexane extract of Sun Chlorella (\* $p < 0.01$ ) (but less than the standard) followed by water and ethyl acetate extracts. The results also revealed the scavenging potential of the extracts in a dose-dependent manner.  $IC_{50}$  values for the standard and the various extracts are 4 and 6mg/mL, respectively.

### Hydroxyl radical ( $OH^-$ ) scavenging activity

To attack the substrate deoxyribose, hydroxyl radicals were generated by reaction of Ferric-EDTA together with  $H_2O_2$  and ascorbic acid. Hydroxyl radical scavenging activity of different solvent extracts of Sun Chlorella determined using the 2-deoxy ribose assay is shown in (Table 2). The effectiveness of the different extracts of Sun Chlorella in inhibiting 2-deoxy ribose degradation was less than

Concentration (mg/mL)	DPPH Radical Scavenging assay					Reducing Power				
	2	4	6	8	10	0.2	0.4	0.6	0.8	1
Standard	24±1.53	31±1.25	35±1.72	46±1.89	55±2.89	0.04±0.003	0.08±0.004	0.11±0.005	0.16±0.004	0.21±0.005
Aqueous extract	22±36*	25±97*	33±0.85*	42±0.59*	52±1.25*	0.03±0.003	0.06±0.003	0.0944±0.004	0.12±0.005	0.15±0.005
Hexane extract	10±37	11±57	16±0.9	19±0.45	24±0.64	0.02±0.003*	0.045±0.004*	0.07±0.004*	0.09±0.004*	0.11±0.005*
Ethylacetate extract	15±76	21±89	26±0.67	29±0.69	33±0.84	0.02±0.002	0.04±0.002	0.06±0.003	0.08±0.002	0.1±0.003

Results are mean  $\pm$  S.D. of five parallel measurements of different plant extracts

\* $p < 0.01$  statistically significant when compared between the three extracts

**Table 1:** DPPH radical scavenging activity and Reducing power of the aqueous extract and the organic extracts (hexane and ethylacetate) of Sun Chlorella.

Concentra-tion (mg/ mL)	NO radical inhibition assay					Hydroxyl radical scavenging assay					Superoxide anion scavenging activity				
	2	4	6	8	10	2	4	6	8	10	2	4	6	8	10
Standard	17±1.08	27±1.54	39±1.8	45±1.89	54±1.48	42±1.78	51±2.26	59±1.49	66±1.67	76±2.1	39±2.13	47±2.45	59±3.56	65±4.78	94±5.74
Aqueous extract	14±0.42*	22±1.13*	29±0.97*	38±1.45*	46±1.79*	34±1.68*	41±1.75*	52±0.91*	59±1.23*	67±1.57*	29±2.45	35±1.13	49±1.34	54±2.78	78±2.56
Hexane extract	13±0.45	20±0.58	26±0.96	34±1.12	41±1.58	29±1.23	36±1.72	42±0.75	49±1.45	57±1.93	34±1.21*	43±1.36*	52±1.45*	62±2.12*	85±2.58*
Ethylacetate extract	10±0.15	14±0.38	19±0.61	26±0.96	33±1.35	21±1.12	29±1.25	36±1.49	42±2.12	47±1.98	21±1.19	35±1.23	41±1.38	52±1.58	67±1.97

Results are mean  $\pm$  S.D. of five parallel measurements of different plant extracts

\* $p < 0.01$  statistically significant when compared between the three extracts

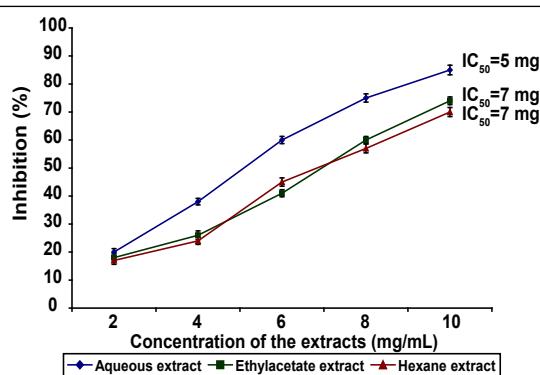
**Table 2:** Superoxide scavenging activity, NO radical scavenging activity and hydroxyl radical scavenging activity of the aqueous extract and the organic extracts (hexane and ethylacetate) of Sun Chlorella.

S.No	Parameters	Different type of extracts		
		Aqueous Extract	Hexane Extract	Ethylacetate Extract
1	Total Phenols (mg/ g dry weight)	2.47 0.12*	1.64 $\pm$ 0.13	1.32 $\pm$ 0.09
2	Flavonoids (mg/ g dry weight)	1.86 $\pm$ 0.06*	1.39 $\pm$ 0.08	1.23 $\pm$ 0.07
3	Total Antioxidant Capacity (µmoles of trolox/g)	19.2 $\pm$ 0.754	10.5 $\pm$ 0.643	8.58 $\pm$ 0.532

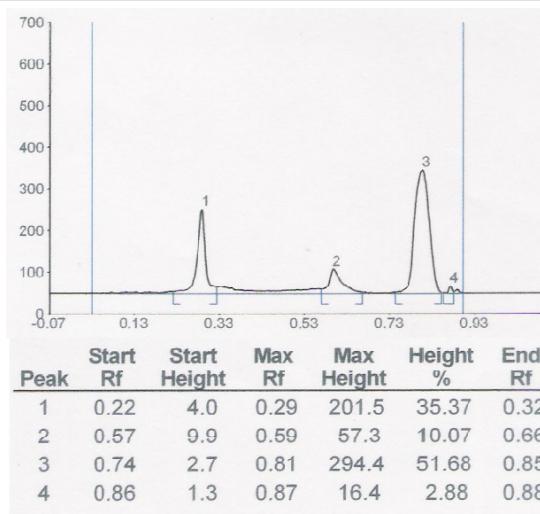
Results are mean  $\pm$  S.D. of five parallel measurements of different plant extracts

\* $p < 0.01$  statistically significant when compared between the three extracts

**Table 2:** Phytochemical composition analysis and Total antioxidant capacity of the aqueous extract and the organic extracts (hexane and ethylacetate ) of the green algae Sun Chlorella.



**Figure 1:** *In vitro* study on the lipid peroxidation inhibition potential of aqueous extract and the organic extracts (hexane and ethylacetate) of the green algae *C.pyrenoidosa*. Results are mean  $\pm$  S.D. of five parallel measurements of different plants.



**Figure 2:** HPTLC chromatogram of the phytochemicals from the aqueous extract of the green algae Sun Chlorella.

that of vitamin E and decreased in the order of aqueous > hexane > ethyl acetate. The scavenging ability of the extracts of Sun Chlorella was dose dependent and increased proportionately with extracts concentration. Hence, according to this result the hydrophilic phenolics are dominant in Sun Chlorella which are attributed to its radical scavenging properties.

#### Nitric Oxide (NO) radical scavenging activity

The NO· radical scavenging activity of the different solvent fractions of Sun Chlorella is shown in (Table 2). The maximum NO· radical inhibition property was observed with aqueous extract of Sun Chlorella ( $IC_{50}$  9 mg) compared to the organic extracts but was less than the standard. Among the organic extracts, the hexane fraction showed greater NO· radical inhibition property than the ethyl acetate fraction. The NO· radical scavenging potential of the extracts were also dose dependent and increased proportionately in relation with the concentration. The results suggest that hydrophilic antioxidants are abundantly present being responsible for this chemical properties.

#### Reducing power

The reducing activity of compound depends on the presence of reductones which exhibits antioxidative potential by breaking

the chain reaction of free radicals. The presence of reductants (antioxidants) in the fraction of Sun Chlorella extract causes the reduction of the  $Fe^{3+}$ /ferricyanide complex to the ferrous form, so the  $Fe^{2+}$  can be monitored by measuring the formation of Perl's prussian blue at 700 nm. The reducing power of different solvents extracts of Sun Chlorella decreased in the order of aqueous > hexane > ethyl acetate (Table 1). The reducing ability of each fraction was also dose-dependent, the results indicates the presence of more hydrophilic phenolic in all the extracts, the values being directly correlated with reducing capacities.

#### ABTS<sup>+</sup> radical cation scavenging activity

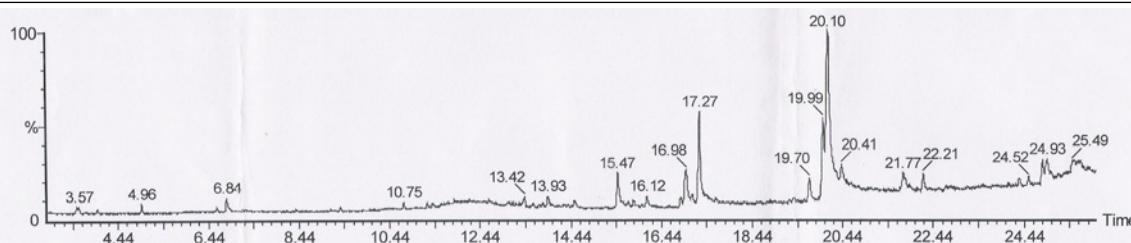
Total antioxidant capacity of different fractions of the Sun Chlorella was determined with ABTS<sup>+</sup> radical decolorisation assay and illustrated in (Table 3). This assay which is based on the ability of a compound to scavenge ABTS<sup>+</sup> radical indicated that the aqueous extract of Sun Chlorella possessed excellent TAC or ABTS<sup>+</sup> radical scavenging activity compared to organic extracts. Comparison between organic fractions revealed greater TAC level in ethyl acetate fraction than hexane fraction. The results further indicated that though the scavenging effect of Sun Chlorella extracts were lesser than the standard trolox, the antioxidant activity increased in a dose dependent manner with increasing sample concentration.

#### Measurement of antioxidant capacity using thiobarbituric acid reactive substances formation inhibition studies

During lipid peroxidation, low molecular weight end products, probably malondialdehyde, are formed by oxidation of polyunsaturated fatty acids that can be reacted with two molecule of thiobarbituric acid to give a pinkish red chromogen. Inhibition of lipid peroxidation potential in fish liver homogenate was used to measure the antioxidant activity of different solvent extracts of Sun Chlorella. The  $CuSO_4$  induced TBARS formation inhibiting effect of the aqueous extract of Sun Chlorella was significantly higher than that of organic extracts. Within the organic fractions, ethyl acetate showed greater lipid peroxidation inhibition property compared to hexane extract. The antioxidant capacity was found to be more for the aqueous extract 85 % and its  $IC_{50}$  = 5mg which is highly significant when compared to ethylacetate 74 % ( $IC_{50}$  = 7 mg) and hexane 70 % ( $IC_{50}$  = 7 mg) extracts (Figure 1). Also, the inhibition effect observed with the extracts of Sun Chlorella was dose dependent. At the concentration of 2-4mg/mL, the lipid peroxidation inhibition % of all the three extracts were found to be below 30 %. At 10mg/mL concentration, 85 % inhibition is observed with the aqueous extract while 74% and 70% inhibition was exhibited by ethylacetate and hexane fractions. This is a significant finding as the aqueous extract can provide a maximum inhibition at low concentration than the organic extracts. The result suggest that the antioxidant compounds that are present in the aqueous extract of Sun Chlorella are absorbed and would be used to prevent invitro lipid peroxidation and hence statistically significant antioxidant capacity when compared to organic extracts.

#### HPTLC analysis of the phytochemical constituents of Sun Chlorella extracts

Figure 2 represents the HPTLC of various phytochemical constituents of aqueous extracts of Sun Chlorella. The fingerprint shows the presence of 4 peaks at various Rf values like 0.22, 0.57, 0.74 and 0.86. The number of peak represents the number of different biologically active phytochemical constituents and the major peak area compounds may belong to the polyphenols and flavonoids.



**Figure 3:** GC-MS analysis Spectrum of the phytochemicals from the ethanolic extract of the green algae Sun Chlorella.

### GC-MS analysis of the phytochemical constituents of sun chlorella extracts

The GC separated compounds of Sun chlorella extracts which were identified from the recorded mass spectra by comparison with the mass spectra from the NIST library is summarized in (Figure 3). The constituents identified in the Sun chlorella are about 14 and the main constituents are 1,2-Benzenedicarboxylic acid, 9,12,15-Octadecatrienoic acid, n-Hexadecanoic acid, Octadecanoic acid and 9,12-Octadecadienoic acid etc.,

### Total phenolic and flavonoid contents and identification of phytocomponents

Phenolic compounds such as flavanoids, phenolic acids and tannins are considered to be major contributors to the antioxidant capacity of plants. For microalgae product like Sun Chlorella there is very limited information on their phenolic contents. In the present investigation, the total phenolic compound of the aqueous extract was about 2.47mg TAE/g (Table 3) and the phenolic content was quite large being significantly higher ( $^{**}p<0.001$ ) in the aqueous extract when compared to hexane and ethyl acetate extracts.

Qualitative analysis performed in the aqueous extract of Sun Chlorella demonstrated the presence of phytocomponents like phenolic compounds, flavonoids, reducing sugar, carotenoids, tannins, alkaloids anthraquinones and quinines. Glycosides was found to be absent in the extract.

### Correlation studies of antioxidant capacity assay or (TAC) with phenolic content

A relationship between the antioxidant activity and phenolic content of green algae was noted in the current study. To our knowledge this is the first report on green algae and its antioxidant activity. The correlation coefficient ( $R^2$ ) between the antioxidant capacity and the phenolic content of different extract of chlorella was determined. The correlation coefficient between the antioxidant capacities and the phenolic contents was found to be very small for hexane ( $R^2 = 0.0162$ ) and ethyl acetate ( $R^2 = 0.0395$ ) when compared to aqueous extract ( $R^2 = 0.0995$ ) fraction Thus the phenolic compounds were the major contributors to the antioxidant capacities of Chlorella.

### Discussion

Algae constitute a valid alternative to other protein foods of animal or vegetal origin [16]. They actually contain high concentration of the basic nourishment and are a source of high biological value proteins, minerals, vitamins and polyunsaturated fatty acids; like  $\gamma$ - linoleic acid. An algal antioxidant –mediated mechanism was hypothesized as a contributing factor for enhanced enzyme activity and reduced

lipid peroxides during inhibition of carcinogenesis as the antioxidant properties of plants could be correlated with oxidative stress defense in different human diseases [17,18]. This study is first of its kind to evaluate the antioxidant activity of different solvent extracts of the green algae Sun Chlorella in a comprehensive manner employing a variety of invitro methods. The results demonstrated that an aqueous extract compared to organic extracts of hexane and ethyl acetate from Sun Chlorella was more active in scavenging stable free radical DPPH, OH radicals, as well as quenching of NO<sup>•</sup> radical and ABTS<sup>+</sup> radical cation in separate model systems.

The ability of aqueous extract of Sun Chlorella to reduce the DPPH<sup>•</sup> radical by donating an electron or hydrogen atom was more than other extracts and many folds weaker than vitamin C in the present study. This ability to reduce and quench free radicals over a longer period of time may have benefits for extending shelf-life of processed foods during distribution and storage [19].

In the PMS/NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The decreased absorbance at 560 nm in the presence of antioxidants indicates the consumption of superoxide anion in the reaction mixture and hence directly correlates with the greater O<sub>2</sub><sup>•-</sup> anion scavenging property, attributed by various phenolics present in Sun Chlorella fractions. Therefore it can be suggested that the superoxide anion scavenging activity of aqueous extract of Sun Chlorella is beneficial to decrease toxicity of not only superoxide anion but also of hydroxyl radicals. Further the study results also revealed that though hexane extract had much higher O<sub>2</sub><sup>•-</sup> scavenging potential, aqueous extract also exhibited demonstrable effect on O<sub>2</sub><sup>•-</sup> scavenging and its potential almost becoming equal with hexane extract at 10mg/mL concentration.

In an individual experiment demonstrated to measure the hydroxyl radical scavenging potential, all the fractions of Sun Chlorella exhibited strong OH radical scavenging effect in a dose dependent manner; the excellent scavenging effect being observed with the aqueous extract of Sun Chlorella when compared with other organic solvent extracts. Both aqueous and organic extracts were able to prevent deoxyribose damage associated with the direct binding of iron to deoxyribose and the subsequent attack by OH radicals generated via the Fenton reaction.

Nitric oxide (NO<sup>•</sup>) in association with O<sub>2</sub><sup>•-</sup> react to produce reactive peroxynitrite (ONOO<sup>•</sup>) exerting a toxic effect on biomolecules like lipids, proteins and nucleic acids [20]. It has been reported that the interaction of nitric oxide with polyphenolic antioxidants is highly relevant in physiological and pathological cellular mechanism [21]. Hence the plant extracts rich in NO<sup>•</sup> scavenging properties can be beneficial under stress situation. In accordance with this, the ability of aqueous and organic extracts of Sun Chlorella to decrease the

amount of nitric oxide generated from the in vitro decomposition of sodium nitroprusside indicates the NO<sup>-</sup> scavenging potential and there by its protective role under oxidative stress condition.

For the measurements of reductive ability, the Fe<sup>3+</sup> to Fe<sup>2+</sup> transformation was investigated in the presence of hydroalcoholic extract. The reducing power was increased by increasing the amount of extract and the reducing capacity of compound may serve as a significant indicator of its potential antioxidant activity [22]. In this investigation, the aqueous extract of Sun Chlorella exhibited the greater reducing power compared to organic extracts and it was dose dependent. This reflects the presence of content like ascorbate in the extracts with high reducing ability being indicated by increased absorbance as a function of extract concentration.

Decolorization of the ABTS<sup>+</sup> radical cation similar to DPPH reflects the capacity of an antioxidant species to donate electrons or hydrogen atom to inactivate this radical species. The aqueous extracts of Sun Chlorella exhibited greater ABTS<sup>+</sup> cation scavenging activity compared to hexane and ethyl acetate extracts. Though a dose dependent scavenging potential was observed, the activity was less than the standard trolox.

The antioxidant activity of algal compounds are often described by their ability to delay the onset of auto-oxidation by scavenging ROS, or the ability to act as chain breaking antioxidants to inhibit the propagation phase of lipid auto-oxidation [23]. This report supports our observed study results with regard to inhibition of CuSO<sub>4</sub> induced TBARS production by different solvent extracts of Sun Chlorella indicating their lipid peroxidation inhibition potential. The production of TBARS was inhibited by all the fraction of Sun Chlorella, with the strongest effect being observed with aqueous extract compared to organic extracts. Amin [24] has demonstrated in vitro, the ability of green algal (*Chlorella vulgaris*) extracts to prevent lipid peroxidation supporting the current study results [24].

Chemical investigations by HPTLC indicated the presence of biologically active constituents in the extracts of Sun Chlorella and the major peak area compounds may belong to the polyphenols and flavonoids.

Similarly, phytochemical constituents analysis by GC-MS indicated the presence of different compounds like 1, 2-Benzenedicarboxylic acid, diisoctyl ester, 9,12,15 - Octadecatrienoic acid,(z,z,z)-, n-Hexadecanoic acid, Octadecanoic acid, 9,12-Octadecadienoic acid,(z,z)-, n-Hexanoic acid, Decanoic acid, Oleic acid etc., in Sun Chlorella.

Many studies have focused on the biological activities of plant-derived polyphenolic flavonoids as they are well known to exhibit antioxidant activity through a variety of mechanisms including scavenging of ROS, inhibiting lipid peroxidation as well as chelating metal ions while the phenolic compounds are reported as an index of antioxidant function [25]. Flavonoids are potent water soluble antioxidants which prevent oxidative cell damage along with strong anticancer activity and anti-inflammatory activity [26]. Total phenolic and flavonoid contents of different solvents extracts of Sun Chlorella were solvent dependent. Aqueous extracts of Sun Chlorella showed higher amount of phenolics and flavonoids, while their counterparts showed lower concentration. Hence the free radical scavenging activity of extracts of Sun Chlorella can be directly correlated with the presence of phenolic compounds and flavonoids in which the free hydroxyl group is mainly responsible for antioxidant activity [27]. As different solvent extracts of Sun Chlorella exhibited different reactive

oxygen species scavenging activities, there may be different kinds of total phenolic compounds (hydrophilic and hydrophobic) present in different Sun Chlorella extracts. Also greater free radical scavenging potential observed in aqueous extract indicates the prevalence of enormous amount of hydrophilic antioxidant. Apart from the presence of total phenols and flavonoids, a preliminary phytochemical analysis of Sun Chlorella revealed the presence of different constituents like steroids, phenolic compounds, reducing sugar, flavonoids, glycosides, saponins, alkaloids, tannins, anthroquinones and catechins in varying concentrations in different extracts which can also be correlated to ROS scavenging properties to an extent.

In summary, an aqueous extract derived from Sun Chlorella exhibited DPPH, ·OH, NO, radicals scavenging activity as well as the ability to quench ABTS<sup>+</sup> free radicals in vitro. The O<sub>2</sub><sup>-</sup> anion radical was significantly scavenged by the hexane extract. The antioxidant activity observed over a prolonged period of time may be beneficial in applications to extend product shelf life. The efficacy of the Sun Chlorella extracts in quenching ROS and free radical species was confirmed by the inhibition of TBARS productions of lipid oxidation in fish liver homogenate. The antioxidant potential of Sun Chlorella extract was further demonstrated through total phenolic and flavonoid contents and phytochemical analysis. The current study results therefore support the use of Sun Chlorella as a natural antioxidative source and demonstrate the importance of the measurement of antioxidant activity via various free radical scavenging systems and phytochemical composition analysis. The chlorella species are noted to contain not only labile antioxidant (i.e ascorbate , glutathione) but also more stable molecule such as chlorophyll, carotenoids and a variety of polyphenols which are suggested as the active components with antioxidative activity [28].

Hence the increased consumption of Sun Chlorella may contribute to the improvement in quality of health by increasing the antioxidant defense, delaying the onset of oxidative stress mediated diseases and importantly could have a significant advantage over the synthetic antioxidants in food. Furthermore, the research performed on the Sun Chlorella confirms their completeness and their regenerative antioxidant effects as it inhibits the production of lipid peroxides in fish liver homogenate. The observed results with the aqueous extracts regarding the free radical scavenging potential are advantageous because of their richness in hydrophilic antioxidants and it can be rightly considered as an antioxidant supplement for fish inhabiting contaminated areas and aquaculture farms to alleviate pollutant stress.

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