

The First Record of Biological Activities of the Egyptian Red Algal Species *Compsopogon helwanii*

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Abstract—*Compsopogon helwanii* is a new Egyptian species of the genus *compsopogon*, collected (in 1999) from Ain Helwan Spring. It dominated all over the year. Neither this new red alga species, nor the other known eight *compsopogon* species were subjected to any physiological or biochemical studies. This is the first investigation concerning the biological activities of this new algal species. Methanolic extract of this alga was tested for antimicrobial (G+ve, G-ve bacteria, yeast, fungi), antialgal (green and cyanobacteria), antioxidant (DPPH and ABTS assays) and anticancer (EACC) activities. The obtained results correlated the biological activities to the contents of phycobilin pigments, spermine derivatives (GC/MS) and other active constituents in this algal extract.

Index Terms—Anticancer, antimicrobial, antioxidant, *compsopogon helwanii*, new species.

I. INTRODUCTION

The first record of the genus *Compsopogon* in Egypt was established by [1]. Since that time, there was no other reports of this genus was recorded till 1981, where [2] collected a new species called *Compsopogon aegyptiacus* from Lake Manzalah and considered several species to be synonyms. In the same time [3] collected the same species from Lake Mariut near Alexandria but it was misidentified as *C. coeruleus* Mort. [4] studied the algal flora of lakes and springs of Egypt, but they did not record any species belonging to the genus *Compsopogon*. Later on in 1999 a new *Compsopogon* species was collected from the hot Spring of Ain Helwan, by [5]. They found that the newly recorded species has different morphological characteristics from the eight known species of the genus which were reported from various regions of the world. The authors compared between the newly recorded species and the eight species using 6 comparative morphological characters and followed the key of [6]. The comparison showed similarity relationship between the newly collected species by [5] and other species of *Compsopogon* but different from them in the absence of microspores, the development of several erect filaments in its profuse branching and the formation of a wrapped whip-like structure and separation discs [5]. These different features illustrated that the collected species from Ain Helwan Spring is not identical to *C. coeruleus* or any other species of *Compsopogon*, so, the authors is claimed to treat it as a new species and called it *C. helwanii*.

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[7] recorded *C. helwanii* in all collected and studied samples from Ain Helwan Spring during her screening study (2000-2005) of the algal flora of this hot Spring.

II. MATERIALS AND METHODS

A. Collection of the Algal Species

During the first author screening study of Ain Helwan Spring (2000-2005), seasonal algal and water samples were collected. The water samples were physically (in situ) and chemically analyzed [8] while the algal samples (including *C. helwanii*) were cleaned, separated, identified, lyophilized and kept at -30 °C till use.

This study was the first record to undergo biochemical, physiological and biological studies on one of *Compsopogon* species, the *C. helwanii*

B. Extraction

The lyophilized alga (10 grams) was extracted three times with 80% methanol and the solvent was evaporated using rotary evaporator (40-45 °C). The residue was weighted and redissolved in the least volume of solvent to give a concentrated crude methanolic extract.

C. Biological Activities

1) Antimicrobial activity

The crude methanolic extract was tested against the gram positive bacterial species; *Bacillus subtilis*, *Staphylococcus albus*, *Streptococcus faecalis* and the gram negative bacterial species; *Escherichia coli*, one yeast (*Candida albicans*) and one fungus (*Aspergillus flavus*)

The paper disc diffusion bioassays using either solid bacteria (nutritive agar broth) or fungal (Doxs) media which have been heavily seeded with spore suspension of the tested organism

Incubation of the bacterial cultures was carried out at 35°C for 24-48 hr and fungal ones at 25 °C for 72 hr. [9].

2) Antialgal activity

The green algal species; *Ankistrodesmus falcatus* var. *tumidus* (west and west) G. S. West, *pseudochlorococcum typicum* Archibald, and the cyanobacterial species were; *Microcystis aeruginosa* (Kuet.) Kuetzing, *Aphanothece caldariorum* Eichter were used.

For screening the antialgal activity of *Compsopogon helwanii* extract, paper disc diffusion bioassay using nutritive media of the tested algal species (Bolds basal medium, [10] for green algae, and BG11, [11] for cyanobacteria), inoculated with known volume of tested algal suspension.

Incubation was carried out under controlled culture

conditions of temperature (25°C), light intensity (30 µE/m²/s) and photoperiod (16-8 light/dark cycles) for 7 days.

The clear zones of inhibition (mm) surrounded the paper discs were determined which correspond to the inhibitory power of the extract.

D. Screening for Antioxidant Activity

1) DPPH method

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) test was carried out as described by [12]. One ml of algal extract at different concentration was mixed with 1ml DPPH reagent (0.002% (w/v) /methanol solution). After an incubation period (30 min), the absorbance was measured at 517 nm.

$$\text{Antioxidant Activity\%} = \frac{Ac - At}{Ac} \times 100$$

where

At Was the absorbance of the extract samples and *Ac* was the absorbance of methanolic DPPH solution.

2) ABTS method

This assay was based on the ability of different substances to scavenge 2,2'-azino-bis(ethylbenzthiazoline-6-sulfonic acid (ABTS⁺) radical cation in comparison with a standard (BHT, 100 µg/ml). The photometric assay was conducted on 0.9 ml of ABTS⁺ solution and 0.1 ml of tested samples (in MeOH solution) and mixed for 45 s, measurements were taken at 734 nm after 1 min. The antioxidative activity of the tested samples was calculated by determining the decrease in absorbance at different concentrations by using the following equation:

$$E\% = \frac{Ac - At}{Ac} \times 100$$

where

At And *Ac* are the respective absorbance of tested samples and ABTS⁺, and expressed as µmol [13].

E. Screening for anticancer activity

1) Induction of tumor cell line

Female Swiss Albino Mice, (kept under environment and nutritional condition for 2 weeks) was injected intraperitoneally (i.p) by Ehrlich Ascites Carcinoma Cells (EACC), for the preparation of tumor cell line.

2) Tumor cells (cell line)

A line of Ehrlich Ascites Carcinoma resistant to endoxan has been used. The parent line was first supplied through the courtesy of Dr. G. Klein, Amestrdam, Holland. The tumor line is maintained in the National Cancer Institute, Egypt in Female Swiss Albino Mice by weekly transplantation of 2.5x10⁶ cells. Tumor cells were centrifuged at 1000 xg for 5 min at 4 °C. The pellets were washed with saline (0.9% NaCl) then the needed number of cells were prepared by suspending the cells in the appropriate volume of saline.

3) Viability of tumor

The viability percentage of tumor cells was measured by the modified cytotoxic trypan blue exclusion technique [14].

4) Medium and reagents

The culture medium used was prepared using RPMI media, 10% fetal bovine serum and 10% l-glutamine.

Trypan blue (0.4%) then kept in brown closed glass bottle.

5) Procedure

The viability percentage (V %) of tumor cells (4x10⁶ cells) was measured after incubation (at 37 °C for 2hrs) with the tested algal extracts and Dimethyl sulfoxide (DMSO) as control, Then the number of living cells was calculated using a hemocytometer.

6) RNA extraction, real time PCR analysis and quantification of gene expression

To assess the gene expression, total RNA extraction from cells was performed using RNeasy Mini Kit® (Qiagen Inc. Valencia, CA, USA). Reverse transcription was undertaken to construct cDNA library from different treatments using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The archived cDNA libraries were then subjected to quantitative real time PCR reactions using cyber green fluorophore (Fermentas Inc., Glen Burnie, MD, USA). Primer sequences were as follow: Bcl₂ forward primer GGG-TAC-GAT-AAC-CGG-GAG-AT and reverse primer CTG-AAG-AGC-TCC-TCC-ACC-AC; p53 forward primer CCT-CAC-CAT-CAT-CAC-ACT-GG and reverse primer CTG-AGT-CAG-GCC-CTT-CTG-TC.

F. GC/MS Analysis of Crude Methanolic Extract

The crude methanolic extract of *Compsopogon helwanii* was analyzed by GC-MS for determination of active substances in extract. GC/MS analysis was performed on a Thermoquest- Finnigan Trace GC-MS equipped with a DB-5 (5% (w/v) phenyl) methylpolysiloxane column. The injection temperature was 220 °C and the oven temperature was raised from 40 °C to 250 °C at a rate of 5 °C/min. Exactly 1 µl of sample was injected and helium was used as the carrier gas at a flow rate of 1.0 ml/min. The mass spectrometer was scanned over the 40 to 500 m/z range with an ionizing voltage of 70 eV and identification was based on standard mass library of National Institute of Standards and Technology (NIST Version 2.0) to detect the possible extract components.

III. STATISTICAL ANALYSIS

Data were subjected to an analysis of variance, and the means were compared using the "Least Significant Difference (LSD)" test at 0.05 and 0.01 levels, as recommended by [15].

IV. RESULTS AND DISCUSSION

A. Antimicrobial Activity

The crude methanolic extract showed antibacterial and antiyeast activities, while, the antifungal efficiency was not recorded with *Aspergillus flavus*. The gram positive species exhibited relatively higher antibacterial activity especially with *Bacillus subtilis* (15 mm) followed in descending order by those of *Staphylococcus albus* and *Streptococcus faecalis* (13 mm each). On the other hand *C. helwanii* extract showed moderate antibacterial activity against the gram negative *E. coli* and antiyeast against *Candida albicans* (9 mm and 10 mm respectively) as illustrated in Table I-A.

TABLE I: ANTIMICROBIAL ACTIVITY (A) AND ANTIALGAL ACTIVITY (B) OF METHANOL EXTRACT OF COMPSOPOGON HELWANII (DIAMETER OF INHIBITION ZONES, MM)

A-Antimicrobial activity						
	G+ve bacteria		G-ve bacteria		Yeast	Fungi
Species	<i>Bacillus subtilis</i>	<i>Streptococcus albus</i>	<i>Streptococcus faecalis</i>	<i>Escherichia coli</i>	<i>Candida albicans</i>	<i>Aspergillus flavus</i>
diameter of inhibition zones, mm	15	13	13	9	10	0
B-Antialgal activity						
	Green algal species			Cyanobacterial species		
Species	<i>Ankistrodesmus falcatus</i> var. <i>tumidus</i>	<i>Pseudochlorococcus typicum</i>		<i>Aphanothece coldariorum</i>	<i>Microcystis aeruginosa</i>	
diameter of inhibition zones, mm	14	15		13	13	

B. Antialgal Activity

Compsopogon helwanii methanolic extract showed higher antialgal activity (Table I-B) with the green alga *Pseudochlorococcum typicum* (15 mm) and *Ankistrodesmus falcatus* var. *tumidus* (14 mm). While relatively lower antialgal activity was recorded with the tested cyanobacterial species (*Microcystis aeruginosa* and *Aphanothece coldariorum*, 13 mm each)

Methanol has higher extraction efficiency for pigments and most of the secondary active metabolites which exhibit biological activities

The antimicrobial activity of *C. helwanii* methanolic extract against the tested G+ve, G-ve bacteria and yeast indicated that the crude extract contained active components which exhibited the relatively variable antimicrobial efficiencies depending on the structure and sensitivity of the tested organism.

It was known from literatures and published investigations that phenolic compounds (found in variable percentages in most algal species) showed antimicrobial activity against different bacterial, yeast and fungal species [16] reported that, methanolic extracts of some cyanobacteria and *Eichhornia crassipes* exhibited antimicrobial and antialgal activities which may be due to the presence of phenolic as well as phthalate derivatives in the extracts.

Our results showed conformity with those reported by [17] and [18] who illustrated that the acetone and methanolic extracts of the green and red seaweeds showed antimicrobial activity due to the presence of phthalate derivatives in algal extracts. Also, [19] studied the antimicrobial activity of some cyanobacteria species using agar well diffusion methods. Their results revealed that hexane and methanolic extracts of the tested cyanobacteria (*Scytonema* sp., *Plectonema boryanum* and *oscillatoria* sp.) showed higher activities (19-14 mm diameter of inhibition zones respectively) which may be due to compounds of phenolic nature as flavonoids and triterpenoids.

In the same context, [20] investigated the antimicrobial activity of *laurencia obtusa*, *Laurencia obtusa* var. *pyramidata* extracts (Methanol, chloroform, hexane) using paper disc diffusion bioassay: The obtained results recorded the higher efficiency of the essential oils and phenolic compounds in algal extracts which were responsible for the pronounced antimicrobial activity of the tested red seaweed species.

The recorded antimicrobial activity of *C.helwanii* crude extract may be partly due to permethylspermine and piperazine derivatives recorded by GC/MS in the extract (Table II)

TABLE II: GC/MS OF METHANOLIC EXTRACT OF THE RED ALGA COMPSOPOGON HELWANII

Peak no.	RT	Compounds	Relative conc.
1	3.23	3,4 dimethyl octane	9.50
2	3.31	Octanal	8.97
3	3.38	3-methyl-azaoine-methylester	4.00
4	4.40	Permethyl spermine	47.59
5	6.62	2-methyl-Undecanal	7.72
6	10.76	Piperazine, 2-methyl	2.53
7	10.99	Undecane	2.47
8	13.08	Methyl-docosanoate	3.72
9	14.36	1,4-Oxathian-2-one	2.17
10	14.80	Methyl malonic acid	1.54
11	15.44	(z)-11-hydroxy-9-undecanoic acid	2.01
12	15.88	Carbamic acid	1.48

C. Antioxidant Activity

Compsopogon helwanii inhabit the very exposed and shallow muddy part of the Spring of Ain Helwan, it acquire greenish color and not the red characteristic color of red algae due to its chromatic adaptation. Its persistence all over the year seasons indicated its tolerance to the variable seasonal environmental conditions [7]. This stress tolerant red alga must have more effective defense system against the excessive production of the reactive oxygen species.

It is postulated that marine algae and seaweeds are exposed to intense light and higher oxygen concentration which lead to the formation of free radicals and strong oxidizing agents. These organisms protect themselves from the damaging effects of ROS by producing antioxidative substances.

The antioxidant activity of *C. helwanii* methanolic extract was shown to be concentration dependent. With DPPH method, the activity was 43.5% at 50 µg/ml extract increased to 66% at 100 µg/ml. while on using the sensitive ABTS radical scavenging methods, an increment in antioxidant activity was recorded (55.8% and 74.3% at 50 and 100 µg/ml extract concentration respectively) as shown

in Fig. 1.

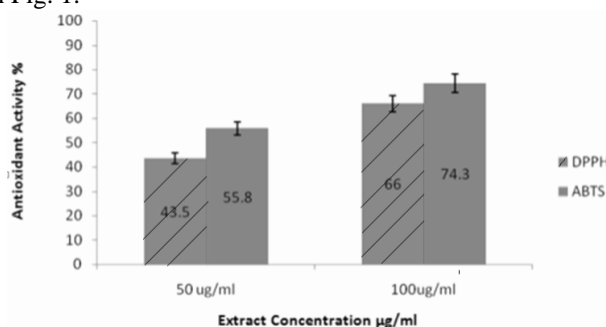


Fig. 1. Antioxidant activity of methanol extract of *Compsopogon helwanii* against DPPH and ABTS radical assay

Methanolic extract of the fresh water red alga (*C. helwanii*) dissolved various naturally occurring pigments and phytochemicals which may have antioxidant efficiency.

The characteristic phycobiliprotein pigments, chlorophylls (chl a, d) and carotenoids were shown to have the capacity to trap free radicals. Phycocyanobilin produced by *Spirulina platensis* [21] was responsible for the majority of antioxidant activity of phycocyanin pigment (of the red algae and cyanobacteria) and may act as effective antioxidant in a living human body. So, these substances may exert the same effect in case of the red alga *C. helwanii* extract. Polyphenolic which covers many different subgroups of phenolic acid and flavonoids were present in variable quantities in all algal groups. Polyphenolics are especially important antioxidants because of their redox potential which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelating potential [22]. [23] and [24] reported that the principle antioxidant components in seaweeds are thought to be polyphenols and studies demonstrated the correlation between polyphenols content and radical scavenging activity. Carotenoids and sesquiterpenoids in the extracts of the red alga *Laurancia obtusa* were antioxidantly effective [25]. It may share also, in the antioxidant activity of red alga *C. helwanii* extract. While [26] focused on the fact that seaweeds and freshwater algae may contain other antioxidative substances as tocopherols, chlorophylls, flavonoids and carotenoids which get partitioned into organic solvents and impart the radical scavenging activity to the extracts from these organisms.

Spectroscopic analysis of methanolic extract of *Compsopogon helwanii* by GC/MS revealed the higher relative concentration of permethylspermine (47.59 %, Table 2). Spermine is found in a wide variety of organisms and tissues, it possesses (*in vitro*) an antioxidant, may act also as pro-oxidant and anti-inflammatory properties [27]. Moreover, [28] reported that spermine analogues were recognized to produce high inhibitory effect on cell growth. So, the highly pronounced antioxidant activity of *Compsopogon helwanii* methanolic extract may be largely due to spermine, phycocyanin, chlorophylls, carotenoids as well as to the phenolic compounds in the red alga. These compounds may act synergistically to induce the recorded antioxidant activity of methanol extract.

These results are in agreement with the results obtained by [29] Who found that, the polyamines Spermidine and spermine are considered as potent antioxidant in

soybean oil and provide higher protective factors than some of the traditional synthetic antioxidant (BHA and BHT).

Also, the same authors concluded that, the antioxidant activity of these compounds was associated with the number of amine group in the molecule, which correlation significantly ($R=98.12$) with the protection factors.

D. Anticancer Activity

Liver as all organs in the body is surrounded by a lipoproteic biomembrane to which is attached certain genes concerned with programming apoptosis process. Under environmental stress conditions, excessive production of ROS led to serious dangerous effects due to alteration of the macromolecules including DNA, protein, lipid—etc.

Any alteration in the structure of these biomolecules induces signals to the apoptosis genes to start expression leading to the death of the cell.

Acute and chronic exposure to environmental chemicals such as pesticides, metals, polycyclic aromatic hydrocarbons (PAHs), solvents and alkalizing agents, has been shown to produce marked toxicity to their target sites. Reactive oxygen species are another important class of damaging agents to cellular macromolecules (DNA, protein, lipids)

The genome is constantly exposed to agents, both exogenous and endogenous that damage DNA leading to mutations involving different base substitution, chromosome breaks, deletions---etc [30]. Consequently it is very important to determine these agents and other agents that protect against them.

The obtained results in this investigation recorded the great effect of methanolic extract of the red alga *Compsopogon helwanii* on the Eherlich Ascites Carcinoma Cells (EACC). The activity was shown to be concentration dependent; where about 50% cytotoxicity was recorded at extract concentration (Fig. 2) 50ug/ml increased to more than 90% on doubling the extract concentration (100 µg/ml). To be sure that cytotoxicity (apoptosis) was due to gene expression, the effect of *C. helwanii* extract on BCL2 & P53 genes expression revealed about four and five folds increase in relative quantity in extracts compared to the control (Fig. 3).

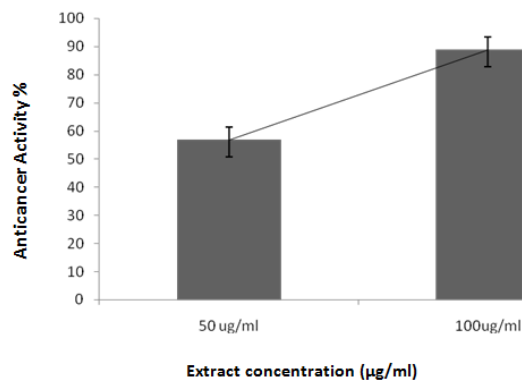


Fig. 2. Anticancer activity of methanol extract from *Compsopogon helwanii* against EACC

The anticancer activity of major compounds in our extract (Spermine derivatives) may be due to the fact that, it bind to nucleic acids, thus interfering with chromatin conformation and gene expression as well to protein and

membrane phospholipids, targeting ion-channel modulation and stability [31]-[32].

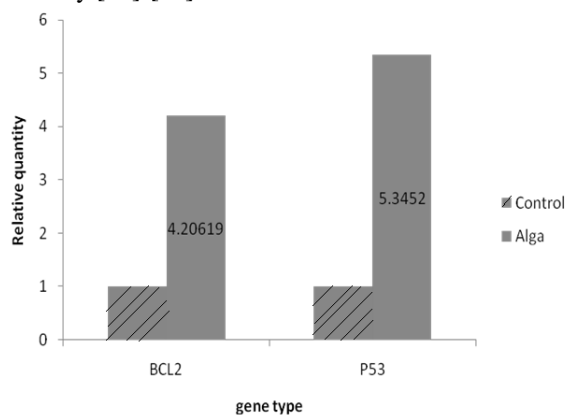


Fig. 3. Effect of *Compsopogon helwanii* methanolic extract on BCL2 and P53 genes (gene expression)

These results are in agreement with the results obtained by [33] who revealed that, the polyamines (putrescine, spermidine and spermine) are indispensable for proliferation and differentiation of cells. Depletion of polyamines is associated with reduced cell proliferation, and the levels of polyamines rapidly increased during cell growth.

Also, [34] reported that, three piperazine derivatives, bromopropionyl piperazine (A 8103) appeared to be most effective and superior to HN₂ or 5-FU against Walker 256 tumor in the rat. In the same context, [35] studied the effect of 1,4-bis-(4-(1H-benzo[d]imidazol-2-yl-phenyl)) piperazine (BIPP) and found that, BIPP causes an intrinsic apoptosis in U937 leukemia cells.

It was reported that water soluble phycobilins, phenolic compounds, polysaccharides induced apoptosis of different cancer cells. In this context, [36] found that polysaccharide fraction from the brown seaweed *Sargassum thenbergii* had antitumor activity against Ehrlich carcinoma (Ascite form). Also, [37] showed that the aqueous extract of *S. kjeldmanianum* had a higher antitumor activity against sarcoma 180. [38] reported that the red algal aqueous extract (mainly contain C-phycoerythrin) exhibited higher antiproliferative effect (82.4%). Several authors reported an increased antioxidant and antimutagenic/anticarcinogenic activities due to the phenolic compounds [39], in different seaweeds [40] and in many plant species and herbs [41]. [42] reported that aqueous extracts of different Cyanobacteria and microalgae exhibited potent antioxidant activity as well as a pronounced anticancer efficiency against Human hepatocellular cancer cell (Hep G2) and Ehrlich Ascites carcinoma cell (EACC) which may be due to the polar algal contents in the aqueous extracts.

Red algae (and cyanobacteria) have the characteristic phycobiliprotein pigments (including phycocyanin and phycoerythrin) and their main cell wall constituent, the sulfated polysaccharides both are polar compounds, dissolved in the methanol extract of the red alga *C. helwanii* and may participated in the well pronounced anticancer activity.

It is worthy to mention that the GC/MS analysis (Table 2) of the methanolic extract recorded the presence of certain content of piperazine derivative which may strengthen the antiproliferative activity of the other mentioned active

components. It was well established that piperazine and epoxide-containing piperazine were effective anticancer agents inducing apoptosis in Leukemia, U937 cells as well as human breast and prostate tumors [35].

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