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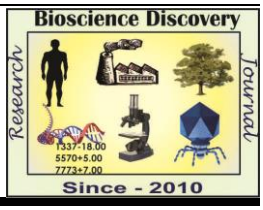

Cultivation, phytochemical screening and quantitative analysis of phytocompounds in *Chlorella vulgaris*

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Abstract

The present investigation is carried out to assess the presence of different bioactive constituents and quantification of primary and secondary metabolites in different solvent extracts of *Chlorella vulgaris*. It also gives information on the solubility of various compounds in different solvents. The presence of phenolics, flavonoids, carbohydrates, saponin and protein were confirmed by routine qualitative studies. The methanolic extract contains both the primary and secondary metabolites in highest concentration when compared to other extracts. Thus the microalgae can be explored for its nutraceutical importance.

INTRODUCTION

Microalgae are a collection of unicellular or basic multicellular photosynthetic microorganisms and have been discovered for their bioactive compounds. They are separated into four groups (red, green, brown and blue green). This taxonomic category, excluded in Plant kingdom yet rather in the Protista kingdom, demonstrates a high photosynthetic performance. In this way, algae can have a high reproductive potential and hencethey can grow very fast (Dineshkumar *et al.*, 2017).

Microalgae are a cause of natural products and have been newly studied for biotechnological applications. The decent variety of microalgae makes a possibly rich source for different chemical products with applications in nutritional, cosmetic, pharmaceutical, and medicinal industries (Wang *et al.*, 2010). Extracts from marine microalgae are abasis of proteins, vitamins, and minerals. Chlorella, a unicellular green algae, contains different valuable proteins (40~60%) and has been broadly utilizedas a part of aquaculture, food and biotechnology industries. The extract from Chlorella contains differentnaturally active compounds including growth factors, anti-inflammatory and wound healing substances, antioxidants, and emollient compounds (Kim *et al.*, 2008).

The creation of free radicals in life forms is directed by variousantioxidants molecules which might be endogenous, for example superoxide dismutase, or may originate from the diet, such as ascorbic acid, α-tocopherol, carotenoids and polyphenols. When there is a restriction in the availability of antioxidants, there may be oxidative harm to the cumulative nature. Among the different classes of obviously occurring antioxidants, phenolic compounds such as simple phenols, phenolic acids (derivatives of benzoic acid and cinnamic acid), coumarins, flavonoids and others, have expected much attention. According to Wang *et al.*,2010 the insulated indigenous *C. Vulgaris* strain extract gained from Supercritical carbon dioxide extraction shows significant antioxidant activities and introduces double restraints to lung cancer cell growth and migration ability, which is the index of cancer metastasis (Wang *et al.*, 2010).

Consequently, microalgae species *C. vulgaris* could have the potential for the improvement of antioxidant and anticancer products. An amendment involving research for creative functional food components from microalgae revealed the particular species of microalgae, the activity of the compounds acquired, and the form of extraction mechanisms used, viewing that the unicellular algae *Chlorella vulgaris*comprisesa lot of bioactive substances with medical properties. Experimental studies carried out under *Chlorella* have established its antitumor effect, cancer chemoprevention properties, anti-inflammatory activity, antioxidant activity, and antimicrobial activity (Wang *et al.,* 2010; Guzman *et al.,*2001; Vijayavel *et al.,* 2007; Makridis *et al.,* 2006).

In the present investigation, the biomass cultivation of *Chlorella vulgaris* was investigated along with the aspect to determine its phytochemical components, primary and secondary metabolites in methanolic extract of *Chlorella vulgaris*.

MATERIALS AND METHODS

1. Cultivation of *Chlorella vulgaris*

Cultures of *Chlorella vulgaris* were grown under continuous light at 20ºC. The growth medium f/2 was prepared as mentioned below.

Growth f/2 medium preparation

This is a common and widely used general enriched seawater medium designed for growing coastal marine algae, especially diatoms. The concentration of the original formulation, termed "f Medium" (Guillard and Ryther 1962), has been reduced by half. To prepare, begin with 950 mL of filtered natural seawater and add the following components. The trace element and vitamin solutions are provided below. Bring the final volume to 1 liter with filtered natural seawater. If the alga to be grown does not require silica, then it is recommended that the silica be omitted because it enhances precipitation. Autoclave(Guillard and Ryther 1962, Guillard 1975).

Component	**Primary Stock Solution**	**Quantity**	**Molar Concentration In Final Medium**
NaNO3	75 g/L dH_2O	1 ml	8.82 x 10-4 M
NaH2PO4 H2O	5 g/L dH_2O	1 ml	3.62 x 10-5 M
Na2SiO3 9H2O	30 g/L dH_2O	1 ml	1.06 x 10-4 M
trace metal solution	(see recipe below)	1 ml	-------
vitamin solution	(see recipe below)	0.5 ml	-------

f/2 Trace Metal Solution

To prepare, begin with 950 mL of dH_2O, add the components and bring final volume to 1 litre with dH_2O. Autoclave. Note that the original medium (Guillard and Ryther 1962) used ferric sequestrene; we have substituted $Na_2EDTA \cdot 2H_2O$ and $FeCl_3 \cdot 6\ H_2O$.

Component	**Primary Stock Solution**	**Quantity**	**Molar Concentration In Final Medium**
$FeCl_3\ 6H_2O$	-----	3.15 g	1.17×10^{-5} M
$Na_2EDTA\ 2H_2O$	-----	4.36 g	1.17×10^{-5} M
$CuSO_4\ 5H_2O$	9.8 g/L dH_2O	1 ml	3.93×10^{-8} M
$Na_2MoO_4\ 2H_2O$	6.3 g/L dH_2O	1 ml	2.60×10^{-8} M
$ZnSO_4\ 7H_2O$	22.0 g/L dH_2O	1 ml	7.65×10^{-8} M
$CoCl_2\ 6H_2O$	10.0 g/L dH_2O	1 ml	4.20×10^{-8} M
$MnCl_2\ 4H_2O$	180.0 g/L dH_2O	1 ml	9.10×10^{-7} M

f/2 Vitamin Solution

First, prepare primary stock solutions. To prepare final vitamin solution, begin with 950 mL of dH_2O, dissolve the thiamine, add 1 mL of the primary stocks and bring final volume to 1 litre with dH_2O. Filter sterilize. Store in refrigerator or freezer(Guillard and Ryther 1962, Guillard 1975).

Component	Primary Stock Solution	Quantity	Molar Concentration In Final Medium
thiamineHCl (vit. B1)	-----	200 mg	2.96×10^{-7} M
biotin (vit. H)	1.0 g/L dH_2O	1 ml	2.05×10^{-9} M
cyanocobalamin(vit. B12)	1.0 g/L dH_2O	1 ml	3.69×10^{-10} M

2. Dry harvesting

The cultures at stationaryphase of growth (10 days) were harvested and collected by centrifuging at 10,000 rpm for 3 min. TheCollected *Chlorella vulgaris* pellets were dried under shade and made into a coarse powder withmechanical grinder for further use. The dried powders of *Chlorella vulgaris* were stored for further studies.

3. Preparation of*Chlorella vulgaris* extracts

The obtained algal biomass was subjected to centrifugation (2500 r/min) for the time duration of 10 min so as to partially dehydrate it. About 25 g algal biomass was subjected to extraction for a time period of 30 min using Soxhlet apparatus, using 150 mL of each organic solvent, i.e aqueous, ethanol, methanol, acetone, pyridine and isopropanol. Respective solvents were used to test phytochemical compound and primary as well as secondary metabolites (Adhoni *et al.*, 2016).

4. Preliminary phytochemical screening

Different extract of *Chlorella vulgaris*were subjected to qualitative tests for the identification of phytochemical constituents such as carotenoids, alkaloids, flavonoids, glycosides, phenols, lignins, saponins, sterols, tannins, reducing sugars, volatile oil, fatty acid, amino acids and carbohydrates according to standard procedures (Martinez Nadal *et al.*, 1963; Hornsey and Hide, 1974; Yao and Moellering , 1995; Benkendroff *et al.*, 2005).

5. Quantitative determination of primary metabolites

Determination of carbohydrate

100 mg of sample was hydrolysed in a boiling tube with 5 ml of 2.5 N HCl in a boiling water bath for aperiod of 3 hours. It was cooled to room temperature and solid sodium carbonate was added untileffervescence ceases. The contents were centrifuged and the supernatant was made to 100 ml using distilledwater. From this 0.2 ml of sample was pipetted out and made up the volume to 1 ml with distilled water. Then 1.0 ml of phenol reagent was added followed by 5.0 ml of sulphuric acid. The tubes were kept at 25-30°C for 20 min. The absorbance was read at 490 nm (Geetha and Geetha, 2014).

Estimation of total chlorophyll content

100 mg leaf tissues were soaked in 10 ml of DMSO: acetone mixture (1:1) for overnight incubation(in the dark) and absorbance read at 663 and 645 nm and total chlorophyll content was calculated using thefollowing equations.

Chlorophyll a (Ca) = (12.25 × OD at 663) - (2.79× OD at 645) ×10/ (1000×wt)

Chlorophyll b (Cb) = (21.50 × OD at 645) - (5.10× OD at 663) ×10/ (1000×wt)

Total Chlorophyll (C) = (7.15 × OD at 663) + (18.71× OD at 645) ×10/ (1000×wt)

Determination of protein

The dried and powdered samples was extracted by stirring with 50 ml of 50% methanol (1:5 w/v) at 25°C for 24 h and centrifuged at 7,000 rpm for 10 min .0.2 ml of extract was pipette out and the volume wasmade to 1.0 ml with distilled water. 5.0 ml of alkaline copper reagent was added to all the tubes and allowed itto stand for 10 min. Then 0.5 ml of Folin's Ciocalteau reagent was added and incubated in dark for 30 min.The intensity of the colour developed was read at 660 nm 23 (Geetha and Geetha, 2014).

Estimation of total lipid content

10 gm sample was used to extract lipids with 150 ml of petroleum ether for 16 hr, at a solvent condensation rate of 2–3 drops/sec according to AOAC approved method with minor modifications of sample size and extraction time. The obtained extract was concentrated and evaporated at room temperature todryness. The weight of extract gives the total lipid content which was expressed as mg/g dry matter (Geetha and Geetha, 2014).

6.Quantitative determination of secondary metabolites

Determination of Total Phenolics and Tannins

The total phenolic content was determined according to the method described by Siddhuraju and Becker, 2003. Ten microliter aliquots of the extracts (2 mg/2 ml) was taken in test tubes and made up to the volume of 1 ml with distilledwater. Then 0.5 ml of Folin-Ciocalteu phenol reagent (1:1 with water) and 2.5 ml of sodium carbonate solution(20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes wereplaced in dark for 40 min and the absorbance was recorded at 725 nm against the reagent blank. The analysis wasperformed in triplicate and the results were expressed as tannic acid equivalents.Using the same extracts the tannins was estimated after treatment with polyvinyl polypyrrolidone(PVPP). One hundred milligrams of PVPP was placed in a test tube and to this 1 ml distilled water and then 1ml of the sample extracts were added. The contents were vortexed and kept in the test tube at 4°C for 4h. Then it was centrifuged (3000 rpm for 10 min at room temperature) and the supernatant was collected. Thissupernatant has only simple phenolics other than tannins (the tannins would have been precipitated along withthe PVPP). The phenolic content of the supernatant was measured as mentioned by Siddhuraju and Becker, 2003 and expressed as thecontent of non-tannin phenolics (tannic acid equivalents) on a dry matter basis. From the above results, thetannin content of the sample was calculated as follows:

Tannin (%) = Total phenolics (%) – Non-tannin phenolics (%)

Determination of Total flavonoids content

The flavonoids content was determined by the use of a slightly modified colorimetric method. A 0.5 ml aliquot of appropriately (2 mg/2 ml) diluted sample solution was mixed with 2 ml of distilled water and subsequently with 0.15 ml of 5 % $NaNO_2$ solution. After 6 min, 0.15 ml of 10% $AlCl_3$solution was added and allowed to stand for 6 min, and then 2 ml of 4% NaOH solution was added to themixture. Immediately, water was added to bring the final volume to 5 ml, and then the mixture was thoroughlymixed and allowed to stand for another 15 min. Absorbance of the mixture was determined at 510 nm versuswater blank. The analysis was performed in triplicate and the results were expressed as routine equivalent.

RESULTS

1. Preliminary phytochemical screening

Chlorella vulgaris was extracted using six different solvents and preliminary phytochemical analysis of different extract was done and result was represented in table 1. The best phytochemical constituents were seen in methanol extract of *Chlorella vulgaris* which shows the presence of flavonoids, phenols, sterols, carbohydrates, saponin, protein and amino acid while tannins and alkaloids was found to be absent. All other extract of *Chlorella vulgaris* showed less phytochemical components when compared to methanol extract (**Table 1**).

2. Quantification of Primary Metabolites

Primary and secondary metabolites were also assessed for different extract of *Chlorella vulgaris.* The maximum amount of primary metabolites such as protein, lipid, carbohydrates and chlorophyll were projected to be 53.18 ± 0.13, 9.38 ± 0.36, 23.46 ± 0.53 and 4.89 ± 0.06 (mg/g dw) for methanol extract followed by ethanol extract (41.56 ± 0.25, 7.81 ± 0.37, 18.21 ± 0.15 and 3.87 ± 0.17 mg/g dw) and acetone extract (28.15 ± 0.69, 5.12 ± 0.38, 15.51 ± 0.86, 2.45 ± 0.58 mg/g dw) respectively. Moderate amount of primary metabolites were obtained by Isopropanol (21.87 ± 0.43, 4.04 ± 0.18, 12.43 ± 0.28, 1.98 ± 0.38, mg/g dw) and pyridine (19.26 ± 0.48, 3.99 ± 0.57, 10.27 ± 0.27, 1.12 ± 0.25mg/g dw) and least amount was gained by aqueous extract of *Chlorella vulgaris* which possess 8.21 ± 0.17 mg/g dw carbohydrates, 1.02 ± 0.37 mg/g dw chlorophyll, 13.15 ± 0.45 mg/g dw protein and 3.25 ± 0.32 mg/g dw lipids (**Table 2**).

Similar value of secondary metabolites were obtained for methanol extract and ethanol extract of *Chlorella vulgaris*which was found to be rich in source of antioxidant such as phenolics which shows 5.34 ± 0.28(mg/g dw) and 4.97 ± 0.27 (mg/g dw) and flavonoids shows 2.48 ± 0.05 (mg/g dw) for methanol extract and 2.16 ± 0.28 (mg/g dw) for ethanol extract. Since tannin was found to absent in phytochemical analysis, it shows only 0.23 ± 0.13 (mg/g dw)for methanol extract and 0.14 ± 0.45 (mg/g dw) for ethanol extract. All other type of extract such as acetone, isopropanol, pyridine and aqueous shows very least amount of phenolics, tannin and flavonoids **(Table 2)**.

Table 1 Phytochemical screening of different extract of *Chlorella vulgaris*

Phytochemical constituents	Extracts						
	Test name	Aqueous	Methanol	Ethanol	Acetone	Pyridine	Isopropanol
Alkaloids	Wagner's test	-	-	-	+	-	-
Flavonoids	Shimoda, Lead acetate test	+	+	+	+	+	+
Phenolics & Tannins	Lead actetate test,	-	+	-	-	-	-
	Ferric chloride test	-	-	-	+	-	-
Steroids & Sterols	Salkowski test	- +	- +	- +	- -	- +	- +
Carbohydrates	Fehlings test,	+	+	+	+	+	+
	Benedicts test	+	+	+	+	+	+
Saponin	Honey comb test,	+	+	+	-	+	+
	Foam test	+	+	+	-	+	+
Glycosides	Glycosides test	+	+	-	+	+	+
Protein & amino acid	Biuret test,	+	+	+	+	+	+
	Ninhydrin test	+	+	+	+	-	+
Anthraquinone	Borntragers test	-	+	+	-	-	-

Table 2 Primary Metabolites of Different Extract of *Chlorella vulgaris*

S.NO	Primary metabolites	Aqueous extract Weight (mg/g dw)	Methanol extract Weight (mg/g dw)	Ethanol extract Weight (mg/g dw)	Acetone extract Weight (mg/g dw)	Pyridine extract Weight (mg/g dw)	Isopropanol extract Weight (mg/g dw)
1	**Carbohydrates**	8.21 ± 0.17	23.46 ± 0.53	18.21 ± 0.15	15.51 ± 0.86	10.27 ± 0.27	12.43 ± 0.28
2	**Chlorophyll**	1.02 ± 0.37	4.89 ± 0.06	3.87 ± 0.17	2.45 ± 0.58	1.12 ± 0.25	1.98 ± 0.38
3	**Protein**	13.15 ± 0.45	53.18 ± 0.13	41.56 ± 0.25	28.15 ± 0.69	19.26 ± 0.48	21.87 ± 0.43
4	**Lipids**	3.25 ± 0.32	9.38 ± 0.36	7.81 ± 0.37	5.12 ± 0.38	3.99 ± 0.57	4.04 ± 0.18

3. Quantification of Secondary Metabolites

Table 3 Secondary Metabolites of DifferentExtract of *Chlorella vulgaris*

S.NO	Secondary metabolites	Aqueous extract Weight (mg/g dw)	Methanol extract Weight (mg/g dw)	Ethanol extract Weight (mg/g dw)	Acetone extract Weight (mg/g dw)	Pyridine extract Weight (mg/g dw)	Isopropanol extract Weight (mg/g dw)
1	**Total phenolic**	2.25 ± 0.17	5.34 ± 0.28	4.97 ± 0.27	4.10 ± 0.17	3.01 ± 0.12	3.97 ± 0.98
2	**Tannin**	0.05 ± 0.47	0.23 ± 0.13	0.14 ± 0.45	0.11 ± 0.39	0.05 ± 0.14	0.09 ± 0.35
3	**Total flavonoids**	0.89 ± 0.33	2.48 ± 0.05	2.16 ± 0.28	1.98 ± 0.25	1.03 ± 0.31	1.56 ± 0.48

As of late, the utilization of algae for extraction of metabolites, bioactive molecules and other industrial important products has been picking up bunches of significance. Some algal species have been utilized for therapeutically practices against different pathogens both in medication and farming for decades.The phytochemical constituents were resolved for the methanol extract of *Chlorella vulgaris* in the present investigation. This shows higher movement of phenols, flavonoids, and alkaloids correspondingly. This is related with the examination of others findings whose result is similar to our outcome which shows the presence of phenolic and alkaloids in trace amount (+), terpenes in moderate amount (++), flavonoids in high amount (+++) and absent of other phytochemical compound such as tannins, terpenoids and saponins for the methanol extract of *Chlorella vulgaris* (Dineshkumar *et al.,* 2017).

Primary phytochemical analysis of methanol extracts of *Chlorella vulgaris* exhibited the presence of phenolic compounds, flavonoids, glycosides, terpenoids and saponins while the absence of tannins and alkaloids (Jayashree and Thangaraju, 2014). Conversely, the recent research by Klejdus*et al.*,2010 indicated that several classes of flavonoids, such as isoflavones, flavanones, flavonols and dihydrochalcones are found in microalgae and cyanobacteria. From this it is established, that though microalgae are more primitive than terrestrial plants, they are accomplished of producing relatively complex polyphenols (Annamalaiand Nallamuthu, 2014).

Distinctive crude organic extracts of *Chlorella vulgaris* were dissimilar in colour creäte on the components presenting in the extracts. High concentrations of carotenoids denoted to as colouring pigments and various fatty acids are present. Sterols, phenols and saponins are also present in good concentrations. Sugars, amino acids and flavonoids are also recognised. Tannins were absent in all extracts. Chloroform and hexane extracts revealed the presence of a larger group of molecules followed by ethyl acetate and methanol extracts. The presence of carbohydrates, amino acids and lipids was confirmed in almost all the organic extracts. Petroleum ether extract showed less significance in the preliminary phytochemical analysis as related to other solvent extracts was also reported (Adoni *et al.,* 2016).

In our research, primary and secondary metabolites were estimated using standard procedure and compared with other findings.The essential components were examined and related among the dry biomass obtained from *Chlorella vulgaris* in the BBM and the Sewage Water, correspondingly. They were exposed to biochemical analysis and their composition was deliberate and matched to check their yield. Comparing all the parameters except carbon content, it was found that the dry biomass from the sewage water showed satisfactory results in the protein content as (36.56 ± 1.28 mg/g), carbohydrates content (42.13 ± 0.85 mg/g), total chlorophyll (35.76 ± 0.61 mg/g), Carotenoids (32.14 ± 0.66 mg/g) in SW followed by lipid content was found to be very similar in the BBM (28.20 ± 0.89 mg/g), and the SW samples (28.68 ± 0.82 mg/g) was reported by Dineshkumar *et al.,* 2017.

Primary and secondary metabolites were also evaluated with the periodic intervals of 5 days respectively. Primary metabolites in both the cultures were established to be synthesized nearly in similar quantities. In *Chlorella vulgaris* to the maximum of protein, the lipid and carbohydrate contents were projected to be 3 µg/mL on 5thday, 47.12 µg/mL on 10thday and 254 µg/mL on 25thday whereas, Secondary metabolites of algae are usually rich source of antioxidants such as phenolics and flavonoids and in *Chlorella vulgaris* the assessed quantity of phenol and flavonoid were 20.8 µg/mL on 20thday and 55.76 µg/mL on 25thday respectively (Annamali and Nallamuthu, 2014).

A microalgae is gaining potential as nutraceutical source of bioactive constituents being used in medicinal applications. The research reports on their phytochemicals are of limited than on seaweeds. The qualitative and quantitative determination of primary and secondary metabolites revealed the appreciable presence of phenolics and flavonoids in *Chlorella vulgaris* and in addition, they are also significantly rich in pigments, protein and carbohydrates. Thus,these microalgae can be explored further as promising food supplement and good natural source of antioxidants.

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