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Bioactive Allelo-chemical Compounds From *Oscillatoria* Species (Egyptian Isolates)

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ABSTRACT

Three *Oscillatoria* species; *O. hamelii*, *O. platensis* and *O. rubescens* were isolated from the water samples of Ain Helwan thermal spring. Antibiotic activities (antibacterial, antifungal & antialgal) of the three diethyl ether *Oscillatoria* extracts were carried out by paper disc diffusion bioassays. Antimicrobial activity was tested against four bacteria (Gram –ve; *Escherichia coli* & three Gram +ve; *Bacillus subtilis*, *Staphylococcus albus*, *S. faecalis*) one yeast (*Candida albicans*) and one fungus (*Aspergillus flavus*). Diethyl ether extracts of the three *Oscillatoria* species exhibited great antibacterial and antifungal activities especially *O. rubescens,* which demonstrated the highest activity followed by *O. hamelii* and *O. platensis*. Antialgal efficiencies of the three *Oscillatoria* extracts were more pronounced in the eukaryotic green algae especially in *Ankistrodesmus falcatus var. tumidus* than in either *Pseudochlorococcum typicum* or in the two cyanobacterial species (*Microcystis aeruginosa* PCC 7806 (toxic), *Aphanothece caldariorum*). Chromatographic analyses by GC/MS and GLC of extracts of the three *Oscillatoria* species revealed the presence of the tetraamine spermine, piperazine derivatives, saturated and un-saturated fatty acids, which may act synergistically and induced the antibiotic activity.

Key Words: *Oscillatoria* spp; Antibiotic activity; Fatty acids; Bioactive compounds

INTRODUCTION

Several bioactive metabolites produced by cyanobacteria and algae have been discovered by screening programs, employing target organisms quite un-related to those for which the metabolites evolved (Smith & Doan, 1999). Many of these chemicals have diverse range of biological activities and chemical structures, which affect many biochemical processes within the cells (mainly directed against photosynthetic process). Such chemicals are presumably related to the regulation and succession of algal and bacterial populations and can be involved as natural herbicide or bio-control agents (Jüttner, 1987).

The existence of algicidal and bacteriocidal properties of cyanobacteria is to be expected in the light of the co-occurrence of these organisms in aquatic natural communities, where an inhibitory interactions occurred between producers and competitors within the same ecosystem. These allelo-chemicals are therefore expected to be synthesized under stress conditions (Oligotrophic) and low growth rate and released at concentration large enough to be effective (Keating, 1977 & 1978).

A pronounced reduction of gram positive bacteria in lakes during the occurrence of cyanobacterial water blooms was reported by Chrost (1975) and the production of antibacterial substances may be one reason for this phenomenon. Although there are reports of algicidal activity in species such as *Microcystis aeruginosa* (Ikawa *et al*., 1996), algicidal properties have generally been observed in a restricted number of genera as reported by Schlegel *et al*. (1999).

The screening study of Flores and Wolk (1986) in which 65 filamentous cyanobacteria were tested against related strains, revealed the presence of 7 antibiotic-producing organisms, confined to *Fischerella*, *Nostoc* and *Anabaena*. Moreover, Rippka *et al*. (1979) reported that the nitrogen-fixing filaments of *Scytonema* and *Oscillatoria* have algicidal activity.

Cyanobacterial bioactive allelo-chemicals that have been characterized as algicides are directed against photosynthesis (photosystem II) and therefore are termed natural herbicides. Light-dependent processes are unique to both prokaryotic cyanobacteria and eukaryotic algae and are therefore logical targets for a bioactive producer organism in competing with other such organisms (the targets) in the same habitat. The aim of this investigation was to screen for antibiotic-producing *Oscillatoria* species, which were isolated from the thermal spring of Ain Helwan and its role in algal dominance and succession.

MATERIAL AND METHODS

Algal isolation and growth conditions. Three *Oscillatoria* species were isolated from the thermal Spring of Ain Helwan (Egypt) in October 2004 (Autumn) and were identified, according to Bourrelly (1970) and Prescott (1978), as the Egyptian isolates of *Oscillatoria hamelii* Frémy, *O. platensis* (Nordst.) Geiller and *O. rubescens* De Cand. Unialgal cultures of these species were incubated at temperature 25 ± 1˚C under continuous illumination (cool

white fluorescent light) of 30 μE m^{-2} s^{-1}. At the late exponential growth phase, algae were harvested, lyophilized and kept at -20˚C till use.

Extraction of active components. A 500 mg of each lyophilized *Oscillatoria* species was extracted separately by diethyl ether. Evaporation of the solvent under vacuum using rotary evaporator (at 40˚C) was followed by dissolution of residue in the least volume of solvent (Gromov *et al*., 1991).

Separation and purification of active components. Precoated silica gel plates F_{254} were spotted with the concentrated *Oscillatoria* extracts and developed using carbon tetrachloride/ethyl acetate (95:5 v/v). The separated fractions that fluoresced under ultraviolet lamp (Rf o.93, 0.97 & 0.95 for *O. hamelii*, *O. platensis* and *O. rubescens*, respectively) were eluted with diethyl ether, tested for biological activities and analyzed chromatographically by GC/MS and GLC (Chauhan *et al*., 1992).

Screening for antibiotic activities. Screening for antibiotic activity of *Oscillatoria* extracts, paper disc diffusion bioassays were used, where the sterilized filter paper discs saturated with algal extract (s) were placed on the surface of plates containing either solid bacterial (nutrient agar broth) or fungal (Dox's) media, which have been heavily seeded with spore suspension of the tested organism. Four bacteria species (Gram –ve; *Escherichia coli* & three Gram +ve; *Bacillus subtilis*, *Staphylococcus albus*, *Streptococcus faecalis*), one yeast (*Candida albicans*) and one fungus (*Aspergillus flavus*) were used as test organisms. Incubation of bacterial cultures was carried out at 35˚C for 24 - 48 h and fungal ones at 25˚C for 72 h (Grayer & Harborne, 1994; Muanza *et al*., 1994).

Screening for the antialgal activity of the three *Oscillatoria* extracts, also the paper disc bioassay was used, where the discs were saturated with each of the extracts and placed on the surface of solid nutritive media [Bold's basal medium (Bischoff & Bold, 1963) for green algae and BG_{11} (Rippka, 1988) for Cyanobacteria] inoculated with known volume of tested algal suspension [(green algae; *Ankistrodesmus falcatus* var. tumidus (West & West) G.S. West; *Pseudochlorococcum typicum* Archibald) and (Cyanobacteria; *Microcystis aeruginosa* PCC 7806 Kuetzing; *Aphanothece caldariorum* Richter)]. Incubation of plates was carried out for 7 – 10 days at the same previously mentioned algal culture conditions.

The diameter of the clear inhibition zones surrounding the paper discs saturated with algal extracts were taken as a measure of the inhibitory power of each *Oscillatoria* extract against the particular test organism. Each data is the mean of triplicates.

Identification of the biologically active compounds. Methylated extracts (using anhydrous methanol & ethereal diazomethane) were analyzed by GC/MS. Saponification of another extract using ethanolic 20% KOH at room temperature, over night then acidification with HCl and extraction by ether) followed by Methylation of the fatty acid containing ether extract and were analyzed by GLC according to Vogel (1975) and then the method described by Farag *et al*. (1986) was applied for the determination of active compounds. Separation of active compounds by GC / MS (Hewlett-Packard Co., Palo Alto, CA) and fatty acid methyl esters by GLC (UNICAM PRO-GC) were compared with and identified by a mixture of standard fatty acids (Carried out at Principal Central Lab., Faculty of Agriculture, Cairo University).

RESULTS AND DISCUSION

Growth of all microorganisms, bacteria, candida, cyanobacteria and green algae, used as test organisms was inhibited by the separated fluoresced fractions on TLC and

Table I. Antibiotic activities of extracts of three *Oscillatoria* species, expressed as diameter of inhibition zones (mm) using paper disc diffusion bioassay, (A) antibacterial and antifungal activity, (B) antialgal activity

(A)

Species	Bacteria				Fungi	
	Gram –ve	Gram +ve				
Samples	*Escherichia coli*	*Bacillus subtilis*	*Staphylococcus albus*	*Streptococcus faecalis*	*Candida albicans*	*Aspergillus flavus*
Control (diethylether)	0.0	0.0	0.0	0.0	0.0	0.0
O. hamelii,	15	14	14	12	12	0.0
O. platensis	13	11	16	11	14	0.0
O. rubescens	18	14	12	13	17	0.0

(B)

Species	Algae			
	Cyanobacteria		Chlorophyta	
Samples	*Microcystis aeruginosa* PCC 7806	*Aphanothece caldariorum*	*Ankistrodesmus falcatus* var. tumidus	*Pseudochlorococcum typicum*
Control (diethylether)	0.0	0.0	0.0	0.0
O. hamelii,	14	15	22	14
O. platensis	13	14	21	21
O. rubescens	13	12	19	16

Table II. Bioactive compounds isolated by Gas chromatography/Mass spectrometry (GC/MS) of the methylated *Oscillatoria* species

Bioactive compounds	Retension times (min.)	Relative concentration (%)
Spermine derivative	4.42, 4.45, 4.47, 4.50, 5.29, 6.28	31.4
Piperazine derivative	11.82, 12.99	7.09

Fig. 1. GC/MS fragmentation patterns of (A) spermine, (B) piperazine derivatives isolated from the methylated algal extracts of the three *Oscillatoria* species

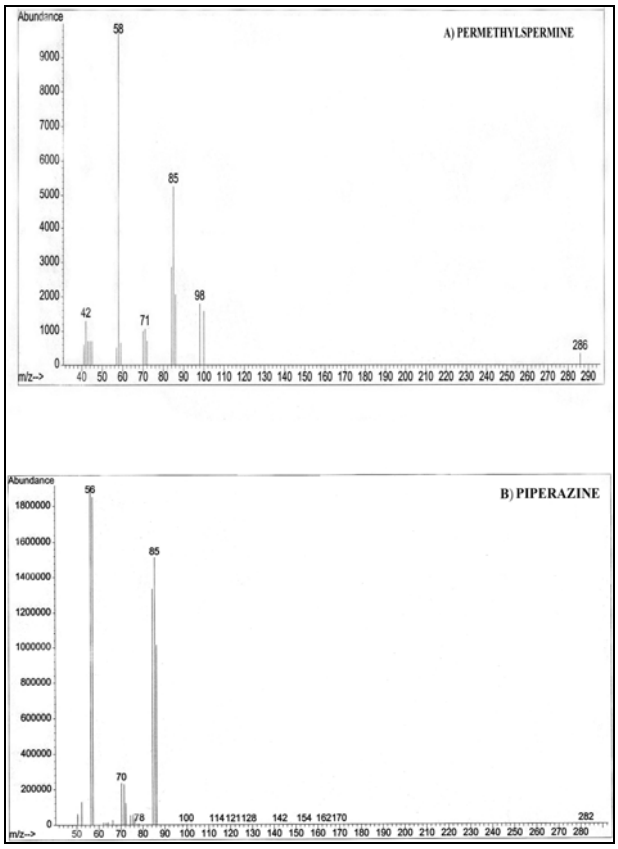
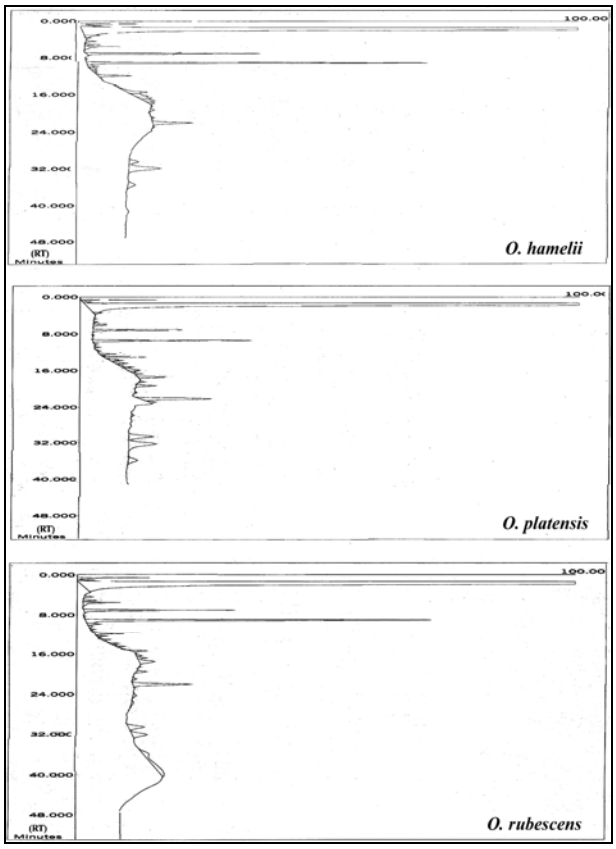


Fig. 2. Total ion chromatograms of GLC analyses of the three *Oscillatoria* extracts after saponification and methylation and compaired with mixture of standard fatty acids



eluted by diethyl ether of the three *Oscillatoria* species. Antibacterial activity against both G - ve and G + ve species and anticandida were clearly manifested by the three *Oscillatoria* species as illustrated in Table IA especially by *O. rubescens*, followed by *O. hamelii* and *O. platensis*. On the other hand no antifungal activity was detected with *A. flavus* as a fungal test organism.

Concerning the antialgal activity, Table IB illustrated that all *Oscillatoria* species exerted an antialgal activity against the four tested algae, with greater activity detected against the eukaryotic green algae than the prokaryotic cyanobacteria species. *A. falcatus* var. tumidus demonstrated the largest inhibition zones when treated with *O. hamelii* extract followed by that of *O. platensis* and *O. rubescens* (maximum diameter of inhibition zones were 22, 21 & 19 mm, respectively).

The green algal species *P. typicum* was similarly affected (as *A. falcatus*) by *O. platensis* extract (21 mm), while the inhibitory activities were less pronounced by *O. hamelii* and *O. rubescens* extracts than in *A. falcatus* (diameter of inhibition zones were 14 & 16 mm, respectively). Both prokaryotic cyanobacterial species; *M. aeruginosa* and *A. caldariorum* demonstrated more or less similar inhibition zones with the three *Oscillatoria* extracts, which were generally much less pronounced than the eukaryotic green algae.

Chromatographic analyses of the three *Oscillatoria* extracts, using GC/MS, revealed presence of the tetraamine spermine (present as permethyl spermine) and piperazine (present as piperazine-2-methyl) in all *Oscillatoria* species (Table II, Fig. 1). This analysis further demonstrated more or less similar total ion chromatograms, which were identified by a mixture of standard fatty acids (Fig. 2). These extracts contained different saturated and un-saturated fatty acids of variable relative percentages (Table III).

Schlegel *et al*. (1999) reported that a given organism may produce more than one bioactive substances targeted against different biochemical processes. Gromov *et al*. (1991), Bagchi and Marwah (1994) and Bagchi (1995) demonstrated that although the chemical structure of cyanobacteria bioactive metabolites differed, they frequently share a common mechanism of action namely inactivation of photosystem II-mediated electron flow in cyanobacteria, green algae and higher plants.

The antibiotic activities (antibacterial, antifungal & antialgal) exhibited by the three *Oscillatoria* species in this investigation were in accordance with the reported antibiotic efficiencies manifested by different cyanobacterial bioactive compounds; Hapalindoles alkaloids, Cyanobacterin,

Table III. Fatty acid content (saturated and unsaturated) in the saponifiable and methylated extract of The Three *Oscillatoria* Species, their retention times and relative concentrations as analyzed by Gas Liquid Chromatography (GLC) and compared with a mixture of standards of fatty acids

Algal species		*O. hamelii*		*O. platensis*		*O. rubescens*	
Fatty acids		Retension Time (RT)	Relative conc. (%)	Retension Time (RT)	Relative conc. (%)	Retension Time (RT)	Relative conc. (%)
Saturated fatty acids							
Caprylic	C_8 : 0	1.900	60.700	1.833	64.181	1.950	61.870
Capric	C_{10} : 0	7.083	5.780	7.100	3.527	7.067	4.940
Undecyclic	C_{11} : 0	8.233	0.061	8.267	0.061	8.233	0.056
Lauric	C_{12} : 0	9.085	11.410	9.417	6.085	9.050	11.610
Tridecylic	C_{13} : 0	9.850	0.464	—	—	9.817	0.539
Myristic	C_{14} : 0	11.883	1.538	11.717	0.188	11.833	1.760
Pentadecylic	C_{15} : 0	15.433	1.731	12.367	0.554	12.417	0.251
Palmitic	C_{16} : 0	16.767	0.856	16.683	0.335	16.767	0.398
Stearic	C_{18} : 0	20.867	0.142	20.817	0.131	20.983	0.249
Total Saturated Fatty Acids			82.682		75.062		81.674
Un-saturated fatty acids							
Palmitoleic	C_{16} : 1 n 7	17.833	0.363	17.417	1.420	17.500	1.288
	C_{16} : 2 n 4	18.567	0.032	18.450	0.251	18.583	0.057
	C_{16} : 3 n 4	19.350	0.276	19.317	1.165	19.517	0.542
Oleic	C_{18} : 1 n 9	22.000	2.609	22.167	4.859	22.050	3.598
	C_{18} : 1 n 7	22.867	0.066	—	—	—	—
Linoleic	C_{18} : 2 n 6	—	—	—	—	24.300	0.304
	C_{18} : 3 n 4	—	—	25.750	0.192	25.817	0.288
α- Linolenic	C_{18} : 3 n 3	30.450	0.991	30.667	3.085	30.550	1.611
Stearidonic	C_{18} : 4 n 3	31.917	4.255	32.235	4.695	32.083	1.767
Arachidonic	C_{20} : 4 n 6	35.517	1.052	35.850	1.438	—	—
Eicosapentaenoic	C_{20} : 5 n 3	—	—	—	—	40.233	3.206
Total Unsaturated Fatty Acids			9.644		17.105		12.661

Nostocyclamide, Cyanobacterin LU-1 and LU-2, Fischerellin A and B and Norharmane produced by various cyanobacteria species (Pignatello *et al*., 1983; Moore *et al*., 1987; Vepritskii *et al*., 1990; Srivastava *et al*., 1998; Jüttner, 1997; Volk, 2005). Both spermine and piperazine derivatives identified by GC/MS of the three Oscillatoria species exhibited antimicrobial activities (Walters *et al*., 1995; Dash *et al*., 2002; Walters *et al*., 2003; Cushion *et al*., 2004).

The saturated fatty acids caprylic, capric, lauric, myrestic and the un-saturated ones, palmitaleic, oleic, linoleic and linolenic acids (separated also in *Oscillatoria* extracts in the present study) were demonstrated to have antimicrobial activities against G - ve, G + ve bacteria and pathogenic fungi as reported by other studies (Fei *et al*., 2002; Kamenarska *et al*., 2002; Walters *et al*., 2003; Ghazala *et al*., 2004; Kimura & Yokota, 2004; Krasnoff *et al*., 2005).

Different *Oscillatoria* species (*Oscillatoria laetevirens*, *Oscillatori redekei* & other *Oscillatoria* species) were reported to produce secondary metabolites (in their non-polar extract) with two active compounds having long chain saturated fatty acids as a part of their structure (Chauhan *et al*., 1992) and/or un-saturated hydroxylated fatty acids (Mundt *et al*., 2003) produced by these cyanobacteria and inhibited the growth of green algae and other cyanobacterial species including the toxic *Microcystis aeruginosa* PCC 7820.

The great antibiotic activity manifested by *O. rubescens* extract in the present study may be due to its total contents of saturated and un-saturated fatty acids 81.674 and 12.661% followed by *O. hamelii* (82.682 & 9.644%) and *O. platensis* (75.062 & 17.105%) in addition to their contents in spermine (31.4%) and piperazine (7.09%), which may act synergistically and induced the pronounced antibiotic activity characteristic of the three *Oscillatoria* species. These compounds appear to be synthesized by these species as a defense reaction against the cohabitants (cyanobacteria & green algae) and leading to its predominance in the aquatic ecosystem.

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