

Investigating the Taxonomy and Bioactivity of an Egyptian *Chlorococcum* Isolate

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Abstract: The taxonomic designation of a green microalga isolated from Helwan governorate was investigated. The alga was coccoid, non-buoyant and mostly colonial with no other distinguishing features. Due to the lack of clear-cut diagnostic phenotypic characters and in order to accurately place the isolate in its correct taxonomic position, a polyphasic approach was applied. This involved the combination of both morphological and molecular methods coupled with phylogenetic analysis using small subunit rDNA as a marker. Accordingly, the isolate was identified as *Chlorococcum* (green alga). Other types of markers such as fatty acid composition, vitamin C content, phytochemical screening for secondary metabolites were also used. To further investigate the biochemical activities of this isolate, fractions of the lipophilic extract were tested for their antimicrobial effect. The fraction that showed highest bioactivity was purified and its structure elucidated using UV, FTIR, GC-mass and proton NMR. The bioactive compound from *Chlorococcum* sp. has a molecular weight of 256 and was identified as Hexadecanoic fatty acid. The possible antimicrobial effect of this compound is discussed. The study highlights both the importance of combining traditional classification methods with modern taxonomic techniques such as phylogenetics and chemotaxonomy. The study also emphasises the on importance of exploring the bioactive metabolites and their antimicrobial activity of microorganisms for future biotechnological applications.

Key words: *Chlorococcum*, polyphasic description, fatty acid composition, vitamin C, phytochemical screening, antimicrobial screening and hexadecanoic acid.

INTRODUCTION

The taxonomy of green algae in particular and algae in general is in a state of flux (McCourt, 1995). The traditional morphological-based methods of taxonomy, long employed in algal systematic, have led to misidentification of different algae as later was revealed when modern molecular techniques were used (McCourt, 1995; Michalopoulos *et al.*, 2000). A case study exemplifying this was reported on chlorophyceae (Lewis and McCourt, 2004). This class of green algae, represents an example of how modern molecular techniques revolutionized algal taxonomy and amended the taxonomic errors resulting from the use of morphology as the sole base of classification. This class includes diverse forms of microscopic green algae, ranging from unicellular to colonial. When 18S rDNA sequence data were collected from members of this class and compared, reclassification of several members including members of chlorococcales had to be made. This was due to the reliance of old classification on phenotypic characters that resulted in the inclusion of several morphologically similar yet genetically-distinct taxa in the same taxonomic clade. *Chlorococcum*, currently a distinct genus of chlorophyta, was first described as vegetative cells solitary or in temporary groups of indefinite form, never embedded in gelatin. Cells ellipsoidal to spherical with smooth cell walls and variable size. These morphological characters are commonly shared by many algal taxa and erroneous identification can ensue if only those plain phenotypic characters were used (Lewis and McCourt, 2004). Therefore, we use here 18S rDNA as a taxonomic marker coupled with phylogenetic analysis to confirm the identity of *Chlorococcum* strain originally isolated from the River Nile, Helwan, Egypt. This isolate was initially identified according to its phenotypic characters. Using a polyphasic approach in which

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the morphological, molecular and chemical data (reflected in fatty acid composition) are integrated, we aim to accurately place the isolate in its correct taxonomic position.

Moreover, we aim to explore the biotechnological potential of that isolate through investigating the presence of bioactive and antimicrobial compounds in that isolate. Vitamin C in particular is investigated here. Despite this vitamin's biological importance, its presence in green algae was hardly investigated (Aaransonet *et al.*, 1977; Runnger *et al.*, 1994).

Another group of bioactive compounds investigated here is natural secondary metabolites namely: tannins, flavenoids, saponins and alkaloids. Reports on the detection of these compounds in green algae and their bioactivity are also sparse despite their biological importance (Ghazala and Shameel, 2005; Schloz and Libezeit, 2006) and therefore their presence is going to be addressed here. Furthermore, we aim to explore the antimicrobial bioactivity of lipophilic extract from that alga. Ohta *et al.*, (1995) found that extracts from *Chlorococcum* strain, containing α -linolenic fatty acid as active substance, strongly inhibited the growth of a strain of methicillin-resistant *Staphylococcus aureus* (MRSA). Finally, through the current study we hope to highlight the importance of characterizing local microorganisms and their bioactive metabolites for future biotechnological exploitation.

MATERIALS AND METHODS

Materials:

Water samples were collected in triplicates, from the photic zone of the banks of The Nile, Maadi area, Helwan Governorate in sterile containers.

Methods:

Initial microscopic examination showed the dominance of green algae and diatoms with few observations of cyanobacteria and chrysophytes. Samples were spun down, and the pellets were spread over different solidified media (1.5 % agarose, w/v of growth medium) for green algae including ASM medium (Gorham *et al.*, 1964), modified Bold's medium (Nichols, 1973) and Oscillatoria (Feuillade, 1994). Colonies were picked up, examined under microscope and used to establish monospecific cultures through successive culturing and purification steps. Oscillatoria medium gave the best growth and was used for growing cultures.

Morphological Identification of Samples:

Morphological identification was initially performed using light microscopy and aided by following identification keys detailed in (Prescott's, 1982). Determination of cell dimensions was performed using a microscope digital camera (3.34 x10⁶ pixels; Q-imaging digital camera (Micropublisher 5.0 RTV and Q capture, Quantitative Imaging Corporation) supplied with Image analysis system (Simple PCI 5.3.1, Compix Inc., Cranberry, Pennsylvania, USA). The camera was fitted to a Leitz Orthoplan microscope (Wetzlar, Germany) equipped with a 40 x PHACO and 100 x Oil immersion lenses. Dimensions were taken in ten replicates and pixels were equated to microns using the software package and means and standard deviations were calculated using the MINITAB statistical software package (release 13.32, MINITAB Inc., USA). The images were taken at the School of Biological Sciences, Faculty of Science, University of Bristol, Courtesy of Professor Paul Hayes, Former Head of the School of Biological Science, University of Bristol, United Kingdom.

Genomic DNA Extraction, Small Subunit rDNA Sequence Analysis and Phylogenetic Reconstruction:

Genomic DNA was extracted using DNAeasy extraction kit (Qiagen, The Netherlands) following the protocol used for green plants. A partial fragment was amplified from purified genomic DNA, using Eukaryotic primers Euk algae F: GGAGAGGGAGCCTGAGARA designed specifically from alignment of several 18S sequences from different *Chlorococcum* strains and EukR: tgatccttctgcagggtcacctac designed by (Delong, 1992).

The 18S rRNA gene sequences were imported from GenBank and aligned in Clustal W tool within alignment function of MEGA 4 Phylogenetic package. Phylogenetic trees were reconstructed using different methods integrated in the MEGA 4 software (Tamura *et al.*, 2007) such as minimum evolution, maximum parsimony and neighbor-joining using both consensus and linearised tree approaches. Bootstrap values from 500 resamplings were calculated for each set of data and all trees were rooted using the *Scenedesmus abundance* as an outgroup. All resulting trees had similar topologies in which our isolate clustered together with other *Chlorococcum* isolates and away from *Scenedesmus* indicating similar

phylogenetic relationships. Bootstrapped-consensus Maximum parsimony tree inferred from 6 optimal trees was chosen for presentation. Branches corresponding to partitions reproduced in less than 50% trees are collapsed. The percentage of parsimonious trees in which the associated taxa clustered together is shown next to the branches. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar, 2000) at a search level of 3. The Neighbor-joining algorithm (Saito and Nei, 1987) was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the dataset. Phylogenetic analyses were conducted in MEGA 4 (Tamura *et al.*, 2007).

Fatty Acid Analysis:

A modified method of (Gunasekaran and Hughes, 1980) was applied. 0.5 gram of lyophilised cells was treated with methanolic HCL in the presence of 2, 2-dimethyl propane. The reaction proceeded for two hours at 80 °C followed by addition of 0.9 %M NaCl to the reaction mixture. 300 µl of n-Hexane were added and the sample was spun at 3000 rpm for five minutes. The methyl esters in n-hexane layer were analyzed on a Hewlett-Packard Model 5830A gas chromatography. The flow rate of N₂ was 40 ml/minute and the column temperature programmed to increase linearly from 160 °C to 230 °C at 30 °C min. The esters were identified by co-chromatography with standards.

Vitamin C content:

Lyophilised cyanobacterial filaments (1 g) were extracted in 80% (v/v) methanol and vitamin C content was quantified by its UV absorbance at 254 nm using reverse phase HPLC on a C18 column (Perkin-Elmer Phenomenex HPLC (C18 Ultracarb 7.0 Column ODS 20, dimension 250 x 4.6 mm). The mobile phase was methanol: water (97: 3), added under isocratic conditions with a flow rate of 0.5 ml min⁻¹ (Jeffe, 1984). The identity and quantity of vitamin C was confirmed by co-chromatography of known concentration of authentic standards (Sigma).

Phytochemical Screening:

Approximately 1 gram of lyophilised sample was extracted in 80% ethanol for two days. The extract was used for the following qualitative tests:

Test for Tannins (Claus, 1967):

Two ml of the alcoholic extract was added to 2 ml distilled water and filtered. 1ml of 5% ferric chloride was added to the filtrate. The development of a yellowish green colour indicates tannins presence.

Test for Saponins (Wall et Al., 1954):

Saponins were detected by their ability to develop a froth that is stable for a period of 30 min and longer. Two ml of alcoholic extract were added to 1 ml of distilled water then filtered. The filtrate was vigorously shaken.

Test for Alkaloid (Scholz and Liebezeit, 2006):

The lyophilised sample was boiled in water with 5ml 2M HCl solution and the filtrate was treated with Mayer's reagent which precipitates alkaloids. The presence of compounds of this class is established by the occurrence of turbidity or precipitation.

Test for Flavenoids (Scholz and Liebezeit, 2006):

Flavenoids presence is detected by the addition of a small magnesium ribbon to alcohol followed by dropwise addition of concentrated HCl. Their presence is indicated by the development of orange to red color within 1-2 min .

Antimicrobial Screening:

Extraction and Column Chromatography of Lipophilic Fractions:

Microalgal biomass (1 g fresh wt.) was collected and lyophilized. The lyophilised cells (0.5 g dry wt.) were extracted twice with 100 ml chloroform: methanol (2: 1, v/v) HPLC grade for two days, centrifuged (14,000×g) for 30 min using Hettich-cooling microcentrifuge, Germany). The supernatant was left to evaporate to dryness and was redissolved in 3 ml chloroform: methanol (2: 1v/v) modified from (Doan *et*

al., 2000). The sample was applied on a silica gel G60 column (1.5 x 25 cm) prepared from slurry of 30 g of precipitated Silica gel G60 (Merck, UK). The column was developed using the following solvent systems sequence in (Table 1).

Paper Disk-agar Diffusion Antibacterial Bioassa:

The pathogenic microbial strains were *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhae*, *Bacillus subtilis* in addition to pathogenic fungus *Candida albicans*. The lipophilic extracts were concentrated before applying to 6 mm paper disks for testing. The paper was left to dry and evaporate the solvent before using in the antimicrobial test. Sensitivity of these bacterial strains to the extracted fractions was assessed by using the slightly modified Kirby Bauer Disk Diffusion Susceptibility method (Bauer *et al.*, 1966). The bacterial suspension was then added to 20 ml nutrient agar (Mueller Hinton agar) and poured into petri dishes after well-mixing. The plates were incubated for 18 h at 37 °C, the diameter of the inhibition zone was measured in triplicates and the average and standard deviation was recorded. Disks containing chloroform: methanol (2:1, v: v) were left to evaporate and then used as controls.

Subfractionation of Active Fraction:

Using silica gel (Merck, 60) packed-column, dimensions: 25 x 1.5) the active fractions 10 and 11 for *Chlorococcum* (Table 2) were further subfractionated in using the solvent system detailed in (Table 3). After subfractionation, the fractions were further tested for their antimicrobial bioactivity. The fraction that showed bioactivity was further analyzed.

Table 1: Column fractionation of lipophilic extract from *Chlorococcum* isolate :

Fraction	Eluting agent	Elution volume	Order of fractions	Fractions received
	Chloroform: Methanol (v:v)			
I	(100: 0)	75 ml	1-8	7
II	(95: 5)	75 ml	9-16	7
III	(90:10)	75 ml	17-23	7
IV	(85: 15)	75 ml	24-30	7
V	(80: 20)	75 ml	31-36	6

Table 2: Antimicrobial activity profile of highest bioactive fractions of *Chlorococcum*. Numbers in brackets indicate the diameter of the inhibition zone in cm \pm standard deviation).

Fraction	Pathogenic microbial strain					
	<i>Pseudomonas aeruginosa</i>	<i>Escherichia coli</i>	<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>	<i>Salmonella typhae</i>	<i>Candida albicans</i>
10	+ve (1.5 \pm 0.2)	+ve (1.5 \pm 0.2)	-ve	+ve (1.3 \pm 0.3)	-ve	+ve (1.7 \pm 0.2)
11	+ve (1.5 \pm 0.2)	+ve (1.5 \pm 0.3)	-ve	+ve (1.3 \pm 0.2)	-ve	+ve (1.3 \pm 0.2)

Table 3: Solvent system used in sub-fractionation of fraction number 10 from *Chlorococcum*.

Fraction	Eluting agent	Composition (v: v)	Total volume	Fractions received
I	Acetone: Isopropanol: water	(80: 10: 10)	10 ml	2*
II	Acetone: Isopropanol: water	(70: 20: 10)	10 ml	2
III	Acetone: Isopropanol: water	(60: 30: 10)	10 ml	2
IV	Acetone: Isopropanol: water	(50: 40: 10)	10 ml	2

This fraction showed all the bioactivity when used in second antimicrobial screening on the same microbial pathogens and was used for further chemical analyses.

Elucidation of the Structure of the Bioactive Compounds:

Gas Chromatography, UV and FTIR, Proton NMR Analyses:

The GC-mass spectra were recorded on a Shimadzu-QP2010-plus with library search at electron voltage 70 ev and EI ionization mode. The UV-spectrum was recorded with a UV-VIS spectrophotometer (Shimadzu UV 200) and the FTIR spectrum was recorded on a Shimadzu R-5300. For proton NMR, 5 mg of the lyophilised sample was dissolved in DMSO and the NMR spectra were recorded on a JEOL AL300 NMR spectrophotometer. The chemical shifts were recorded in ppm at 300 MHZ using microtube.

RESULTS AND DISCUSSION

Growth and Morphological Identification:

The coccooid green forms were initially identified as *Chlorococcum* (Figure 1). According to (Prescott's, 1982), *Chlorococcum* was described as aggregates of cells are usually covered with very thin general mucous membrane mostly indistinguishable with no central mucous area. Colonies are formed of spherical cells of approximate diameter range ($5.8\mu\text{m} \pm 1.3$) Single chloroplast is nearly covering the entire cell.

18S rDNA Sequence and Phylogenetic Reconstruction:

The 18S rDNA sequence of the isolate showed 91% of similarity to other *Chlorococcum* isolates. The phylogenetic analysis revealed that the Egyptian isolate was clustered with *Chlorococcum* sp. 101-99 (FJ559386) with bootstrap value of 100%. They were clustered together with the rest of *Chlorococcum* strains in a single clade with bootstrap value of 67% and away from the outgroup taxon *Scenedesmus abundance* (Figure 2). Different treeing approaches resulted in similar topologies, indicating similar phylogenetic relationships and confirming the generic identity of the isolate as *Chlorococcum*.

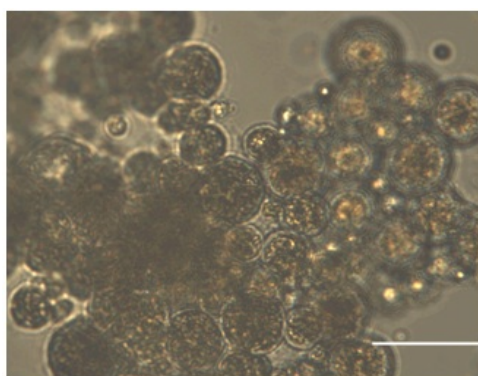


Fig. 1: Light micrograph of *Chlorococcum* cells (scale bar 10 μm).

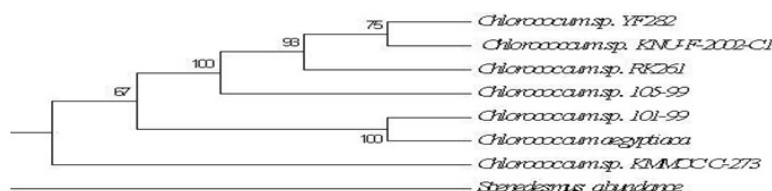


Fig. 2: Phylogenetic Maximum parsimony bootstrapped-tree. Accession numbers for the taxa used in the tree reconstruction are: *Chlorococcum* sp. 105-99 (AF514408); *Chlorococcum* sp. KMMCC C-273 (FJ559386); *Chlorococcum* sp. RK261 (AB490286); *Chlorococcum* sp. YF282 (AB490287); *Chlorococcum* sp. KNU-F-2002-C1 and *Chlorococcum* sp. 101-99 (AF514407). The green alga *Scenedesmus abundance* (X73995) was used as an outgroup taxon to root the tree. The isolate under study is designated *Chlorococcum aegyptiaca*.

The Fatty Acid Profile:

Regarding *Chlorococcum* strain fatty acid composition, it was found to be mostly dominated by saturated fatty acids followed by monounsaturated fatty acids (Table 4). The most dominant fatty acid was Hexadecanoic acid (64% followed by linoleic acid (23%). Polyunsaturated fatty acid were minor in the fatty acid composition and only represented by γ -Linolenic whereas Docosahexaenoic acid DHA fatty acid was completely absent. The pattern of fatty acids in Chlorococcales Ahlgren *et al.* 1992. is mainly characterized by the abundance of palmitic fatty acid (16:0), the presence of considerable amounts of linolenic acid (18:3 ω 3), linoleic acid 18:2 ω 9/12 and oleic acid 18:1 ω 9 and the lack of DHA C22:6. The pattern obtained from the *Chlorococcum* isolate was identical to that description.

Table 4: Composition of fatty acids in the *Chlorococcum* showing double bond position and percentage of each fatty acid.

Fatty acid	Number of carbon atoms	Number of double bonds	Position of double bonds	%Total fatty acid composition
Caproic	10	0	-	00.37
Lauric	12	0	-	01.77
Myristic	14	0	-	01.62
Palmitic	16	0	-	64.83
Palmetoleic	16	1	9	00.66
Oleic	18	1	9	03.89
Linoleic	18	2	9,12	23.11
γ -Linolenic	18	3	6, 9, 12	03.75
Arachidic	20	0	-	ND
Erucic	22	1	13	ND
DHA	22	6	4,7,10,13,16,19	ND

*ND: not detected

One of the important fatty acids revealed is palemetoleic acid. This fatty acid was found in diatom *Phaeodactylum tricorutum* and proved to be powerful antibacterial agent against multidrug resistant *Staphylooccus aureus* (MRSA) Desbois *et al.*, 2008. Other reported that unsaturated fatty acids had antibiotic activity against MRSA Ohta *et al.*, 1995. Also found that extract from some marine algae containing fatty acids showed strong bactericidal effect Ghazala *et al.*, 2004. Regarding the Hexadecanoic acid, it was found that this particular fatty acid played a constant role in increasing the endurance of the cyanobacterial strains to stress induced through temperature variations. This fatty acid predominated in the lipids of thylakoid membranes regardless of the temperature applied Iliev *et al.*, 2006.

Phytochemical Screening and Vitamin C:

For *chlorococcum* the flavenoids and alkaloids were both detected, however both saponins and tannins were absent. Vitamin C was present and its quantity estimated to be 6 $\mu\text{g}/\text{mg}$ dry weight for *Chlorococcum* (Figure 3)

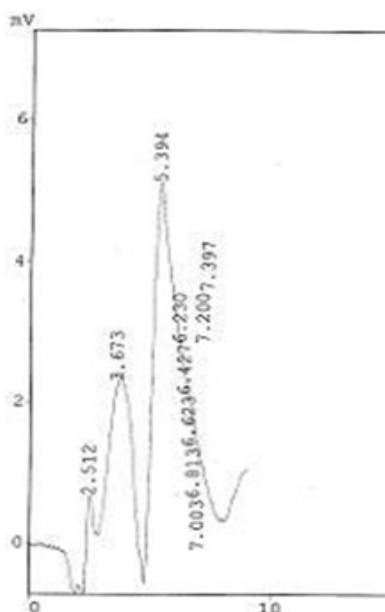


Fig. 3: HPLC graph of vitamin C in *Chlorococcum*

Antimicrobial Screening:

The antimicrobial activity was highest for fractions number 10 and 11 (Table 2). Since fraction 10 showed greatest diameter of inhibition zone of the pathogenic bacteria *Eschrichia coli* (Figure 4). Therefore, fraction 10 was used for further purification.

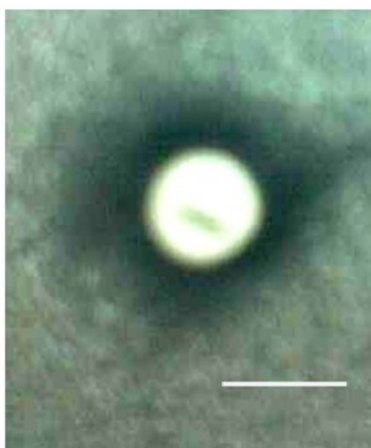


Fig. 4: Antimicrobial effect of fraction 10 from *Chlorococcum* on *Escherichia coli*. Scale bar 1.0 cm.

Eluci Elucidation of the Structure of Bioactive Compound

1-UV Spectroscopy:

The compound showed a single band at 322 nm in the UV range of 200-1000. This indicate single un-conjugated aliphatic fatty acid that has —COOH group (Figure 5).

2-infrared Spectra:

The spectrum was subdivided into different sections mainly; 4000–2800, 2800–2000 and 2000–1000 and 1000-400 cm^{-1} . The representative curve is shown in (Figure 6) which was divided into regions to allow identification of functional groups

a) Absorption in the 4000–2600 cm^{-1} region:

In this region, a broad band appears at 3404 cm^{-1} band indicating OH group. Also -CH aliphatic stretching vibration was detected where bands appeared to the right of the major band at 3000 at 2970, 2931 and 2614 cm^{-1} indicating the aliphatic nature of the long un-conjugated fatty acid.

b) Ab) Absorption in the 2600–1800 region:

This region showed no bands were detected in this region indicating the lack of ring system and substituted functional groups.

c) Absorption in the 2000–1000 region:

This region comprises several bands where clear band due to the stretching vibration of the C =O group at 1716 cm^{-1} . -CH₃ bend are also observed at 1379 cm^{-1} . whereas CH₂ bend is observed at 1462 cm^{-1} . C-O vibration characteristic of carboxylic acid is found at 1160, 1128 cm^{-1} and 1010 cm^{-1} .

d) Absorption in the 1000–400 region:

e) Long chain band is clearly observed at 812 cm^{-1} . Back ground signals of the solvent system are observed below this point.

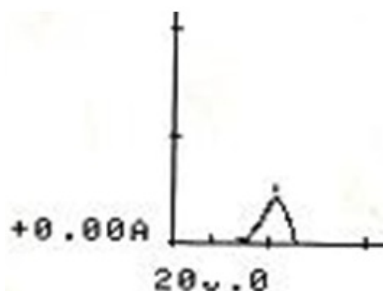


Fig. 5: The UV spectra of the bioactive compound from *Chlorococcum*.

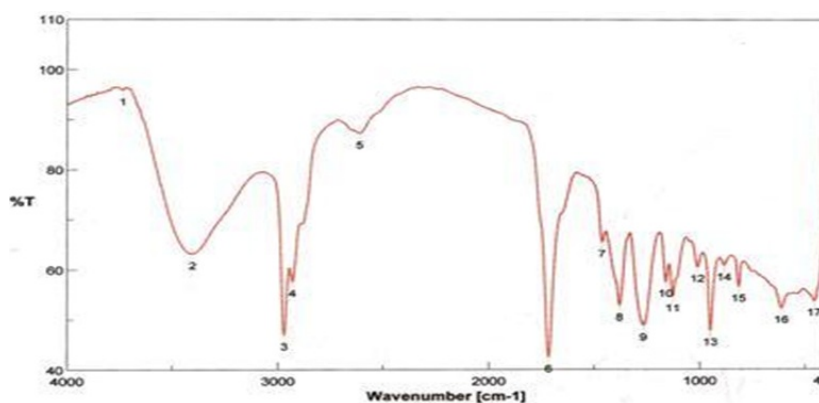


Fig. 6: FTIR for the bioactive compound from *Chlorococcum*.

3- The proton NMR Spectrum:

The characteristic signals in the ¹H NMR spectrum are shown in (Figure 7). The ¹H-NMR spectrum showed signals concentrated in the range 0.8-1.2, Characteristic of aliphatic compounds. Signals at 2.4 and 3.1 characteristic of DMSO and water.

4r- Gas Chromatography-Mass Spectra:

The mass spectrum fragmentation pattern of the compound under investigation is shown in (Figure 8). It reveals the presence of peak at *m/z* of 265 as molecular weight of the molecule. The library search indicated that this is hexadecanoic fatty acid

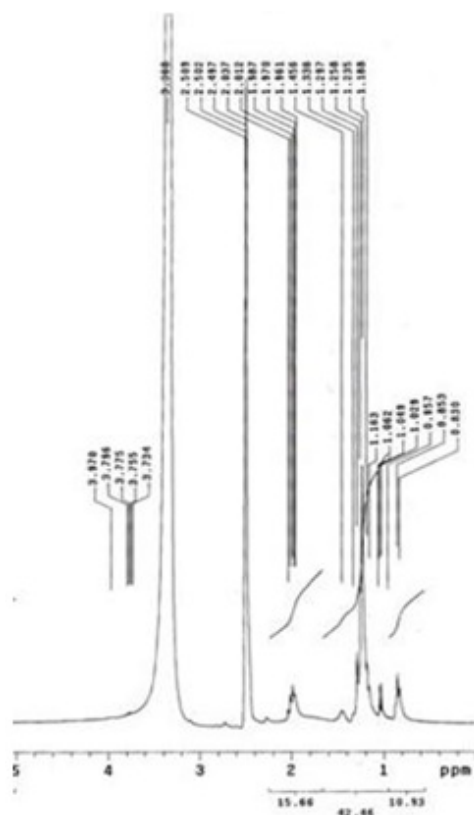


Fig. 7: proton-NMR of the bioactive compound from *Chlorococcum*

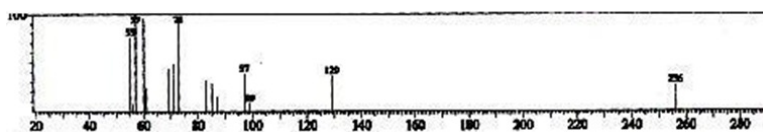


Fig. 8: GC-Mass of bioactive compound from *Chlorococcum*

This fatty acid, has previously been reported to have significant antimicrobial activity Benkendorff *et al.*, 2005. Copyright © 2004 Phycological Society of America The algae *Botryococcus braunii* (green alga) has toxic effects on a variety of aquatic organisms. Blooms of this alga, which typically occur in autumn, are associated with fish deaths in this lake Chiang *et al.*, 2004. Experiments indicated that plankton showed various susceptibilities to *B. braunii* that ultimately lead to their disappearance thus suggesting allelopathic effects that this green alga exhibits on neighbouring planktons The allelochemicals characterised were a mixture of free fatty acids, including α -linolenic, oleic, linolic, and hexadecanoic acid. Compared with other phytoplankton isolates, *B. braunii* produced significantly higher amounts of free fatty acids, which ultimately favored dominance of *B. braunii* in the natural environment. The mechanism of action that fatty acids might exhibited on other microorganisms was discussed by Wu *et al.*, 2006. They suggested that fatty acids exhibit cytotoxic activity against microbial planktons through damaging the plasma membranes. When these organisms were treated with deleterious concentrations of fatty acids, a remarkable elevation of extracellular potassium (K^+) was detected in the culture medium; indicating leakage of intracellular K^+ as a result of damage to the plasma membranes. The suggested sequence of cytotoxic effects is that plasma membranes are affected, leading to a change in membrane permeability. Severe damage to the plasma membranes would give rise to a disruption of the stressed cells.

Similarly Mundt *et al.*, 2003 suggested that fatty acids are able to change the permeability of the cell membrane, interact with proteins and lipids of the cell membrane, inhibit special enzymes or form a layer around the cells. All these mechanisms could result in bacteriostatic and/or bactericide activity and improve the survival of microalgae in their environment. In context with these reports, Ibrahim *et al.*, 1991 showed that lysozyme can be modified to be highly potent antimicrobial agent against gram negative bacteria that possess a large lipopolysaccharide layer thus hindering the entry of the lytic action of lysozyme. if linked to with Hexadecanoic fatty acid emphasizing on the effect of the latter on bacterial cell wall as well. Consistently, It is reported that the antibiotic activity of some algal species could be attributed to the presence of a mixture of organic acids such as: lauric, linoleic, myristic, hexadecanoic acid, stearic Kanas *et al.*, 1992. The free fatty acids were also reported to be potent allelopathic agents Ramsewak *et al.*, 2001. Another aspect of free Hexadecanoic fatty acid is that it can act as a metal-binding ligand through hydrophobic interactions Sandrin and Maier, 2003, a character that might help scavenging metal activators needed by the enzymatic system of the susceptible/exposed organism. Moreover, an interesting aspect of the bioactivity of Hexadecanoic fatty acid was reported by Harda *et al.*, 2002. They showed that an extract from a marine red alga, *Amphiroa zonata* which contained Hexadecanoic fatty acid showed selective cytotoxic activity to human leukemic cells, but no cytotoxicity to normal human dermal fibroblast (HDF) cells *in vitro*. The investigation on the effect of hexadecanoic acid showed that concentrations ranging from 12.5 to 50 $\mu\text{g/ml}$, had selective cytotoxicity to human leukemic cells, but no cytotoxicity to normal HDF cells. Furthermore, Hexadecanoic fatty acid induced apoptosis (programmed cell death) in the human leukemic cell line MOLT-4 (cancer Cell line) at 50 $\mu\text{g/ml}$. Hexadecanoic fatty acid also shows *in vivo* antitumor activity in mice. One molecular target of hexadecanoic fatty acid in tumor cells is DNA topoisomerase I, however, interestingly, it does not affect DNA topoisomerase II at low concentrations, suggesting that hexadecanoic fatty acid may be a bioactive compound of anticancer drugs. Since The topo I of both prokaryotes and eukaryotes are the type I topoisomerase, it is possible that it can affect this enzyme, if supplied in a concentrated pure form, in both prokaryotes such as pathogenic bacterial strains and eukaryotic such as pathogenic fungal strain, thus damaging DNA replication through inhibiting the enzyme responsible for removing supercoils.

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