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Spectral characterization of bioactive compounds from microalgae: *N. Oculata and C. Vulgaris*

P. Bhuvana[a], P. Sangeetha[a], V. Anuradha[a,*], M. Syed Ali[b]

[a] PG and Research Department of Biochemistry, Mohamed Sathak College of Arts & Science, Sholinganallur, Chennai, Tamil Nadu, India
[b] PG and Research Department of Biotechnology, Mohamed Sathak College of Arts & Science, Sholinganallur, Chennai, Tamil Nadu, India

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ABSTRACT

Microalgae are unicellular eukaryotic organisms that thrive in a wide variety of habitats such as fresh, salt water and marine environment. Microalgae are considered to be a good source of fatty acids, pigments and various other bioactive metabolites. Hence, the current study attempts to identify bioactive metabolites from two microalgae species: *Nannochloropsis Oculata* and *Chlorella Vulgaris.* This diverse group of autotrophic organisms can rapidly produce large biomass per surface area. These phyto-compounds were qualitatively analysed using biochemical methods. Solvent extracts of fresh samples were subjected to preliminary identification by UV spectra and thin layer chromatography. Furthermore, GC-MS and HPLC analysis were conducted to identify and characterize pharmacologically potent molecules. Major Phytochemicals such as phenolic, flavonoids, pigments, protein, and carbohydrates were present in both the samples. This was primarily subjected to UV spectral analysis and TLC chromatogram. The chemical diversity of compounds was further confirmed using GC MS and HPLC.

1. Introduction

Microalgae are one of the most primitive microscopic living organisms on earth which can grow in diverse environments (Hellebust, 1965). The green algae are the most diverse group of algae with more than 7000 species growing in a variety of habitats (Stengel et al., 2011). These produce many bioactive compounds such as proteins, polysaccharides, lipids, vitamins, enzymes, sterols, and other high-value compounds with pharmaceutical and nutritional importance that can be employed for commercial use (Priyadarshani and Rath, 2012). They have diversified use ranging from fatty acids and vitamins as fish feed in aquaculture systems (Yoshimatsu et al., 1997; Hamasaki et al., 1998; Gapasin et al., 1998), daily nutritional supplements, components in foodstuffs, and in the cosmetics industry. The nutritional value of microalgae is influenced by their size, shape, digestibility and biochemical composition (Webb and Chu, 1983; Brown et al., 1989). Understanding extra cellular secretions is also important for deeper insights into laboratory cultured ecosystems of algae, their growth condition and nutritional requirements. This leads to the optimization of growth conditions that favor the release of secondary metabolites with pharmacological importance.

Nannochloropsis oculata is a species of genus *Nannochloropsis*. These unicellular small green algae are identified by ovoid or spherical shaped cells (Gwo et al., 2005) and found to be present in both marine and freshwater habitats (Hibberd, 1981; Karlson et al., 1996). These algae have diameters ranging between 2 and 5 lm (Hu and Gao, 2003) and chlorophyll *b* and cellular xanthophyll pigment are absent (Whittle and Casselton, 1975, Volkman et al., 1993 and Adl et al., 2005). The main pigment violaxanthin and chlorophyll *a* are present, without pyrenoid. Proteins, lipids and polysaccharides are packed in an amorphous mucilaginous material. The encrusting substances sometimes contain silica, calcium carbonate, or sporopollenin (Barsanti and Gualtieri, 2006). This extremely protein rich microalga is used for aquaculture feed (Osinga et al., 2001 and Ferreira et al., 2009) because of high quantities of pigments, and polyunsaturated fatty acids (Volkman et al., 1993; Lubian et al., 2000; Lee et al., 2006). The Nannochloropsis has also been considered a viable proponent for biofuel production (Rodolfi et al., 2009 and Griffiths and Harrison, 2009).

The Chlorella vulgaris is a genus of single-celled green algae, belonging to the phytum Chlorophyta, which contain green photosynthetic pigments chlorophyll *a* and *b* in its chloroplast. The bioactive components of this unicellular alga make it an excellent candidate for various medical uses. The *Chlorella vulgaris* exhibit medical properties such as antitumor effect, hepato-protective, antioxidant, and antibacterial properties. These algae also include many dietary antioxidants such as lutein, α -carotene, β-carotene, ascorbic acid and α -tocopherol.

* Corresponding author.
E-mail address: vanuradha2712@gmail.com (V. Anuradha).

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These bioactive compounds have the capacity to scavenge free radicals (Vijayavel et al., 2007). Moreover, the biomass of these algae is rich in vitamin B complex, especially B12, which are vital in the formation and regeneration of blood cells. Most essentially, the Chlorella has a GRAS certificate issued by the FDA which makes it ideal for food production in a hygienic manufacturing environment (Costa et al., 2006, 2013).

The antioxidative properties of many microalgae species have been screened in multiple studies using different in vivo and in vitro assays whereas only few published studies consider the identification and quantification of phenolic composition in microalgae species (Herrero et al., 2006; Jaime et al., 2005; Klejdus et al., 2009; Chaudhuri et al., 2014). The present study aims to investigate the potential compounds such as secondary metabolites as a vital resource for screening of novel therapeutics by UV–Visible spectral analysis and thin layer autobiogram as easy and rapid technique to identify the type of compounds and subsequent analysis by HPLC and GC-MS to confirm their occurrence by specific band spectra.

2. Materials and methods

2.1. Sample preparation

The microalgae were cultivated in f/2 basal medium (Guillard, 1975) for 45 days and the biomass was collected by centrifugation. The filtrate was continuously washed with distilled water to remove the salt remnants. The samples were then dried, lyophilized and used for further analysis. 1 g dried powder of *Chlorella vulgaris* and *Nannochloropsis oculata* were subjected to sohxlet extraction using methanol as solvent [1:10 w/v]. This extract was collected and subjected for the qualitative determination of secondary metabolites.

2.2. Phenolic compounds

The samples were ground into a fine powder, and then 50 mg samples were soaked in 5 mL of pure methanol and shaken vigorously for 30s. Then tubes were put in the dark and at room temperature for 45 min. Then samples were centrifuged at 7500 × *g* for 10 min and supernatants were separated. The extraction process was repeated with another 5 mL portion of pure methanol. Collected supernatants were combined and stored at 20 °C. For analysis of tocopherols, one milliliter of the extract was evaporated under a stream of nitrogen and then redissolved in one milliliter of n-heptane. For analysis of phenolics, the rest of the methanolic extract solution was diluted with pure methanol to various concentrations (mg algae biomass dry weight/mL) for each test (Hamed Safafar et al., 2015).

2.3. Pigments

Freeze-dried samples were ground into a fine powder, after which 20 mg samples were soaked in 5 mL of methanol containing 0.025 μg/mL of BHT (Butylated Hydroxy Toluene) as standard. Then, the tubes were shaken vigorously for 30 seconds and put in a sonication bath at a temperature lower than 5 °C for 15 min. Subsequently, the tubes were centrifuged at 5000 × *g* and the supernatants were separated. The extraction was repeated with 5 mL portion(s) of solvent until a nearly colourless biomass was obtained. Supernatants were combined and used immediately for the analysis by TLC (Hamed Safafar et al., 2015).

2.4. Tocopherols

One milliliter of the methanolic extract was evaporated to dryness in darkness and under a stream of nitrogen and then re-dissolved in a mixture of isopropanol: heptane (0.5:99.5, v/v). Then the solution was filtered and analysed spectrophotometrically with the excitation wavelength set at 290 nm and emission wavelength at 330 (Hamed Safafar et al., 2015).

2.5. Characterization of Chlorella vulgaris and Nannochloropsis oculata

The extracts obtained by above procedure was analysed for the characterization of bioactive compounds through UV Spectroscopy, TLC, HPLC and GC-MS techniques to confirm their presence in *Chlorella vulgaris* and *Nannochloropsis oculata.*

The extract was dissolved in methanol at a concentration of 100 μg/mL and its UV spectrum was determined in the UV region (200–400 nm) and in UV- Vis spectrophotometer (Shimadzu 1800) against methanol blank. The samples were spotted in silica gel plate to obtain TLC chromatogram. The pure compound was evaporated by drying and the precipitated residue dissolved in HPLC grade methanol. The extract was injected into an LC- 8A Shimadzu 72 C18 column with HPLC grade acetonitrile- water gradient system over 15 min at a flow rate of 0.5 mL/min with detection at 254 nm. Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns.

3. Results and discussion

The isolated pigments, phenolic and tocopherol extracts from both *Chlorella vulgaris* and *Nannochloropsis oculata* were subjected to different characterization techniques.

3.1. Characterization using UV–Visible spectra

Absorbance spectra of all plants, including algae, are maximal in the blue (and to a lesser extent, the red) portion of the visible spectrum. The qualitative UV–Vis spectra showed sharp peak and the nature of secondary metabolite corresponds to specific wavelength at which band is obtained. The UV–visible spectrum of *chlorella vulgaris* shows two sharp peaks of phenolic compound at 407.96 nm and 669.79 nm. Similar peak for phenolic compounds were not observed in *Nannochloropsis oculata* (Fig. 1). The absorption maximum at 390–430 nm represents for aurones (Santos–Buelga et al., 2003) and sharp peak around 660 nm corresponds to the presence of alkaloids. On comparison with literature value, it is confirmed that the extract has some similar alkaloid, flavonoids, and flavonol compounds as reported (Michalak, 2006, Sahaya Sathish and Janakiraman, 2012; Niara Moura et al., 2016).

Various forms of chlorophyll have absorption maximum at different wavelengths. All organisms able to conduct photosynthesis have chlorophyll *a*, which absorbs red light at approximately 662 nm and violet light at approximately 430 nm. Chlorophyll *b* absorbs strongly the flame light at 642 nm and blue light at 453 nm. Plants with more complex chlorophyll systems absorb light in wider ranges, and in that manner the process of photosynthesis are much more effective. From Fig. 2 UV- visible spectra confirms the presence of the pigment chlorophyll A by predominant peaks at 402.21 nm and 664.02 nm in *chlorella vulgaris* and 411.24 nm and 666.97 nm in *Nannochloropsis oculata.* During photosynthesis process light is absorbed by chlorophylls below 480 nm and between 550 and 700 nm. Similar results were also reported in pigments extracted with ethanol (Lichtenthaler, 1987; Jeffrey, 1972). Computational analysis of chlorophyll structure and its UV absorbance spectra were also revealed by Magdalena Makarska-Bialokoz and Kaczor (2014).

The structure of tocotrienols differs from tocopherols by the presence of three trans- double bonds in the hydrocarbon tail but both series contain a polar chromanol ring linked to an isoprenoid derived hydrocarbon chain. Thus, α, β, γ and δ species of both tocopherols and tocotrienols differ with regard to the number and position of methyl groups on the chromanol ring (Sayago et al., 2007). The α tocopherol was found to be present in Chlorella vulgaris and in Nannochloropsis oculata which was confirmed by two peaks at 408.36 nm and 398.65 nm respectively (see Fig. 3). The additional peak in Chlorella vulgaris at 669.12 nm may correspond to lycopene. Zandomeneghi et al. (2005) have revealed that tocopherols dissolved in methanol

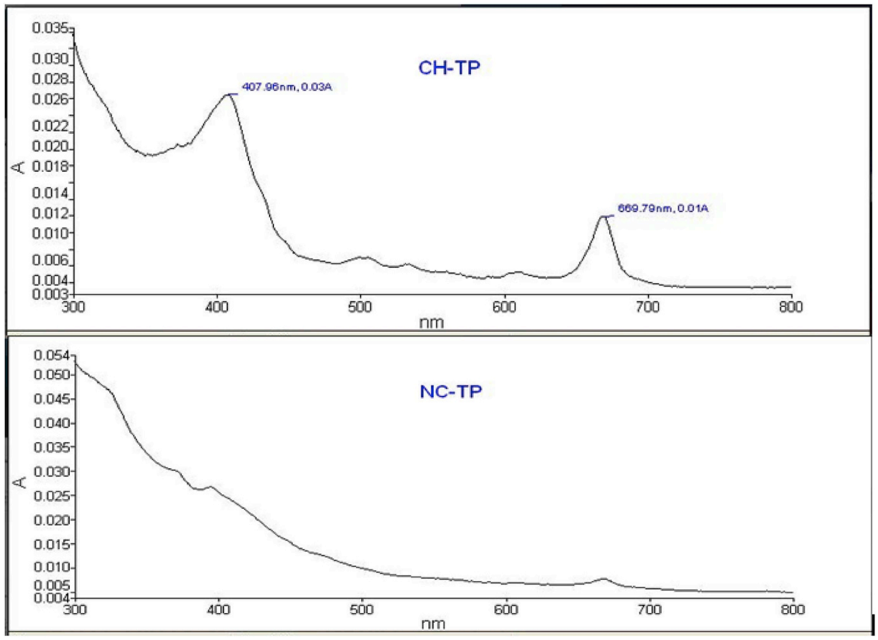

Fig. 1. Determination of Total Phenolics in *Chlorella vulgaris and Nannochloropsis oculata* by UV–Vis spec analysis.

represent a unique band that goes from 300 nm to 400 nm.

3.2. Characterization by thin layer chromatogram

TLC was used for analysing mixtures in the solid support separated using mobile solvent phase. TLC helps to determine the presence of various components like phenolics, pigments and tocopherols in algae mixture by Rf values. Thin layer chromatogram of methanolic extract of both *Chlorella vulgaris* and *Nannochloropsis oculata* are given in Fig. 4 and its corresponding Rf values along with name of identified compounds are given in Table 1.

From the results it is observed that the Rf value 0.43 stands for the presence of pigment chlorophyll *b* in *chlorella vulgaris* and the Rf value 0.33 denotes the pigment chlorophyll *a* in *Nannochloropsis oculata.* The Rf values 0.55 and 0.57 reveal the presence of α-tocopherol in *Chlorella vulgaris* and N*annochloropsis oculata* respectively. Phenolics were confirmed by the spot having Rf value of 0.71 and 0.73 which revealed the presence of octadecane in chlorella vulgaris and tetradecane in Nannochloropsis oculata. Rf values were compared with the previously reported literature as referred by Jeffrey (1974), Quach et al., (2004) and Sherma and Fried, 2003 and reported by Sathya (2017).

3.3. Characterization by HPLC and GCMS

HPLC and GCMS are rapid and versatile techniques used for analytical characterization of compounds. The compounds are identified

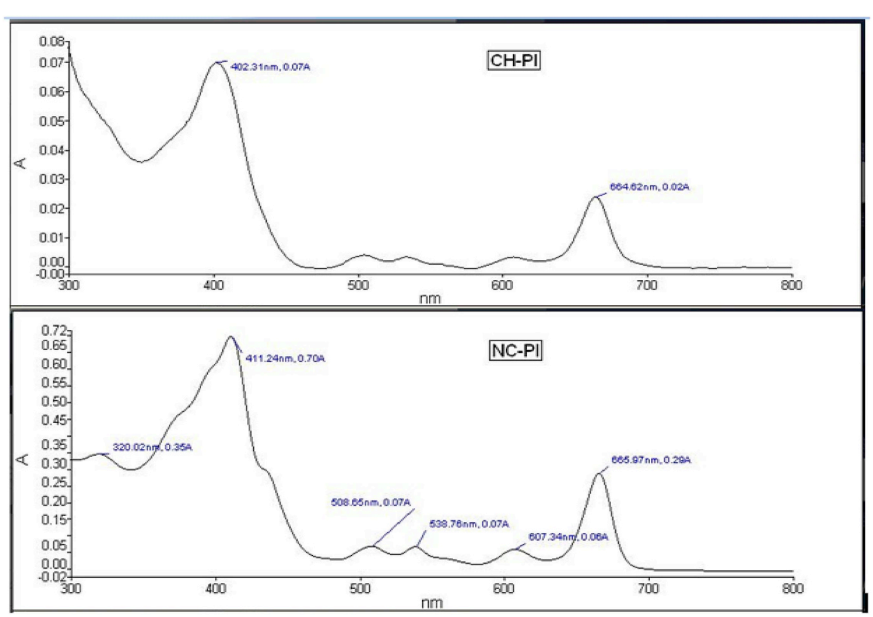

Fig. 2. Determination of Pigments in *Chlorella vulgaris and Nannochloropsis oculata* by UV–Vis spec analysis.

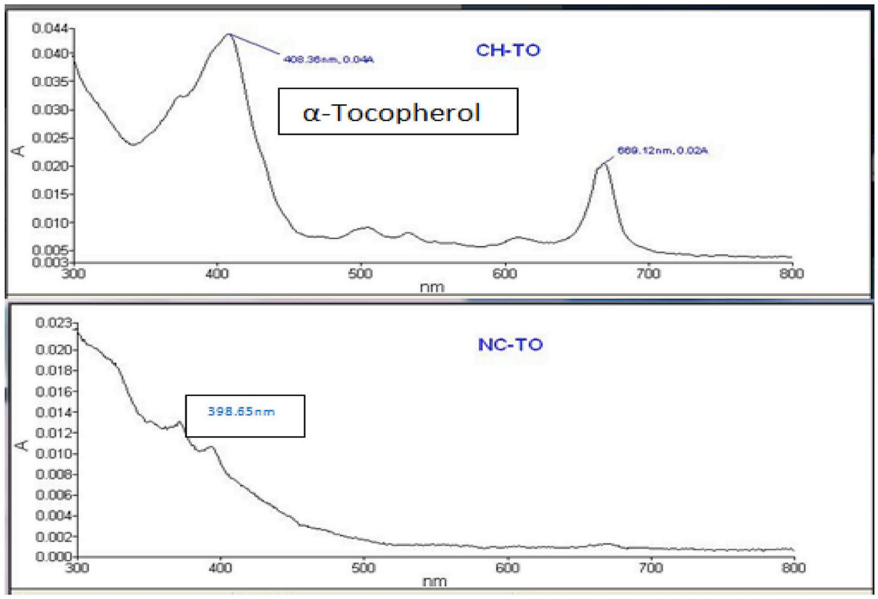

Fig. 3. Determination of Tocopherol in *Chlorella vulgaris and Nannochloropsis oculata*by UV–Vis spec analysis.

from peaks obtained in the spectra and by retention time. The peak represented in Figs. 5–7 for HPLC and Fig. 8 for GCMS chromatogram. Similarly, the compounds identified by HPLC are given in Tables 2–4 and the compounds that identified by GCMS are provided in Table 5.

From HPLC chromatogram of extracted phenolics of *Chlorella vulgaris,* the bands with retention time of 43.14 represents chicoric acid and at the retention time 34.80 depicted the presence of Caffeic acid derivative where as in *Nannochloropsis oculata* the highest three peaks at retention time of 28.687, 30.798 and 32.20 represents the presence of Quinic acid derivative, Quercetin pentosidehexoside and Luteolin 7-0-glucoside respectively (Fig. 5 and Table 2). Characterization of phenolic acids in microalgae species has been carried out in other studies, which can confirm some of the results of this study. One study reported the presence of highly-polar phenolic compounds of C6-C11 or C6 phenolic skeletons (Jaime et al., 2005), single phenols including protocatechuic, p-hydroxybenzoic, vanillic, syringic, caffeic, chlorogenic acid, 4-hydroxybenzaldehyde, and 4-dihydroxybenzaldehyde in *Spirulin*a (Klejdus et al., 2009), hydroxycinnamic acids (ferulic acid, p-coumaric acid) in Chlorella vulgaris, Haematococcus pluvialis, Diacronema lutheri, Phaeodactylum sp., Tetraselmis suecica, and Porphyridium purpureum, and p-hydroxybenzoic, protocatechuic, vanillic, syringic, caffeic, and chlorogenic acid; 4-hydroxybenzaldehyde and 3,4-dihydroxybenzaldehyde in Spongiochloris spongiosa and Spirulina platensis, Anabaena doliolum, Nostoc sp., and Cylindrospermum sp (Goiris et al., 2014).

The pigments chlorophyll *a* and *b*, violaxanthin, fucoxanthin and lycopene were predominantly observed in Chlorella vulgaris whereas peridinine, Zeaxanthin and chlorophyll a alone is present in Nannochloropsis oculata. Similar pigment composition in microalgae has been reported to possess various pharmacological activity as reported by hammed Safafar et al. (2016) and sin-ichiro et al. (1999). All xanthophylls synthesized by higher plants e.g., violaxanthin, antheraxanthin, zeaxanthin, neoxanthin, and lutein, can also be synthesized by green microalgae; however, these possess additional xanthophylls, e.g., loroxanthin, astaxanthin, and canthaxanthin. Diatoxanthin, diadinoxanthin, and fucoxanthin can also be produced by brown algae or diatoms (Barredo, 2012).

The presence of tocopherols was also confirmed by HPLC. The various species of chlorella contain varied composition of tocopherol which depends on cultivation condition and nutrient parameters. Total tocopherol content was higher in some green algae (Chlorophyceae) compared to others (Eustigmatophyceae and diatoms). Only few publications reported the tocopherol composition of microalgae. The effects of nitrogen source, concentration, and growth phase on tocopherol

Chlorella vulgaris ***Nannochloropsis oculata***

Fig. 4. Thin layer chromatogram of *Chlorella vulgaris and Nannochloropsis oculata*.

Table 1
Rf values obtained for *Chlorella vulgaris* and *Nannochloropsis oculata*.

Compounds	Rf value with component name	
	Chlorella vulgaris	*Nannochloropsis oculata*
Pigment	0.36 (chlorophyll *a*) 0.43 (chlorophyll *b*)	0.33 (chlorophyll *a*)
Phenolics	0.71 (octadecane)	0.73 (tetradecane)
Tocopherols	0.55 (α-tocopherol)	0.57(α tocopherol)

concentration in Nannochloropsis occulata was reported by Durmaz, 2007).

The GCMS spectrum indicated the presence of some fatty acids and small organic compounds as given in Table 5. The presence of mono and poly unsaturated fattyacids in *chlorella lobophora* has been previously reported (Santhoshkumar et al., 2015). Such fatty acid composition in microalgal species are well explored for biodiesel production (Stansell et al., 2012). Thus GCMS remains an alternative to FAME analysis for identification and quantification of phytocompounds.

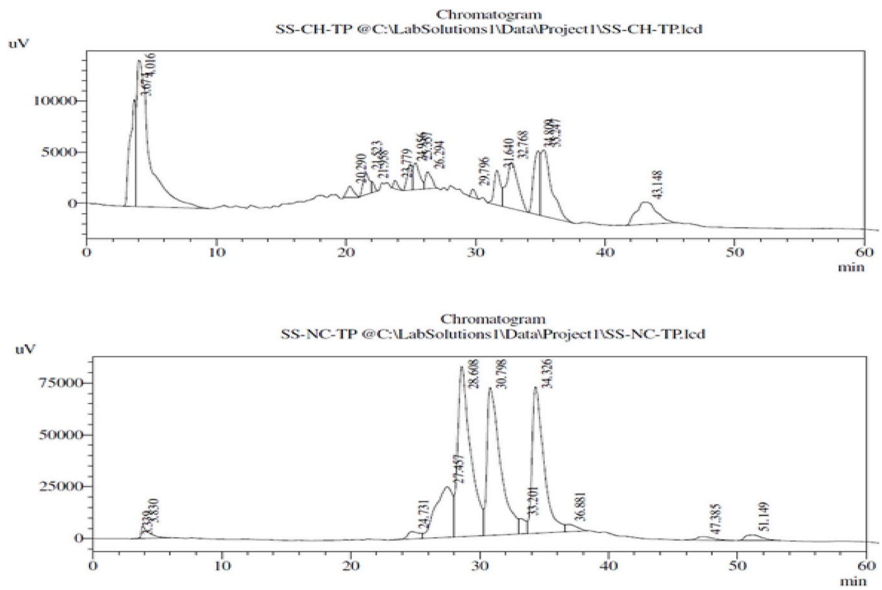

Fig. 5. HPLC for Total phenolic compound in *Chlorella vulgaris* and *Nannochloropsis oculata*.

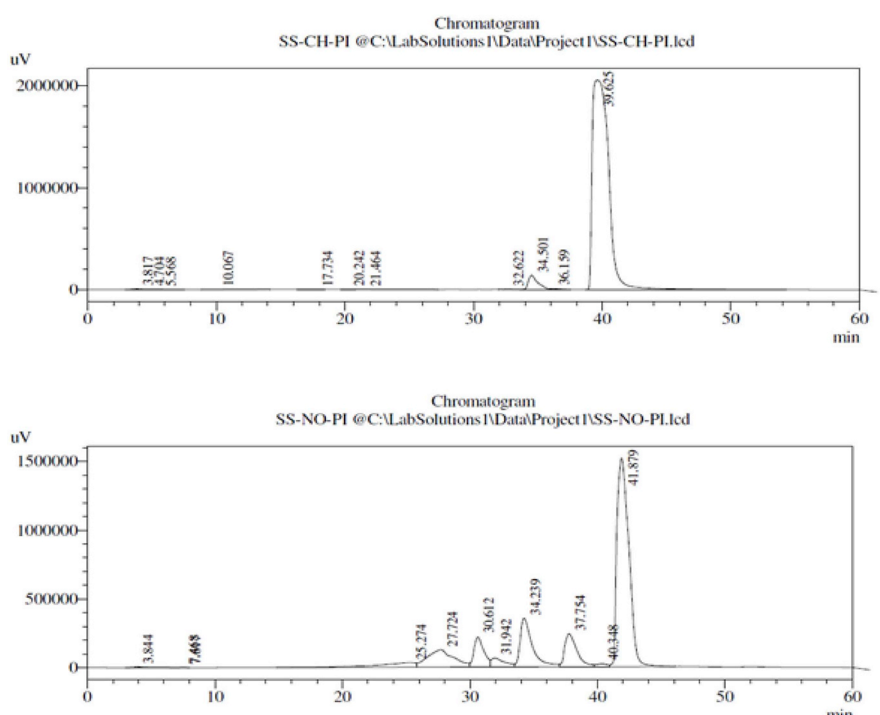

Fig. 6. HPLC for pigments in *Chlorella vulgaris* and *Nannochloropsis oculata*.

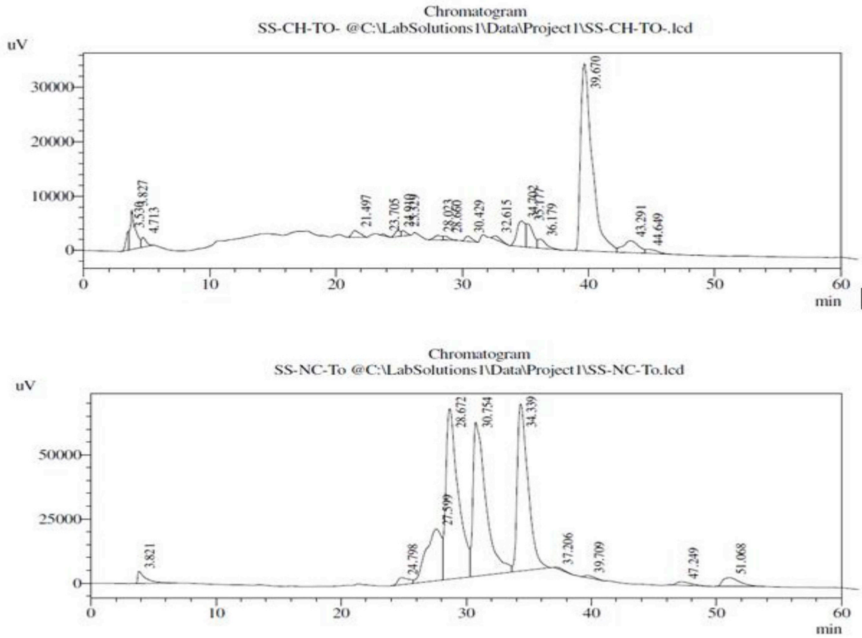

Fig. 7. HPLC for tocopherol in *Chlorella vulgaris* and *Nannochloropsis oculata*.

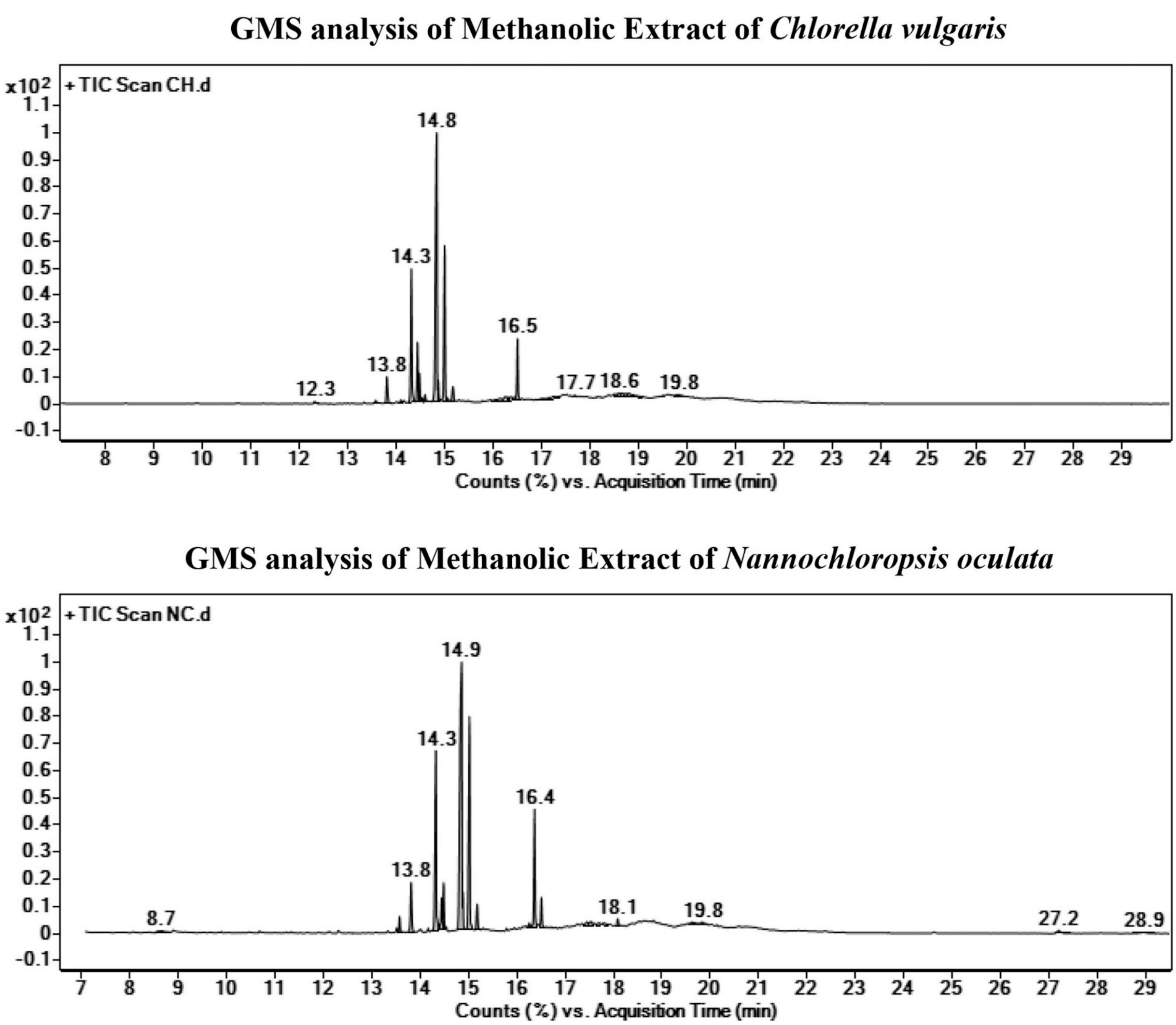

Fig. 8. GCMS analysis of Methanolic Extract of *Chlorella vulgaris and Nannochloropsis oculata*.

Table 2
HPLC Retention time with identified Phenolic Compounds in *Chlorella vulgaris* and *Nannochloropsis oculata*.

Chlorella vulgaris	
Retention time	Identified Compounds
20.290	Chlorogenic acid
23.779	Caffeic acid
25.357	Hydroxycinamicacid derivative
26.294	Quercetin pentosidehexoside
31.640	Caffeoyl dihydroxy phenyl lactoyl tartaricacid
32.768	Quercetin -7-o-hexoside3-o-hexoside
34.809	Caffeic acid derivative
35.247	Luteolin 7-0-rutinoside
43.148	Chicoric acid
Nannochloropsis oculata	
27.457	Protocatechuric acid hexoside
28.687	Quinic acid derivative
30.798	Quercetin pentosidehexoside
32.204	Luteolin 7-0-glucooside
47.385	Chicoric acid derivative
51.149	Caffeylhexoxidedeoxyhexoside

Table 3
HPLC Retention time with identified Pigments in *Chlorella vulgaris* and *Nannochloropsis oculata*.

Chlorella vulgaris	
Retention time	Identified Pigments
5.568	Chlorophyll *a*
10.067	Violaxanthin
17.59	Lycopene
21.462	Fucoxanthin
39.625	Chlorophyll *b*
Nannochloropsis oculata	
7.463	Peridinine
27.724	Zeaxanthin
41.879	Chlorophyll *a*

Table 4
HPLC Retention time with identified Tocopherol in *Chlorella vulgaris* and *Nannochloropsis oculata*.

Chlorella vulgaris	
Retention time	Identified Tocopherol
3.530	α tocopherol
28.660	δ tocopherol
34.702	γ tocopherol
39.670	α tocopherol
Nannochloropsis oculata	
24.798	α tocopherol
28.672	δ tocopherol
30.754	α tocopherol
34.399	γ tocopherol

4. Conclusion

In the present study, the active constituents of both the algal samples were identified and characterised. Analytical characterization revealed the presence of fatty acids, bio-organic acids, pigments and tocopherol in both the species of microalgae. The samples should be further column fractionated and subjected to LCMS to elucidate the hidden compounds. This would enhance the chances for screening and purification of pharmacologically active compounds from the under explored marine resources.

Table 5
Identification of Tocopherol present in *Chlorella vulgaris and Nannochloropsis oculata* by GCMS.

Chlorella vulgaris	
RT	Compound Name
9.9664	Tetradecane
10.5336	Decane, 2,3,5,8-tetramethyl-
10.62	Pentadecane
11.3061	Hexadecane
12.138	Heptadecane, 2,6,10,14-tetramethyl-
13.8072	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester
14.3041	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester
14.477	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester
14.8119	Dibutyl phthalate
14.9848	1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester
15.1685	Diamyl phthalate
Nannochloropsis oculata	
9.2533	Trichloroacetic acid, pentadecyl ester
10.5335	Decane, 2,3,5,8-tetramethyl-
10.6092	Pentadecane
10.8306	Dodecane, 2,6,11-trimethyl-
10.9063	1-Decanol, 2-hexyl-
11.3006	Hexadecane
11.4789	Dodecanoic acid, 1-methylethyl ester
12.1271	Heptadecane
12.5539	Eicosane
13.1265	Hexadecane
13.9692	Phytol
14.4392	Hexadecanoic acid, methyl ester
16.3515	Phytol

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101094.

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