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Antioxidant potential phytochemicals from methanol extract of *Chlorella vulgaris* and *Chlamydomonas reinhardtii*

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Abstract

Microalgae are indigenous source of phytochemicals potent of being used in medicinal applications and in numerous natural health products but the research reports on their phytochemicals are of less count than on seaweeds. The purpose of the present study is to evaluate the metabolites in the growth phase of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* and to determine the phytochemicals present in methanol extract. Cell density, pigments, protein, carbohydrate, lipid, flavonoid and phenols during the growth phase of microalgae was quantified and the algal cells were extracted for phytochemical analysis using methanol. The results from preliminary phytochemical tests, thin layer chromatographs, and antioxidant assays suggested these two microalgae to be potential natural antioxidant source in food supplements and pharmaceutical industries.

Introduction

Algae are rich source of structurally novel and biologically active metabolites which finds various application in pharmaceutical industries. The chemical compounds synthesized by algae are usually sorted into primary and secondary metabolites based on chemical class, biosynthetic origin and functional groups. Primary metabolites make up the physical integrity of a cell and are involved in metabolic process like building and maintaining of living cells while secondary metabolites are not vital for the survival of a cell. Both of these metabolites are pool of antioxidants such as carotenoids, astaxanthin, phenol and flavonoid derivatives. With the increasing concern on algae currently, microalgae are paid more attention as nutraceutical and health food in the markets. Many research studies suggest that biological composition of microalgae such as protein, carbohydrate, minerals and bioactive compounds have potential medicinal value which influence the nutritional value (Brown and Jeffrey 1992; Fuentes et al., 2000). Industrial and developmental sectors of natural product chemistry i.e., chemotaxonomy are also initiating the use of pigments as natural food dyes (Campo et al., 2000). In the present urge to discover novel and effective drugs against resistant pathogenic strains; algal derived compounds have a broad range of biological activities such as antibiotic, antiviral, antioxidant, antifouling, anti-inflammatory, cytotoxic and antimitotic activities which could be explored more (Salvador, 2007). Bioactive compounds: polyphenols, catechin, flavonols, glycosides, and phlorotannins discovered from methanol extract of red, green and brown algae are been reported to have uniqueness in their molecular skeleton and structures contributing to the strong antioxidant activity (Khoddami et al., 2013). Polyphenols for instant, uses its phenol rings as electron traps for free radicals (Zakaria et al., 2011). In the present investigation, the primary and secondary metabolites in *Chlorella vulgaris* and *Chlamydomonas reinhardtii* were studied and their phytochemicals present in the methanol extract was evaluated.

Materials and methods

Test organism and culturing condition

Algal strains *Chlorella vulgaris* and *Chlamydomonas reinhardtii* were collected from Algal Culture Collection, Center for Advanced Studies in Botany, University of Madras, Chennai, India and were inoculated in an inorganic Bold Basal medium. These cultures were maintained at 24 ± 1 ºC in a thermostatically controlled room and illuminated with cool inflorescence lamps (Phillips 40 W, cool day light 6500 K) at an intensity of 2000 lux in a 12:12 h light dark regime.

Measurement of primary and secondary metabolites

Growth of the algal cultures were monitored at the regular intervals of 5 days. Measurement of growth and synthesis of primary and secondary metabolites included the determination of cell density at 678 nm (Robert, 1979), chlorophylls, carotenoids, astaxanthin and total pigments according to Lichtenhaler (1987), total carbohydrates (Dubois et al., 1951), proteins (Bradford, 1976) lipid (Folch et al., 1956) Phenols (McDonald et al., 2001) and flavonoids (Chang et al., 2002).

Collection of algal mass

The log-phase culture of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* were used as feeder cultures for outdoor cultivation and were carried out at terrace in wide mouthed plastic tubs by inoculating 1500 mL of cultures in 15.0 L of medium separately; pH were adjusted to 8.5. *Chlorella vulgaris* and *Chlamydomonas reinhardtii* biomass were harvested and collected after 25 days.

Preparation of algal methanol extract

Ten g of fresh algal biomass of each culture equal to 1g of dried one were completely homogenized and extracted with100 mL of methanol solvent. Clarification of algal mixture were carried out by filtration method using Whatman No.1 filter paper. The clarified extracts were evaporated under a vacuum at 50˚C using rotary evaporator. The crude extracts were stored in the dark in a vial and kept at 4˚C until further analysis.

Preliminary phytochemical studies

The above condensed methanol extracts of algae, *C.vulgaris* and *C.reinhardtii* were preliminarily assessed for the phytochemicals such as phenol, flavonoid, saponins, glycosides, alkaloids, tannins and terpenoids (Trease and Evans, 1978).

Thin layer chromatographic study

Methanol extracts of both the cultures were used for chromatographic study to qualitatively determine the phytochemicals: phenols, flavonoids and saponins using silica gel 60 F254 – TLC aluminium sheets (Merck, Germany). Flavonoid compounds were separated using the solvent system of toluene: ethyl acetate (7:3) and separated fractions were detected under UV-365 nm light (Harborne, 1998). Phenolic compounds were separated using the solvent system of chloroform: methanol (9:1) and separated fractions were detected by spraying Folin-Ciocalteu reagent (Harborne, 1998). Saponins were separated using the solvent system of ethyl acetate: n-hexane (1:9) and were detected by incubating the plate in glass chamber saturated with iodine vapors. The characteristic colored spots were observed under visible light (Sangeeta and Kameshwara, 1993). The hRf values of all the three phytochemicals with characteristic colored spots were calculated and noted.

Antioxidant assays

Hydrogen peroxide scavenging assay

The ability of methanol extracts of algae *C.vulgaris* and *C.reinhardtii* to scavenge hydrogen peroxide was determined by 100 mM hydrogen peroxide. One mL of different concentration of extracts in distilled water was added to the 2 mL of hydrogen peroxide solution. Absorbance was determined at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of scavenging activity by different concentrations of the extracts were calculated from $[(A_c-A_s)/A_c] \times 100$, where A_c is the absorbance of the blank and A_s is the absorbance of sample/standard taken as Ascorbic acid (Gulcin et al., 2005).

Thiocyanate method

The peroxy radical scavenging activity of methanol extracts of algae *C.vulgaris* and *C.reinhardtii* was determined by thiocyanate method. One mL of different concentration of extracts were mixed with 2.5 mL of 0.02 M linoleic acid emulsion (in 0.04 M phosphate buffer, pH 7.0) and 2 mL of 0.04 M phosphate buffer (pH 7) and incubated in darkness at 37 ºC. Absorbance was determined at 500 nm by the amount of peroxide formed with the development of red colour on addition of 0.1 mL of 30 % ammonium thiocyanate solution and 0.1 mL of 20 mM ferrous chloride in 3.5 % hydrochloric acid against blank containing water; replacing the extract. The percentage of scavenging activity by different concentrations of the extracts were calculated by the formula: $[(A_c-A_s)/A_c] \times 100$, where A_c is the absorbance of the blank and A_s is the absorbance of sample/standard, Ascorbic acid (Karagözler, et al., 2008).

Result and discussion

Growth curve of the microalgae, *C. vulgaris* and *C. reinhardtii* showed a steady increase in slope up to 25th d (Fig.1). Among pigments of both the cultures, *C.vulgaris* cells were revealed to be rich in pigments than *C. reinhardtii*. The high estimated values were chlorophyll a- 7.59 mg/mL on 10th d, chlorophyll b-7.85 mg/mL on 20th d, carotenoid- 5.35 mg/mL on 15th d and

astaxanthin- 0.3 mg/mL on 15th d in *C. vugaris* whereas 2.92 mg/mL on 25th d, 4.03 mg/mL on 15th d, 1.78 mg/mL on 25th d and 0.05 mg/mL on 20th d in *C. reinhardtii* (Fig.2 a and b).

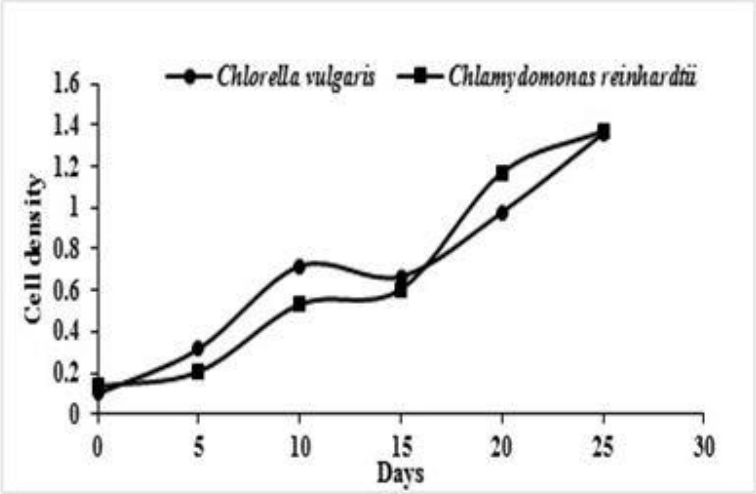

Fig. 1. Growth curve of *C. vulgaris* and *C. reinhardtii*

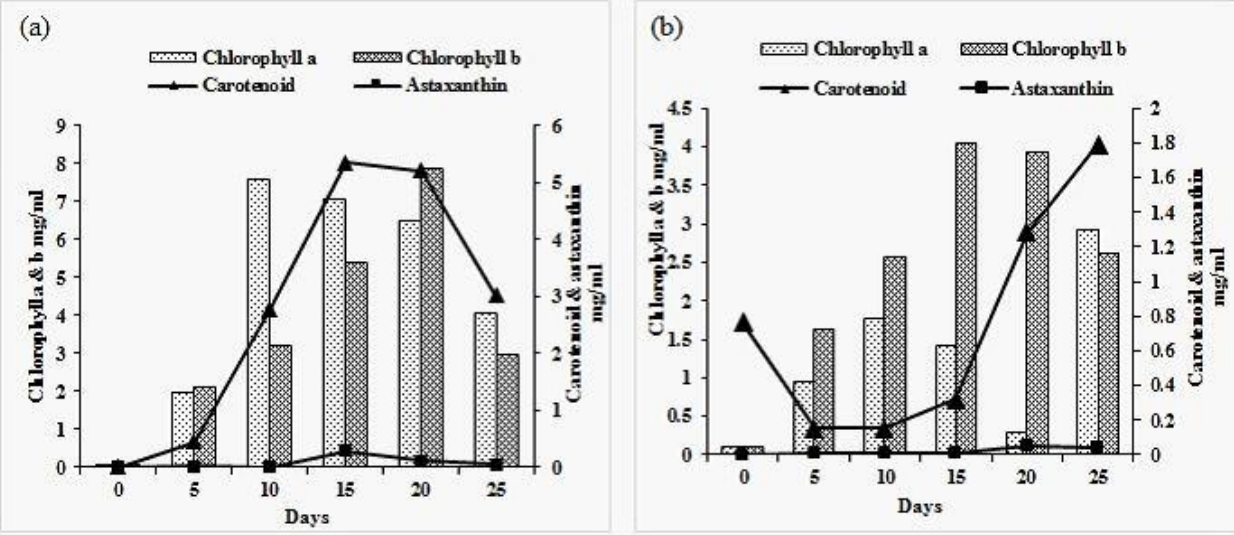

Fig 2. Pigment variation in (a) *C. vulgaris* and (b) *C. reinhardtii*

Primary and secondary metabolites were also assessed with the periodic intervals of 5 days respectively. Primary metabolites in both the cultures were found to be synthesized nearly in similar quantities. In *C.vulgaris* to the maximum of protein, lipid and carbohydrate contents were estimated to be 3 µg/mL on 5th d, 47.12 µg/mL on 10th d and 254 µg/mL on 25th d whereas in *C. reinhardtii* 3 µg/mL on 15th d, 45.97 µg/mL on 20th d and 203 µg/mL on 25th d (Fig 3) Secondary metabolites of algae are usually rich source of antioxidants such as phenolics and flavonoids. In *C.vulgaris* the estimated quantity of phenol and flavonoid were 20.8 µg/mL on 20th d and 55.76 µg/mL on 25th d whereas in *C. reinhardtii* 20 µg/mL on 25th d and 58.46 µg/mL on 25th d (Fig 3 a and b).

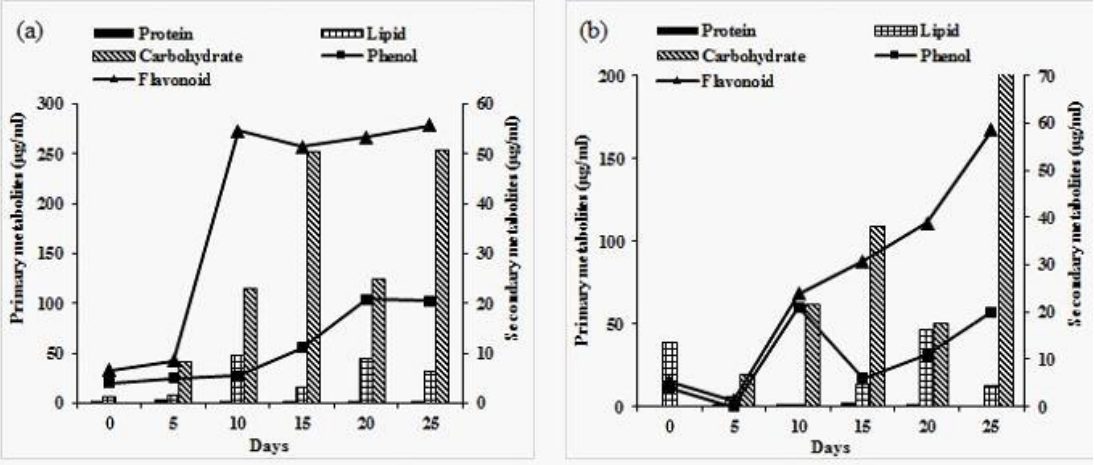

Fig 3. Biochemical contents in (a) *C. vulgaris* and (b) *C. reinhardtii*

Preliminary phytochemical analysis of methanol extracts of both the cultures showed the presence of phenolic compounds, flavonoids, glycosides, terpenoids and saponins while the absence of tannins and alkaloids (Table 1). However very scarce information is available on phenolic compounds in microalgae, one of the recent research by Klejdus et al. (2010) showed that several classes of flavonoids, such as isoflavones, flavanones, flavonols and dihydrochalcones are found in microalgae and cyanobacteria. This indicates that though microalgae are more primitive than terrestrial plants, they are capable of producing relatively complex polyphenols.

Table 1. Preliminary phytochemicals in methanolic extract of *C.vulgaris* and *C.reinhardtii*

Tests	*Chlorella vulgaris*	*Chlamydomonas reinhardtii*
Phenolics		
Phenol test	+	+
Ellagic acid test	+	+
Flavonoids		
Shinoda test	+	+
Ferric chloride test	+	+
Lead acetate test	+	+
Saponins		
Foam test	+	+
Glycosides		
Killar Killani test	+	+
Alkaloids		
Mayer's test	-	-
Wagner's test	-	-
Dragendorff's test	-	-
Tannins		
Ferric chloride test	-	-
Gelatin test	-	-
Terpenoids		
Salkowski test	+	+

Thin layer chromatographic analysis for the methanol extract of *C.vulgaris* and *C.reinhardtii* were performed for medicinally important phytochemicals such as flavonoids, phenols and saponins (Fig. 4). Flavonoids being the largest group of phenolic compounds are known to contain a broad spectrum of chemical and biological activities including antioxidant and free radical scavenging properties (Kahkonen et al., 1999). Flavonoids mainly include flavonols, flavones, catechins, proanthocyanidins, anthocyanidins and isoflavonoids (Ndhlala et al., 2007). In the present study, flavonoids showed eighteen spots and the spots detected at hRf values 14.2, 18.5, 57.1 and 71.4 were identical in both the extracts (Table 2).

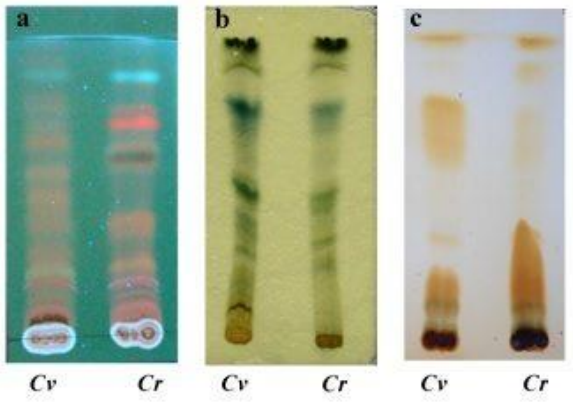

Fig. 4. TLC profile of (a) flavonoid, (b) phenol and (c) saponin in the methanol extracts of Chlorella vulgaris (Cv) and Chlamydomonas reinhardtii (Cr)

Table 2: TLC profile of flavonoids

Colour of spots	hRf values	*Chlorella vulgaris*	*Chlamydomonas reinhardtii*
Brown	7.1	+	-
Fluorescent orange	7.6	-	+
Fluorescent orange	10.0	+	-
Fluorescent orange	14.2	+	+
Fluorescent orange	18.5	+	+
Fluorescent yellow	21.4	+	-
Fluorescent orange	28.5	+	-
Fluorescent yellow	32.8	-	+
Fluorescent orange	35.7	-	+
Fluorescent orange	42.8	+	-
Fluorescent orange	57.1	+	+
Brown	61.4	-	+
Fluorescent orange	64.2	-	+
Fluorescent orange	65.7	+	-
Deep fluorescent orange	71.4	+	+
Fluorescent orange	78.5	-	+
Deep fluorescent olive green	87.4	-	+
Fluorescent olive green	97.1	+	-

In the study for phenol showed thirteen spots among which spot of *C.vulgaris* at hRf value 46.3 showed similar blue coloured spot to that of standard hydroquinone. Blue coloured spot of hRf at 78.9 was identical in both the algal extracts (Table 3). Phenols are known to reduce oxidative damage and act as antioxidants in trapping free radicals directly or scavenge them through a series of coupled reactions with antioxidant enzymes (Lewis et al., 1993). Recently saponins are identified to be an important phytochemical exhibiting anti-cancer activity, our extracts showed eight spots among which hRf at 15.3 and 98.4 were identical in both the extracts (Table 4).

Table 3: TLC profile of phenols

Colour of spots	hRf values	*Chlorella vulgaris*	*Chlamydomonas reinhardtii*	Standard
Grey	10.5	+	-	--
Grey	21.0	+	-	--
Grey	26.3	-	+	--
Grey	28.4	+	-	--
Grey	35.7	-	+	--
Grey	36.8	+	-	--
Blue	38.9	+	-	--
Blue	46.3	+	-	Hydroquinone
Blue	47.3	-	+	--
Grey	70.5	-	+	--
Blue	78.9	+	+	--
Grey	86.3	+	-	--
Grey	91.5	-	+	--

Table 4: TLC profile of saponins

Colour of spots	hRf values	*Chlorella vulgaris*	*Chlamydomonas reinhardtii*
Dark brown	15.3	+	+
Brown	23.0	+	-
Brown	34.6	+	-
Brown	42.3	-	+
Brown	76.9	-	+
Brown	80.7	+	-
Brown	92.3	-	+
Brown	98.4	+	+

Antioxidant activity of the methanol extracts of *C. vulgaris* and *C. reinhardtii* were determined in terms of IC_{50} value based on the percentage of free radical scavenging activity. In both the extracts, higher scavenging activity was observed at the concentration of 1000 µg/mL. IC_{50} value for the *C. vulgaris* determined by H_2O_2 and Thiocyanate assay were 26.31 µg/mL and 28.18 µg/mL whereas for *C. reinhardtii* 27.48 µg/mL and 58.05 µg/mL respectively (Table 5). The presence of flavonoids and phenols in the methanol extract might been responsible for free radical scavenging activity individually or by synergistic action.

Table 5. Scavenging activity of methanol extracts of *C.vulagris* and *C.reinhardtii*

Concentration (µg/mL)	% scavenging activity of crude methanolic extract				
	C. vulgaris		*C. reinhardtii*		Standard (ascorbic acid)
	H_2O_2 assay	Thiocyanate assay	H_2O_2 assay	Thiocyanate assay	
15.5	29.4±0.68	27.5±0.64	28.2±0.65	17.2±0.40	9.0±0.21
31	58.9±1.37	55.0±1.28	56.4±1.31	34.4±0.80	18.0±0.42
62	59.3±1.38	67.4±1.57	66.4±1.54	53.4±1.24	23.8±0.56
125	61.5±1.43	70.6±1.64	75.3±1.75	71.2±1.60	49.0±1.14
250	69.7±1.62	83.2±1.94	76.5±1.78	86.5±2.01	64.5±1.50
500	74.1±1.72	90.0±2.1	80.2±1.87	88.6±2.06	78.6±1.83
1000	79.6±1.85	91.5±2.1	82.8±1.93	94.3±2.20	94.0±2.20
IC_{50} (µg/mL)	26.31	28.18	27.48	58.05	127.55

Results are given as the average of triplicate determination ± standard deviation

Conclusion

The quantitative and qualitative determination of primary and secondary metabolites revealed the presence potent antioxidants β-carotene, astaxanthin, phenols and flavonoids in *Chlorella vulgaris* and *Chlamydomonas reinhardtii*. In addition, they are also significantly rich in pigments, protein and carbohydrates. Thus these mircroalgae can be promising food supplement and good natural source of antioxidant in pharmaceutical industries.

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