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Detection of Chemical Compounds of Two Green Algae *Chlorococcum humicola* and *Chlorella vulgaris* Using GC–MS

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Abstract

Microalgae have been identified as excellent sources of various bioactive compounds for applications in pharmaceuticals, nutraceuticals, and biofuels. The objective of this study was to evaluate the chemical composition of *Chlorococcum humicola* and *Chlorella vulgaris* using Gas Chromatography-Mass Spectrometry (GC-MS) to give us some useful insights regarding their chemical and metabolic potentials. Algal samples were collected from the sewage water pond of the Samarra Pharmaceutical Industries Company, located in Samarra, Iraq. The water in this pond represents treated industrial wastewater rich in organic and inorganic nutrients. The samples were collected from a single collection site within the treatment basin during the study period and transferred to the laboratory for further purification. The dried algal biomass was subjected to solvent extraction using analytical-grade ethanol. The extraction process was carried out to isolate bioactive compounds prior to GC–MS analysis. Then used the resulting chromatograms to calculate retention times, relative abundances, and the molecular structures of bioactive compounds. The chemical characterization of *C. humicola* identified Eugenol, Hexadecanoic acid methyl ester, and Caryophyllene as the most prolific compounds. In *C. vulgaris*, the major components were Eucalyptol, Camphor, and Endo-Borneol, which exhibited superior aroma. Both species contained minor and other compounds of chemical interest, including sesquiterpenes, phenolics, and (methyl) fatty acid esters. The GC–MS chromatogram revealed several compounds, with Eugenol, Hexadecanoic acid methyl ester, and Caryophyllene showing the highest relative abundance in C. humicola, while Eucalyptol and Camphor were dominant in C. vulgaris. The results suggest that both *C. humicola* and *C. vulgaris* possess diverse bioactive natural compounds that have different chemical compositions useful for value-added industrial, pharmaceutical, and nutraceutical purposes.

Keywords: *Chlorococcum humicola*; *Chlorella vulgaris*, Chemical composition, GC–MS analysis, Bioactive compounds, Green microalgae**.**

1. Introduction

Algae represent a diverse array of photosynthetic organisms that find themselves in various aquatic and terrestrial settings. They are critical to the ecological balance because they play a significant role in global carbon fixation and oxygen production[1]. In addition to ecosystem services, the ecological roles of algae are less valued than their biochemistry, which has attracted increasing scientific and industrial attention for their biochemical diversity and their ability to create many important natural products[2]. Algal bio-products include lipids, proteins, carbohydrates, pigments, polyunsaturated fatty acids, and many bioactive ingredients with antioxidant, antimicrobial, and anti-inflammatory activities[3]. Accordingly, algae have been

recognized to have potential as renewable resources for multiple biotechnologies, nutraceuticals, pharmaceuticals, cosmetics, and bioenergy[4].

C. humicola and *C. vulgaris* represent important microalgal groups, which have attracted much attention due to their fast growth rate, plastics, and metabolic composition[5]. *C. vulgaris* is a green unicellular alga that has been evaluated as a food source due to its composition of proteins, vitamins, minerals, and essential fatty acids. It is also used in dietary supplements and bioremediation due to its ability to absorb heavy metals and other pollutants[6]. Although *C. humicola* has not been studied extensively, it has been recognized for its potential to produce lipids and secondary metabolites that can serve as biofuels and pharmacologically active compounds[7]. Thus, a comparative study of the two algae can give us some useful insights regarding their chemical and metabolic potentials[8].

Gas Chromatography–Mass Spectrometry (GC–MS) is an extremely sensitive and reliable lab method for the qualitative and quantitative analysis of complex organic mixtures[9]. It effectively combines the separation abilities of gas chromatography equipment with the structural identification capabilities of mass spectrometers. GC–MS can accurately measure the presence of both volatile and semi-volatile compounds[10]. The technique is well-suited to the analysis of algal extracts and allows for the identification of fatty acids, hydrocarbons, sterols, terpenes, and other metabolites that create that algal species' unique chemical fingerprint[11]. GC–MS profiling can improve our understanding of the metabolic pathways and biochemical differences amongst algal species, and more broadly, evaluate their potential industrial uses[12].

Various previous studies have shown that algae chemical composition can significantly vary by species, environmental factors, and cultivation parameters[13]. Thus, there is importance to complete a chemical characterization for *C. humicola* and *C. vulgaris* in order to evaluate the bioactive potential and identify significant compounds that are responsible for biological activity[14, 15]. Recognition of this variability not only provides insight into the metabolism of algae but also opens up the possibilities of uniquely employing each species to develop sustainable and value-added products[16].

Therefore, the objective of this study was to investigate and compare the chemical composition of *C. humicola* and *C. vulgaris* using GC–MS in order to identify their major bioactive compounds and evaluate their potential industrial and pharmaceutical applications.

1. Materials and Methods

2.1. Collection of algal Samples

Water samples were taken from the treating pond of Samarra Pharmaceutical Laboratory, as per the predetermined spatial coordinates (34.1959° N ،43.8857° E.) **(Figure 1)**. Algal samples were collected using a plankton net with a mesh size of approximately 25 µm. After that, they were transferred into sterilized plastic bottles that were labeled by the date and location of the collection and stored until analysis[17]. The studied algae belong to the division Chlorophyta. *C. vulgaris* and *C. humicola* are unicellular green microalgae classified within the class Trebouxiophyceae and Chlorophyceae, respectively, according to modern algal taxonomic classification[18].

2.2. Examination and preservation of algae

The collected samples were viewed under a compound light microscope at 40x and 100x magnification to recognize and classify the algae using standard taxonomic references[19-22]. The target genera, *C.humicola* and *C.vulgaris*, were captured using a microscope camera and referenced against images that it was found (**Figure 2**). The samples were then preserved in CH-13 medium and supplemented with Logal solution (prepared from 10 g potassium iodide and 20 g glacial acetic acid, filled to 200 mL with distilled water)[23].

Figure 1. Location of sewage water pond of Samarra pharmaceutical laboratory in Iraq.

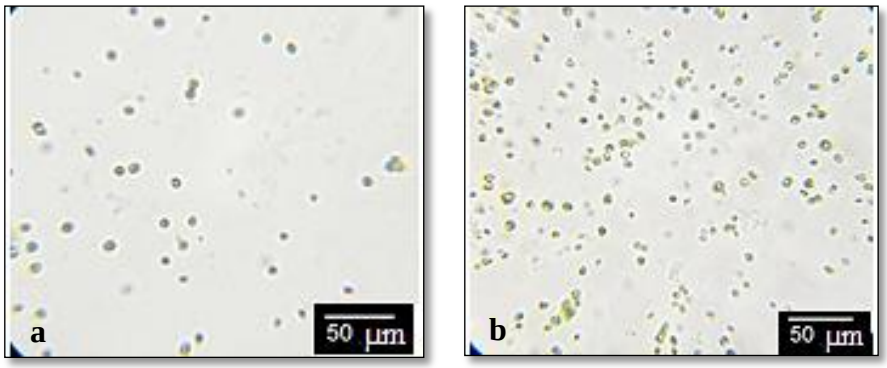

Figure 2. Microscopic image of a. *Chlorococcum humicola* and b. *Chlorella vulgaris*, 40x.

2.3. Isolation and purification of algae

Algal strains were isolated by the streaking method[24] and dilution method[25] on semi-solid media by adding 1.5-2% agar to liquid CH-13 medium. The algal isolates were cultured in BG-11 medium under laboratory conditions. The cultures were maintained at a temperature of approximately 25 ± 2°C with a light–dark cycle of 12:12 hours and continuous aeration when necessary. Axenic cultures were prepared according to [26] to be sure of removing bacteria and fungi. The cultures were incubated in a laboratory incubator under controlled temperature and light conditions. The purified algal cultures were maintained in culture media under laboratory conditions for further growth and experimental analysis. The collected samples were purified using repeated sub-culturing and microscopic examination to obtain unialgal cultures.

The cultures were allowed to grow under controlled laboratory conditions until they reached the exponential growth phase, at which point the biomass was harvested for chemical extraction and GC–MS analysis.

2.4. GC–MS Analysis of Algal Extracts

The structure of the algae was examined via an Agilent GC–MS system at the Ministry of Industry and Minerals, Industrial Research and Development Authority, Ibn Al-Bitar Research Center, Baghdad, Iraq. The algae were cultivated in liquid culture medium in sterile flasks under

controlled environmental conditions. Algal cultures were placed in an oven (40–50°C) for 24 hours, scraped, and using a mechanical grinder (Retsch, Germany). Approximately 0.5–1 g of ground algae was mixed with methanol and shaken on a shaker for 30–60 minutes, then centrifuged at a speed of 4000–6000 rpm for 1 minute. The supernatant was filtered through a 0.45 µm membrane filter and placed in a GC vial. An aliquot of 1 µL was injected using a microsyringe into the GC injector (250–300°C). Separation was achieved in a DB-Wax polar column with programmed temperature gradients. Mass detection was done in electron ionization (EI) mode, using helium as a carrier gas at a speed of 1 mL/min. All chromatograms were processed through the software, and peaks were identified based on retention times and matching mass fragmentation patterns from the NIST library[27].

2. Results

3.1. GC-MS Analysis of *Chlorococcum humicola*

GC–MS analysis of *Chlorococcum humicola* revealed a chemically diverse profile dominated by a few major bioactive compounds. The most abundant compound was eugenol (40.70%, RT 10.652 min), followed by hexadecanoic acid methyl ester (methyl palmitate) (16.04%, RT 19.575 min), caryophyllene (15.43%, RT 11.552 min), and unsaturated fatty acid methyl esters (10.14%, RT 21.817 min). In addition, thymol was detected at a lower proportion (5.33%, RT 9.903 min). These major constituents mainly belong to phenolic compounds, terpenes, and fatty acid methyl esters, which may contribute to the biochemical diversity and potential biological activity of the alga (**Table 1** and **Figure 3**).

Table 1. GC–MS Analysis of bioactive compounds identified in *Chlorococcum humicola* with their retention times, molecular formulas, and relative abundances.

No	Retention Time (min)	Compound	Relative Abundance (%)
1	9.903	Thymol (3-methyl-4-isopropylphenol, acetate derivative)	5.33
2	10.652	Eugenol (3-Allyl-6-methoxyphenol)	40.70
3	11.552	Caryophyllene	15.43
4	12.201	1,4,7-Cycloundecatriene, 1,5,9,9-tetramethyl	2.25
5	14.486	No matches found	1.69
6	19.385	Cis-Aconitic Anhydride / N-Trifluoroacetylimidazole	3.18
7	19.575	Hexadecanoic Acid, Methyl Ester (Methyl Palmitate)	16.04
8	21.817	Trans-13-octadecenoic acid, methyl ester / 10-octadecenoic acid, methyl ester / Cis-13-octadecenoic acid, methyl ester	10.14
9	22.120	Heptadecanoic acid, 10-methyl-, methyl ester / 16-methyl-heptadecanoic acid, methyl ester / Methyl stearate	5.24

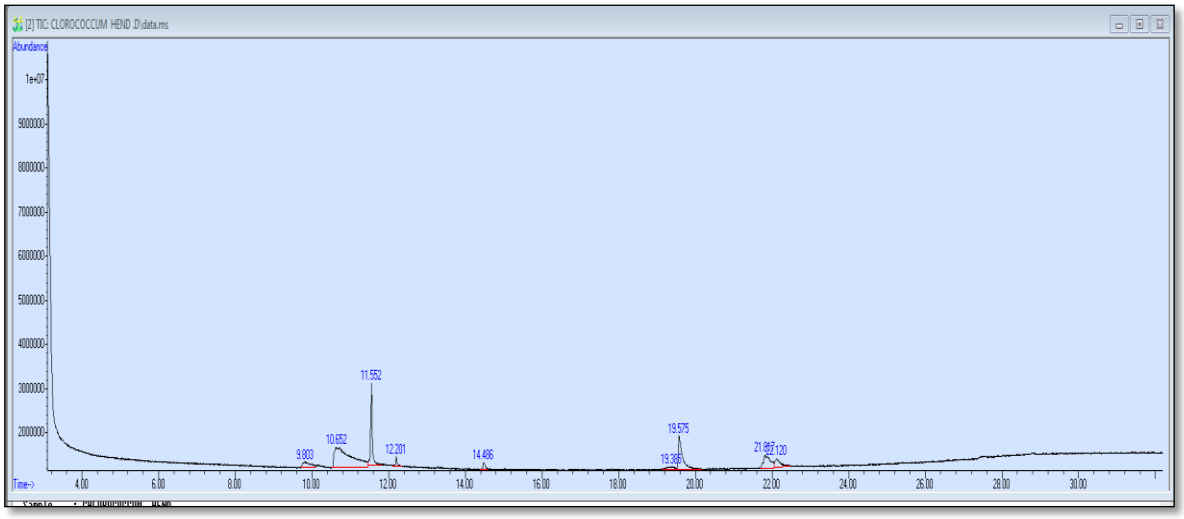

Figure 3. GC–MS chromatogram of bioactive compounds identified in the *Chlorococcum humicola*

3.2. GC-MS Analysis of *Chlorella vulgaris*

GC–MS analysis of *Chlorella vulgaris* showed a heterogeneous chemical profile dominated mainly by monoterpenes, with several compounds present at high relative abundance. The major identified constituents were eucalyptol (37.55%, RT 4.679 min), camphor (19.54%, RT 6.644 min), endo-borneol (11.67%, RT 7.103 min), and α-terpineol (6.74%, RT 7.484 min). Other notable compounds included carene/camphene derivatives (4.79%, RT 3.355 min), o-cymene / 1,3,8-p-menthatriene (4.16%, RT 4.437 min), and eugenol (combined 3.50%, RT 10.470–10.721 min). Overall, the predominance of monoterpenes, together with minor phenolic and sesquiterpene components, supports the potential bioactivity and possible industrial relevance of *C. vulgaris* (**Table 2** and **Figure 4**).

Table 2. GC–MS Analysis of bioactive compounds identified in *Chlorella vulgaris* with their retention times, molecular formulas, and relative abundances.

	Retention Time (min)	Compound	Relative Abundance (%)
1	3.355	3-Carene / Camphene / Tricyclo[2.2.1]heptane,1m3,3-trimethyl	4.79
2	3.874	Bicyclo[3.1.1]heptane,6,6-dimethyl / 1-2-methylene-(IS)-beta-pinene / Bicyclo[3.1.0]hexane,4-methylene 1-methylethyl	1.02
3	4.437	1,3,8-P-Menthatriene / O-Cymene	4.16
4	4.679	Eucalyptol	37.55
5	5.683	1,6-Octadien-3-ol,3,7-dimethyl	0.78
6	6.644	(+)-2-Bornanone / Camphor	19.54
7	6.739	(+)-2-Bornanone / Camphor	1.49
8	7.103	Bicyclo[2.2.1]heptane-2-ol,1,7,7-trimethyl-,(Is-endo) / Endo-Borneol	11.67
9	7.189	Terpinen-4-ol	2.03
10	7.484	Alpha-Terpineol	6.74
11	9.033	Bornyl Acetate / Bicyclo[2.2.1]heptane-2-ol,1,7,7-trimethyl-acetate	1.94
12	9.613	Phenol,2-methyl-5-(1-methyl)	0.98
13	10.470	Eugenol	2.30
14	10.721	Eugenol	1.20
15	11.560	Caryophyllene / Bicyclo[5.2.0]nonane,2-methylene-4,8,8-trimethyl-4-vinyl	1.94
16	12.201	1,4,7-Cycloundecatriene,1,5,9,9-tetramethyl	0.36
17	13.274	Naphthalene derivatives (Hexahydro-4,7-dimethyl-1-(methylthyl))	0.79
18	15.464	Tricyclo[3.3.2.0(3,7)]decane derivatives / 2H-Inden-2-one derivatives	0.38
19	15.750	Cyclohexane derivatives	0.54
20	19.610	Hexadecanoic Acid, Methyl Ester (Methyl Palmitate)	0.38

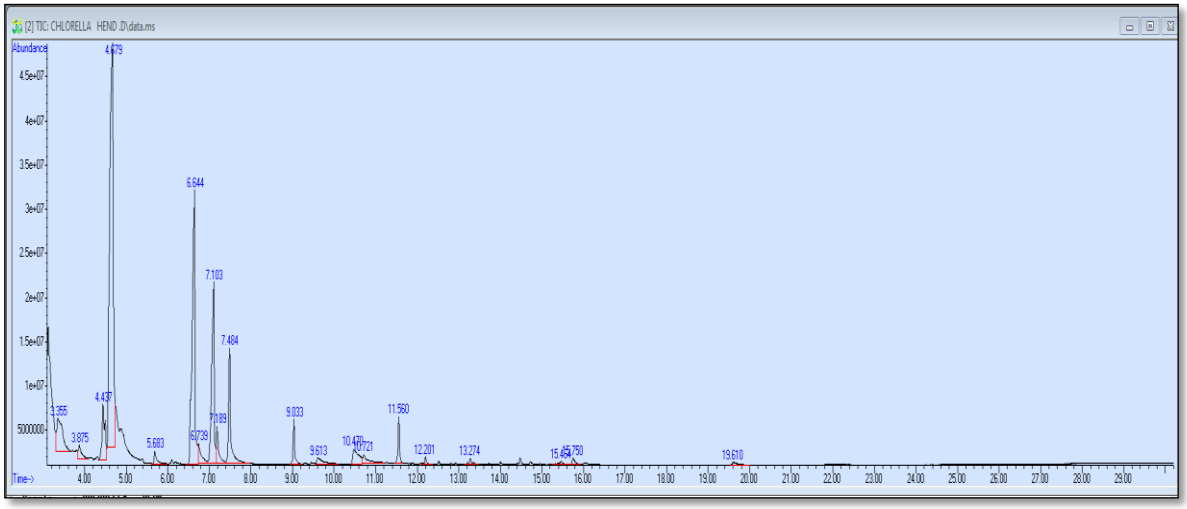

Figure 4. GC–MS chromatogram of bioactive compounds identified in the *Chlorella vulgaris*

3. Discussion

The chemical profile of *Chlorococcum humicola* indicates the prevalence of various bioactive compounds, demonstrating the chemical diversity and biotechnological potential of both groups of algae[28]. Among the compounds in *C. humicola*, Eugenol had the highest percentage at 40.70%, followed by Hexadecanoic acids, methyl ester (16.04%), and Caryophyllene (15.43%). These compounds contain antimicrobial, antioxidant, and anti-inflammation properties, and are currently being used in pharmaceutical products and industrial applications. The occurrence of phenolic compounds such as Thymol and Eugenol aligns with previous studies on green algae that have indicated strong overall antioxidant and antimicrobial activities in phenolic-rich extracts[29]. The detection of the fatty acid methyl esters, also noted by [23], confirms the biodiesel potential of *C. humicola*.

Regarding the compound in this algae that showed no matches, we suspect from comparison to reports of published GC–MS retention data and from the typical biochemical profile of green algae that this peak is Methyl myristate (methyl tetradecanoate), a fatty acid methyl ester that is often found in algal lipid extracts. The retention time for methyl myristate in the GC–MS analyses of algal fatty acid methyl esters (FAMEs) has been reported as 13–15 min[30, 31]. This compound is a potential major chemical class in algal lipids in the biosynthesis of a variety of saturated and unsaturated fatty acids, which serve important roles in energy yield and membrane structure and dynamics[32].

An alternative, but less likely, possibility is that this peak is Diethyl phthalate (DEP), which has also been confirmed at similar retention times (~14.5 min) from GC–MS analyses of marine algal lipids[33]. However, as it is well known that DEP may not be an algal-derived compound and could be identified due to contamination of laboratory plasticware or solvent, an identity here also should be further established by trenching part of the mass spectra matching to the NIST library and, if possible, as previously established by co-injecting an authentic standard[34].

As a result, the RT = 14.486 min peak is most likely related to Methyl myristate, given the natural presence of this lipid in *C. humicola*, and then further confirmation with spectral matching and retention index comparisons.

The chemical analysis of *Chlorococcum humicola* illustrates a diverse array of bioactive compounds, which include phenolic compounds (Thymol, Eugenol) with antioxidant and antimicrobial abilities, terpenes (Caryophyllene and cycloundecatriene derivatives) with anti-inflammatory activities, and fatty acid methyl esters (both saturated and unsaturated) with industrial (potential) and nutritional value. The presence of Eugenol and fatty acid esters indicates the potential of the alga itself as a natural bioactive and industrially relevant source of compounds[16].

In contrast, *C. vulgaris* demonstrated a greater abundance of monoterpenes and cyclic terpenoids. The predominant components were Eucalyptol (37.55%), Camphor (19.54%), and Endo-Borneol (11.67%), indicating a potentially significant aroma and therapeutic profile. Moreover, the Eucalyptol, Camphor, and Endo-Borneol from *C. vulgaris* are well known for their antimicrobial, anti-inflammatory, and analgesic properties, respectively, and are prominent in the pharmaceutical, aromatherapy, and natural flavoring industries. In terms of chemical composition, *C. vulgaris* differs from *C. humicola* in that it has a higher proportion of volatile monoterpenes, and comparatively lower levels of fatty acid methyl esters. Previous research on *Chlorella* species has similarly noted Eucalyptol and Camphor as main volatile compounds contributing to antioxidant and antimicrobial activities[35, 36].

Although there are some differences between the two algae, they have several features in common, including the presence of caryophyllene and other sesquiterpenes that confer some bioactivity that are typical for many green algae. Other minor chemical compounds that exist in small quantities, such as cycloundecatriene derivatives and some naphthalene derivatives, add some diversity to the chemical profile and may potentially be precursors for industries or pharmaceuticals. Comparing the two algae, *C. humicola* was found to contain higher amounts of

phenolics and fatty acid esters, which may render it more appropriate for applications in antioxidant activity or biofuels; however, *C. vulgaris* contained higher amounts of monoterpenes and aromatic compounds that could serve as promising candidates for pharmaceuticals, aromatics or flavor applications[37].

The diversity of bioactive compounds detected in both algae may also be associated with the environmental conditions of the habitat from which they were isolated. The samples were collected from the sewage water pond of the Samarra Pharmaceutical Laboratory, which represents a nutrient-rich and potentially contaminated aquatic environment. Such environments often contain elevated levels of organic matter, chemical residues, and dissolved nutrients that may influence algal metabolism[38]. Microalgae growing under these environmental stresses frequently produce a wide range of secondary metabolites, including phenolics, terpenoids, and fatty acid derivatives, as adaptive biochemical responses. Therefore, the chemical diversity observed in *Chlorococcum humicola* and *Chlorella vulgaris* may reflect physiological adaptations of these algae to the ecological conditions of the sewage water pond environment[39].

4. Conclusion

Chlorococcum humicola and *Chlorella vulgaris* were identified by GC–MS analysis as having potential based on a broad group of bioactive compounds, including phenolics, monoterpenes, sesquiterpenes, and fatty acid methyl esters. *C. humicola* appeared to possess promising antioxidant, antimicrobial, and biofuel value with its major components of Eugenol, Hexadecanoic acid methyl ester, and Caryophyllene. *C. vulgaris* was rich in Eucalyptol, Camphor, and Endo-Borneol with promising aromatic and therapeutic features. The minor components present in each species contributed to the chemical diversity of potential industrial value. Overall, the findings confirm these algal species to be promising sources of bioactive metabolites, which will vary by species for directed applications in pharmaceuticals, nutraceuticals, and biofuels.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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No founding.

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