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Full Length Research Paper

Active substance from some blue green algal species used as antimicrobial agents

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The concept of biological control for health maintenance has received widespread attention during the last few years. Therefore, the main objective of this work was to look for active substances that could be used as antimicrobial agents in an efficient and safe manner. To achieve this target, five different extracts (ethyl acetate, chloroform, diethyl ether, methanol and water) from three blue green algal species (*Anabaena flos aquae* (Linnaeus) Bory*; Anabaena variabilis* (Kützing) and *Oscillatoria angustissima* West and West) were examined. This different algal extracts were tested *in vitro* for their antimicrobial effects against eight Gram +ve and Gram -ve bacteria in addition to two groups of fungi (filamentous fungi and yeast) using agar well diffusion method. The results showed that the ethyl acetate extract of *A. flos aquae* notably inhibited nearly the whole tested bacteria and filamentous fungi with minimal activity against tested yeasts. Furthermore, a significant reduction in sporulation as well as dry weight of the two filamentous fungi was found with ethyl acetate extracts. The toxicity of ethyl acetate extract to *Artemia salina* showed that the effective concentration that would inhibit their growth by 50% (LC_{50}) after 24 h of incubation was 17, 41 and 45 $mg.ml^{-1}$ for *A. flos aquae, A. variabilis* and *O. angustissima*, respectively. The antagonistic materials from ethyl acetate extract of different cyanobacterial species were identified using gas liquid chromatography mass spectrometer (GC-MS). The main components of ethyl acetate extract of *A. flos aquae* consisted of heptadecane and 7-methylheptadecane representing 26.11 and 18.65%, respectively.

Key words: Antibacterial, antifungal, cyanobacteria, ethyl acetate extract, heptadecane.

INTRODUCTION

Fish diseases caused by micro-organisms causes considerable economic losses in aquaculture yearly and it represents a worldwide problem. Members of the genus *Vibrio* are the causative agents of vibriosis, which can cause significant losses in fish culture. *Vibrio* spp causes disease in many fish including the salmon, char and shellfish such as the shrimp (Eguchi et al., 2000; Kent and Poppe, 2002). *Escherichia coli* are a common human pathogen, contaminants of seafood in enormous number and fish usually acquire this pathogen through feeding on food contaminated with feces causing serious life threatening illness within a very short time (Gomez et al., 2008).

Today, antibiotics play a major role in the modern agriculture and aquaculture industries and their use has been on the rise in many developed nations (Boxall et al., 2004; Cabello, 2006; Sarmah et al., 2006). This practice may result in the antibiotics entering the environment by leaching from uneaten feeds, unabsorbed parts in manure, or aquatic animals' excrement (Robinson et al., 2007). For example, in an intensive fish farm, about 70-80% of applied antibiotics, administered to fish as food additives end up in the aquaculture environment. This may result in adverse ecological effects, including the development of resistant bacterial populations, direct toxicity to microflora and microfauna and/or possible risks in the transfer of antibiotics resistances to human pathogenic microbes (Cabello, 2006; Philip et al., 2009).

Therefore, the overuse and misuse of certain antibiotics have led to several adverse environmental effects and

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raised public concern (Sarmah et al., 2006). There has been a concerted effort to synthesize new agents with improved activities, especially against different microbes. Algae are rapidly proving to be an extremely important source of biologically active secondary metabolites which could be used for the biological control of pathogens. Cyanobacteria (blue green algae) are one of the richest sources of biomedical relevant compounds with extensive therapeutic pharmaceutical applications (Tan, 2007; Gademann and Portmann, 2008; Martins et al., 2008). Various strains of cyanobacteria are known to produce intracellular and extracellular metabolites with diverse biological activities such as antibacterial (Mundt et al., 2003; Rao et al., 2007; Kaushik and Chauhan, 2008), antifungal (MacMillan et al., 2002), cytotoxic (Luesch et al., 2000). Most species of cyanobacteria are free-living, freshwater, marine or terrestrial which could be planktonic, or benthic and comprise major components of microbial mats. There are many reports on the inhibition of human pathogens by algal extracts, only few studies reported effects against fish pathogens (Liao et al., 2003; Bansemir et al., 2006; Abd-Elraouf and Ibraheem, 2008).

Research to identify antimicrobial compounds produced by microalgae against human pathogens as well as other pathogens, has recently received considerable attention as a new source of novel antimicrobial substances. Certain systematic groups of microorganisms are characterized by specific composition of intracellular hydrocarbons; in particular, cyanobacteria are unique in their ability to produce 7 and 8-methyl heptadecanes (Ladygina et al., 2006). Ethyl acetate extract of *Spirulena platensis* consisted of heptadecane and tetradecane which can inhibit some Gram +ve and Gram –ve bacteria and *Candida albicans* (Ozdemir et al., 2004). El-Sheekh et al. (2006) showed that phenolic compound from *Nostoc muscorum* exhibited antagonistic activity against Gram +ve and Gram –ve bacteria. Ghasemi et al. (2004) isolated substances belonging to groups of peptides, polypeptides, amides and alkaloids from *Fischerella ambigua*. *Anabaena spp* produce a number of bioactive compounds, mostly lipopeptidases that have antibiotic, antialgal, anticancer, anti-inflammatory, cytotoxic and enzyme-inhibiting effects (Burja et al., 2001; Fujii et al., 2002). *Oscillatoria* spp. can produce fatty acids, tetraamine, spermine and piperazine derivatives which show antimicrobial activity (Mundt et al., 2003; Shanab, 2007). Matern et al. (2003) isolated two depsipeptide metabolites, scyptolin A and B from cyanobacterium *Scytonema hofmanni* PCC. 7110. Thillairajasekar et al. (2009) showed the presence of fatty acids from hexane and ethyl acetate extract of *Trichodesmium erythraeum* showed antimicrobial activity.

This study was designed to investigate the antimicrobial activity of cyanobacterial species *Anabaena flos aquae* (Linnaeus) Bory; *Anabaena variabilis* (Kützing) and Oscillatoria angustissima West and West. The study was extended to identify the individual components of their ethyl acetate extract by gas chromatography (GC) coupled with mass spectrometer (MS).

MATERIALS AND METHODS

Organisms and growth conditions

Three algal strains were selected for screening of their antimicrobial activity belonging to cyanobacteria (*A. flos aquae* (Linnaeus) Bory*; A. variabilis* (Kützing) and *O. angustissima* (West and West), obtained from Phycology Laboratory, Botany Department, Faculty of Science, Tanta University, Egypt. The algal species were maintained in Allen's and Stanier media (1968) at a temperature of 25 ± 1°C under continuous illumination (3000 lux or 60 µmol.$m^{-2}s^{-1}$) for 12 days. Cyanobacterial cells were collected by centrifugation in the exponential growth phase (after 12 days), washed with distilled water and the pellets were dried at room temperature.

Preparation of cyanobacterial extracts

For the aqueous extract, 0.1 g dry powder was mixed with 2 ml distilled water and shaken for 24 h at 20°C in the dark. The mixture was separated by high speed centrifugation and the extraction procedure repeated twice. The combined aqueous extracts were evaporated to dryness and the residue re-dissolved in 2 ml distilled water to form a stock solution (50 mg/ml). The same process was followed with the four organic solvents (ethyl acetate, chloroform, methanol and diethyl ether) according to Wang et al. (2007).

Microbial indicators and growth conditions

Twelve microorganisms including Gram +ve and Gram -ve bacteria, yeast and filamentous fungi were used in this study. *Bacillus subtilis* 1020, *Bacillus cereus, Staphylococcus aureus, Streptococcus faecalis* (Gram +ve), *E. coli* 1357, *Pseudomonas aeruginosa*. *Aeromonas hydrophila* and *Vibrio fluvialis* (Gram -ve), *C. albicans* and *Candida tropicalis* (yeast), in addition to filamentous fungi *(Aspergillus niger* and *Aspergillus flavus).* Bacterial strains and yeast were kindly provided from Microbiology Laboratory, Botany Department, Faculty of Science, Tanta University, Egypt and Microbiology Laboratory, National Institute of Oceanography and Fisheries, Alexandria, Egypt. The two filamentous fungi were isolated from infected fish and identified to the species level at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt. The fungal isolates were subjected to certain morphological studies by an Image Analysis System using Soft-Imaging GmbH software (analysis Pro ver. 3.0) at RCMB by using the most documented keys in fungal identification (Domsch et al., 1993).

All bacterial strains were maintained on nutrient agar slants and incubated at 30°C. Each bacterial biomass was prepared by inoculating 100 ml of nutrient broth medium. Bacterial cultures were shaken (250 rpm) at 30°C for 24 h. Different inocula were used at a logarithmic phase of growth (A_{550} = 1); the yeast and fungal strains were inoculated into glucose peptone broth amended with rose bengal (a wide spectrum antibiotic) for 5 days.

Testing antimicrobial activity by the agar-well diffusion method

The antagonistic activity of cyanobacteria extracts was determined using cut–diffusion technique in which cut (5 mm) was punched upon the surface of agar plates previously inoculated with each of the above mentioned indicator strains. Each well bottom was sealed with two drops of sterile water agar. About 100 µl of algal extract were transferred into each well. Wells loaded with the

extracting solvents were used as controls, plates inoculated with bacteria were incubated at 37°C for 24 h and those inoculated with fungi and yeast were incubated for 3 days at 30°C. After incubation, the diameter of the inhibition zone was measured with calipers and the results were recorded in mm (Attaie et al., 1987). All tests were performed under sterile conditions in duplicate and repeated three times.

Sporulation count

Czapek-Dox agar was mixed aseptically with the ethyl acetate extracts of cyanobacterial species to produce the required concentrations (50 and 25 mg.ml^{-1}) and poured into Petri dishes. The Petri dishes were inoculated with a 4 mm disk of mycelium of *A. niger* and *A. flavus*, incubated for 10 days at 27°C and the sporulation was counted by a haemocytometer.

Dry weight (DW) method

50 ml of the glucose peptone medium amended with different ethyl acetate concentrations (50 and 25 mg.ml^{-1}) of cyanobacterial species, in 250 ml Erlenmeyer flask were inoculated with 5 mm discs. The flasks were incubated at 25 ± 2°C for 7 days. The fungal mats were removed by filtration and dried at 60 ± 3°C to constant weight and their dry weights (g.L^{-1}) were recorded.

Artemia salina bioassay

Ethyl acetate extract of the tested cyanobacterial species were assessed using *A. salina* (brime shrimp) larvae. About 20 of 1-day hatching larvae of brime shrimp were pipetted into glass culture tubes, which had been dosed with different concentrations of extract (50, 25 and 10mg.ml^{-1}). Each test was made in triplicate and seawater was used as a control. The culture tubes were incubated under fluorescent lamp and at room temperature (25°C). The number of dead larvae were counted after 24 h to calculate percentage mortality and the LC_{50} was determined.

Electron microscopic examination

In order to visualize the morphological changes in the fungal spores after treatment with the ethyl acetate extract of *A. flos aquae*, scanning electron microscopy studies were carried out. Fungal spores were harvested, washed with phosphate buffer and fixed with 2% glutraldehyde followed by 1% osmium tetroxide. After completion of fixation, samples were washed in buffer solution and the washed samples were dehydrated in ascending order of ethanol concentrations. The samples were dried completely and finally coated with gold in JEOL-JSM 5300 scanning electron microscope (SEM) operated at 20 kV with a beam specimen angle of 45° at the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University, Cairo, Egypt.

Chemical composition (GC-MS) analysis

The chemical components of ethyl acetate extracts of different cyanobacterial species were analyzed by GC-MS in the central laboratory of the High Institute of Public Health, Alexandria, Egypt. Identification of the chemical constituents of extracts were made using Hewlett Packard HB 5890 gas liquid chromatography (GLC) coupled with 5989 B series mass spectrometer (MS). Identification of the individual components was performed by comparison of mass spectra with the profiles from the Wiley GC-MS 275 libraries.

Statistical analysis

Each single treatment was replicated three times and the mean value ± standard deviation was considered. The data were statistically analyzed by applying Minitab (2003), using the simple linear correlation and cluster analysis for data upon complete linkage level, to clarify the relation between data.

RESULTS

Antimicrobial activities of different algal extracts

The results showed that ethyl acetate extracts notably inhibited nearly the whole panel of the tested bacteria with the widest inhibition zone (15.6 mm) for *A. flos aquae* against *B. cereus*, *A. hydrophila* and *P. aeroginosa* (Table 1). Two bacterial species, *B. cereus* and *A. hydrophila*, were inhibited by ethyl acetate extracts of *A. variabilis*, with inhibition zones of 11.2 and 11.1 mm, respectively. On the other hand, weak inhibitory effects appeared with the other ethyl acetate extracts.

Considering the chloroform extract, the bacterial species (*B. subtillus, B. cereus, A. hydrophila and V. fluvialis)* were inhibited by that of *A. flos aquae* with inhibition zones of 6.1, 5.1, 6.2 and 7.1 mm, respectively. However, weak activity was detected with the extracts of *A. variabilis* and *O. angustissima* (Table 1).

In comparison with other organic extracts, minimum inhibitions of tested microorganisms occurred with methanol and diethyl ether extracts (Table 1). No inhibition was recorded with aqueous extract.

Regarding the effect of different algal extracts on the fungal species (Table 1), ethyl acetate and chloroform extracts were specific but at varying degrees. The ethyl acetate extract of *A. flos aquae* presented the highest activity against the two fungi (*A. niger* and *A. flavus*), but minimum activity against *C. albicans* and *C. tropicals*.

Sporulation count and dry weight of the two fungal species (*A. niger* and *A. flavus*)

The results presented in Table 2 revealed that the numbers of spores produced were significantly reduced with different ethyl acetate extract (50 and 25 mg.ml^{-1}) of the cyanobacterial species. The lowest numbers of fungal spores (136 x 10^4 and 83 x10^4 for *A. niger* and *A. flavus*, respectively) were recorded using *A. flos aquae* at concentration of 50 mg.ml^{-1} as compared with control. Also the results showed that *A. variabilis* and *O. angustissima* inhibited spores production at the same concentration.

Mycelia dry weights of *A. niger* and *A. flavus* were significantly suppressed by ethyl acetate extract of *A. flos aquae*; *A. variabilis* and *Osc. angustissima* (Table 2), the results revealed that the lowest mat dry weigh obtained in case of *A. flos aquae* with the two applied concentrations (50 and 25 mg.ml^{-1}). It was noticed that, with increasing the concentration of extract, the fungal growth decreased.

Table 1. Antimicrobial activities of ethyl acetate, chloroform, methanol, diethyl ether and aqueous extracts of three cyanobacterial species against twelve pathogenic microorganisms.

Cyanobacterial species	Gram positive bacteria				Gram negative bacteria				Yeast		Fungi	
	B. subtilis	***B. cereus***	***S. aureus***	***S. faecalis***	***E. coli***	***P. aeruginosa***	***A. hydrophila***	***V. fluvialis***	***C. albicans***	***C. trobicalis***	***A. niger***	***A. flavus***
Ethyl acetate extract												
A. flos aquae	13.4 ± 0.3	15.6 ± 0.4	11.6 ± 0.3	11.5 ± 0.4	3.5 ± 0.4	15.6 ± 0.4	15.6 ± 0.4	9.5 ± 0.2	3.4 ± 0.3	4.4 ± 0.2	6.5 ± 0.3	5.5 ± 0.4
A. variabilis	8.1 ± 0.3	11.2 ± 0.3	7.3 ± 0.3	6.1 ± 0.2	2.0 ± 0.2	9.1 ± 0.2	11.1 ± 0.2	6.1 ± 0.2	1.0 ± 0.2	2.1 ± 0.1	6.1 ± 0.4	6.0 ± 0.1
O. angustissima	4.1 ± 0.2	3.0 ± 0.2	6.2 ± 0.3	5.1 ± 0.3	-	7.1 ± 0.3	-	-	-	-	3.2 ± 0.3	3.3 ± 0.4
Chloroform extract												
A. flos aquae	6.1 ± 0.2	5.1 ± 0.3	5.2 ± 0.2	6.2 ± 0.1	-	6.1 ± 0.4	6.2 ± 0.2	7.1 ± 0.2	1.1 ± 0.1	1.1 ± 0.3	4.0 ± 0.2	4.1 ± 0.3
A.variabilis	4.0 ± 0.3	5.0 ± 0.1	4.3 ± 0.3	3.1 ±0.2	1.1 ± 0.1	3.8 ± 0.3	6.0 ± 0.3	3.3 ± 0.4	1.1 ± 0.2	1.7 ± 0.3	3.0 ± 0.2	3.2 ± 0.3
O. angustissima	2.0 ± 0.2	2.7 ± 0.4	3.2 ± 0.3	3.0 ± 0.2	-	1.1 ± 0.3	2.1± 0.3	1.0 ± 0.2	-	-	2.1 ± 0.3	2.2 ± 0.3
Methanol extract												
A. flos aquae	3.1±0.2	-	2.5±0.4	-	1.1 ± 0.2	-	-	2.4 ± 0.4	-	1.1 ± 0.2	2.0 ± 0.2	2.3 ± 0.4
A.variabilis	-	-	-	-	-	2.2 ± 0.1	2.2 ± 0.2	-	-	-	-	-
O. angustissima	-	-	-	-	-	-	-	2.2 ± 0.1	-	-	-	-
Di Ethyl ether extract												
A. flos aquae	-	2.2 ± 0.1	-	4.3 ± 0.3	-	-	-	-	2.3 ± 0.1	-	-	-
A.variabilis	-	-	-	-	-	-	-	-	-	-	-	-
O. angustissima	-	-	-	-	-	-	-	-	-	-	-	-
Aqueous extract												
A. flos aquae	-	-	-	-	-	-	-	-	-	-	-	-
A.variabilis	-	-	-	-	-	-	-	-	-	-	-	-
O. angustissima	-	-	-	-	-	-	-	-	-	-	-	-

Zone of inhibition (mm), including the diameter of the agar-well (5 mm), mean value of three replicates ± standard deviation. –, no activity.

The maximum growth inhibition of *A. niger* was 89% for both *A. flos aquae* and *A. variabilis* extracts at 50 mg.ml^{-1}. The same trend was observed in case of *Osc. angustissima*. In addition, the greatest inhibition in biomass yield of *A. flavus* was 75 and 63% for *A. flos aquae* and *A. variabilis*, respectively.

Artemia salina bioassay

The ethyl acetate extract of *A. flos aquae* killed 50% of the shrimp larvae when their LC_{50} was 17mg.ml^{-1}, whereas extracts of *A. variabilis* and *O. angustissima* LC_{50} was 41 and 45 mg.ml^{-1} (Table 3), respectively.

Electron microscopic examination

Electron microscopic examination of *A. niger* and *A. flavus* spores before and after treatment with ethyl acetate extract of *A. flos aquae* demonstrated morphological alteration and they were greatly transformed by the effect of algal extract as shown in Figure 1.

Chemical analysis of the ethyl acetate extracts of different cyanobacteria species

The GC-MS was used to determine the composition and concentrations of the substances having antimicrobial activity, from ethyl acetate extract of

Table 2. Number of spores (x10^4) and dry weight (g.L^{-1}) of *A. niger* and *A. flavus* as affected by different concentrations of ethyl acetate extracts of *A. variabilis*, *A. flos aquae* and *O. angustissima* after 10 days of incubation.

Algal species	Extract concentration	Spore number		Dry weigh (g)	
		A. niger	***A. flavus***	***A. niger***	***A. flavus***
Control.		1757 ± 31	635 ± 6.0	0.9 ± 0.01	0.8 ± 0.30
A. flos aquae	25 mg/ml	175 ± 04	129 ± 1.3	0.2 ± 0.10	0.4 ± 0.03
	50 mg/ml	136 ± 03	083 ± 1.2	0.1 ± 0.01	0.2 ± 0.02
A. variabilis	25 mg/ml	216 ± 07	140 ± 1.3	0.3 ± 0.02	0.5 ± 0.02
	50 mg/ml	153 ± 05	089 ± 1.2	0.1 ± 0.01	0.3 ± 0.03
O. angustissima	25 mg/ml	293 ± 05	164 ± 1.0	0.4 ± 0.01	0.5 ± 0.10
	50 mg/ml	165 ± 04	096 ± 2.0	0.2 ± 0.02	0.3 ± 0.03

Mean value of three replicates ± standard deviation.

Table 3. Percentage mortality with LC_{50} (mg.ml^{-1}) of *A. salina* for different concentrations of ethyl acetate extract of *A. flos aquae, A. variabilis* and *O. angustissima*.

Algal species	Extract concentration (mg.ml^{-1})	% Mortality	LC_{50} (mg.ml^{-1})
Control	**0**	**0**	
A. flos aquae	50	70 ± 3	17
	25	60 ± 2	
	10	45 ± 3	
A. variabilis	50	59 ± 5	41
	25	28 ± 3	
	10	22 ± 2	
O. angustissima	50	54 ± 4	45
	25	37 ± 4	
	10	18 ± 3	

different blue green algal species. Results present in table (4) revealed that nine component were identified in the ethyl acetate extract of *A. flos aquae*, of which the main common components are heptadecane and heptadecane, 7 methyl (26.11 and 18.65%, respectively) as shown in figure (2). In contrast, the extract of *A. variabilis* (Table 4) contained sixteen component with lower concentrations of the substances having antimicrobial activity that is, hexadecanoic acid (13.30%), octade-canoic acid (11.10%) and tricosane (10.14%). While the extract of *O. angustissima* (Table 4) contains twenty fractions, the major component was 5, 7-diisopropylxanthone-2-carboxyl (15.38%) and tricosane (13.30%).

Statistical analysis

Cluster analysis of a similarity matrix (Figure 3) demonstrated that the profiles of the antimicrobial action produced by ethyl acetate extract of *A. flos aquae* and *A. variabilis* was closely linked with similarity of 96.54%, followed by *O. angustissima* (75.58%). The cluster analysis of the response of different tested pathogenic micro-organisms to the action of ethyl acetate extract are observed in Figure 4, the data showed three main clusters, the first one comprised *A. flavus* (96.41%) while, the second cluster include *A. niger* (98.4%). The third cluster was divided into three sub-clusters, in the first sub-cluster the most closely related pathogens were *B. subtilis* and *C. tropicals* (99.99%). However, the second sub-cluster comprised *B. cereus*, *A. hydrohila*, *V. fluvialis* and *E. coli* with similarity of 99.78%. The third sub-cluster includes *S. aureus, P. aeroginosa, S. faecalis* and *C. albicans* (99.53%).

The results of simple linear correlation coefficients (Table 5) noted that, there were highly significant positive correlation between different cyanobacterial ethyl acetate extracts ($P \geq 0.05$). As presented in Table 6, highly significant positive correlation ($P \geq 0.01$) between different pathogens affected by different cyanobacterial ethyl acetate extracts was observed.

DISCUSSION

Our goal in this study is to reveal the biological production of bioactive compounds by some cyano-

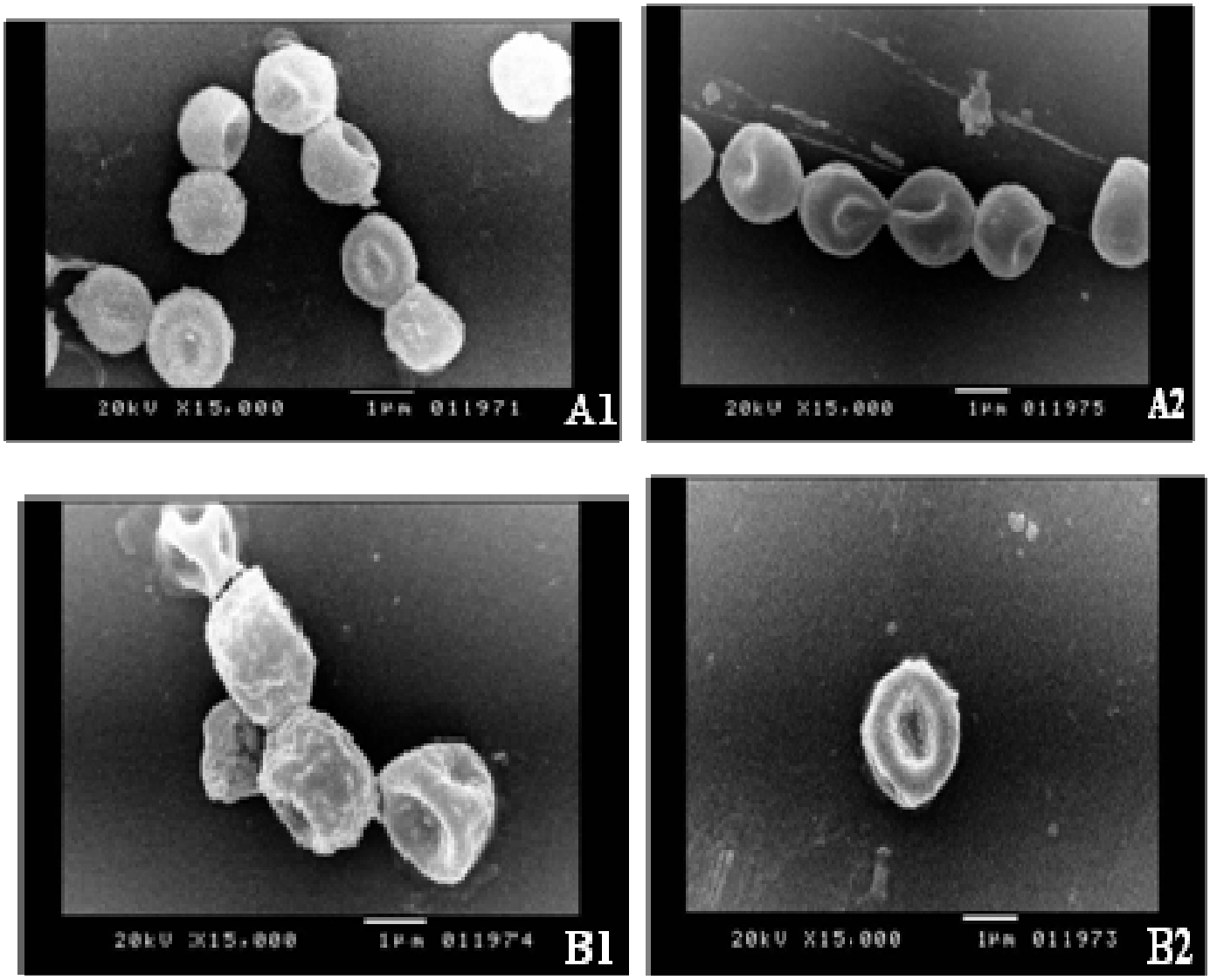

Figure 1. Electron microscopic examination of *A. niger* and *A. flavus* spores, (A_1, A_2), untreated, (B_1, B_2) treated with ethyl acetate extract of *A. flos aquae.*

bacterial species. This study confirms that the ethyl acetate extract of different cyanobacterial species have high activities against the tested microorganisms. The cluster analysis showed that, not all the target strains tested were equally susceptible to antimicrobial metabolites produced from cyanobacterial species. These differences could be attributed to the strain of the bacteria used that could affect the results significantly (Philip et al., 2009). In accordance to our results, Abdel-Raouf and Ibraheem (2008) reported the antibiotic activity of the ethyl acetate extract of two *Anabaena* species against four fish pathogenic *Aeromonas* spp. Also, Thillairajasekar et al. (2009) found that, ethyl acetate extract of *T. erythraeum* can inhibit the growth of *A. flavus* and *A. niger.* In contrast to our results, Ostensvik et al. (1998) found that the methanolic extracts made from *Tychonema bourrellyi*, *A. flos-aquae* and *Cylindospermopsis raciborskii* showed the most pronounced inhibitory effects where an aqueous extracts made from *Microcystis aeruginosa* and *T. bourrellyi* possessed evident antibacterial properties. Kaushik et al. (2009) showed highest effective zone of inhibition by methanol extract of *A. variabilis* against *S. aureus, E. coli*, *P. aeruginosa* and *Salmonella typhi*. The aqueous extract of the tested cyanobacterial species exhibited no inhibitions as compared with the other organic extracts. The present results are in agreement with those of Ghosh et al. (2008) who showed that aqueous extracts are generally less potent in their bioactivity than organic extracts. From previous studies in addition to that of the current results, it could be concluded that the antibiotic

Table 4. GC-MS analysis of the different components in ethyl acetate extracts of blue green algal species.

No.	Compound	Retention time (min)	Area (%)
A. flos aquae			
1	Heptadecane	14.16	26.11
2	Heptadecane, 7 methyl	14.63	18.65
3	4,4-dimethyl-5-ethylcyclopent-2-en	15.64	5.20
4	Dimethylester of 8,8-dimethyl-3,5	16.09	2.95
5	24,24-dimethyl-5.alpha.-cholest-7-	19.07	9.30
6	Beta Carotene	19.94	11.93
7	Tetracosane	20.75	7.56
8	No matches found	21.54	5.58
9	1,2-benzenedicarboxylic acid	22.01	12.72
A. variabilis			
1	3-Glutathionylacetaminophen	3.54	2.61
2	Heptadecane	14.12	5.78
3	(1-Methoxy-2,2-dimethylpropyl)b	14.21	4.43
4	Nonylphenol Isomer	14.41	3.21
5	Potassium Bromo dinitromethane	14.50	3.55
6	Octadecane	14.62	5.84
7	Ethanamine, 1- (2,4-cyclopentadien)	14.82	7.46
8	Hexadecanoic acid	16.88	13.30
9	Phenylalanine, N-trifluroacetyl-4	17.20	5.57
10	Tricosane	18.16	3.84
11	Octadecanoic acid	18.74	11.10
12	Octadecane	19.06	8.49
13	Tricosane	19.91	10.14
14	Tetracosane	20.74	5.46
15	Heneicosane	21.55	5.34
16	1,2-benzenedicarboxylic acid	21.99	4.27
O. angustissima			
1	2.alpha.-nitrocholest-4-en-3-one	11.61	1.86
2	Heptadecane	14.14	2.70
3	2- (N-Butyl) -3,4,5,6- D4-Pyridine	14.28	1.18
4	Perylene, eicosahydro- (CAS)	14.41	2.34
5	Phytol Isomer	14.63	2.30
6	Hexadecanoic acid (CAS)	16.90	5.16
7	Benzenamine	17.06	1.92
8	N- (3-methylbutyl) - acetamide	17.21	5.28
9	8,8,10,10-trtramethyl-8-germa-10-S	17.66	8.63
10	5,7-Diisopropylxanthone-2-carboxyl	18.09	15.38
11	Tetradecanoic acid	18.33	3.42
12	Docosane	19.05	6.66
13	Tricosane	19.93	10.75
14	Docosane, 10-ethyl-10-propyl- (CAS)	20.76	5.41
15	Hexadecane	21.54	4.56
16	2,4-Bis (dimethylbenzyl) phenol	21.79	3.25
17	8-hydroxysclerodin trimethyl ether	21.92	7.76
18	1,2-benzenedicarboxylic acid	21.99	7.83
19	Hexadocosane (CAS)	22.30	2.57
20	3,3-Diethoxy-1,1,1,5,5,5-hexamethyl	23.83	1.03

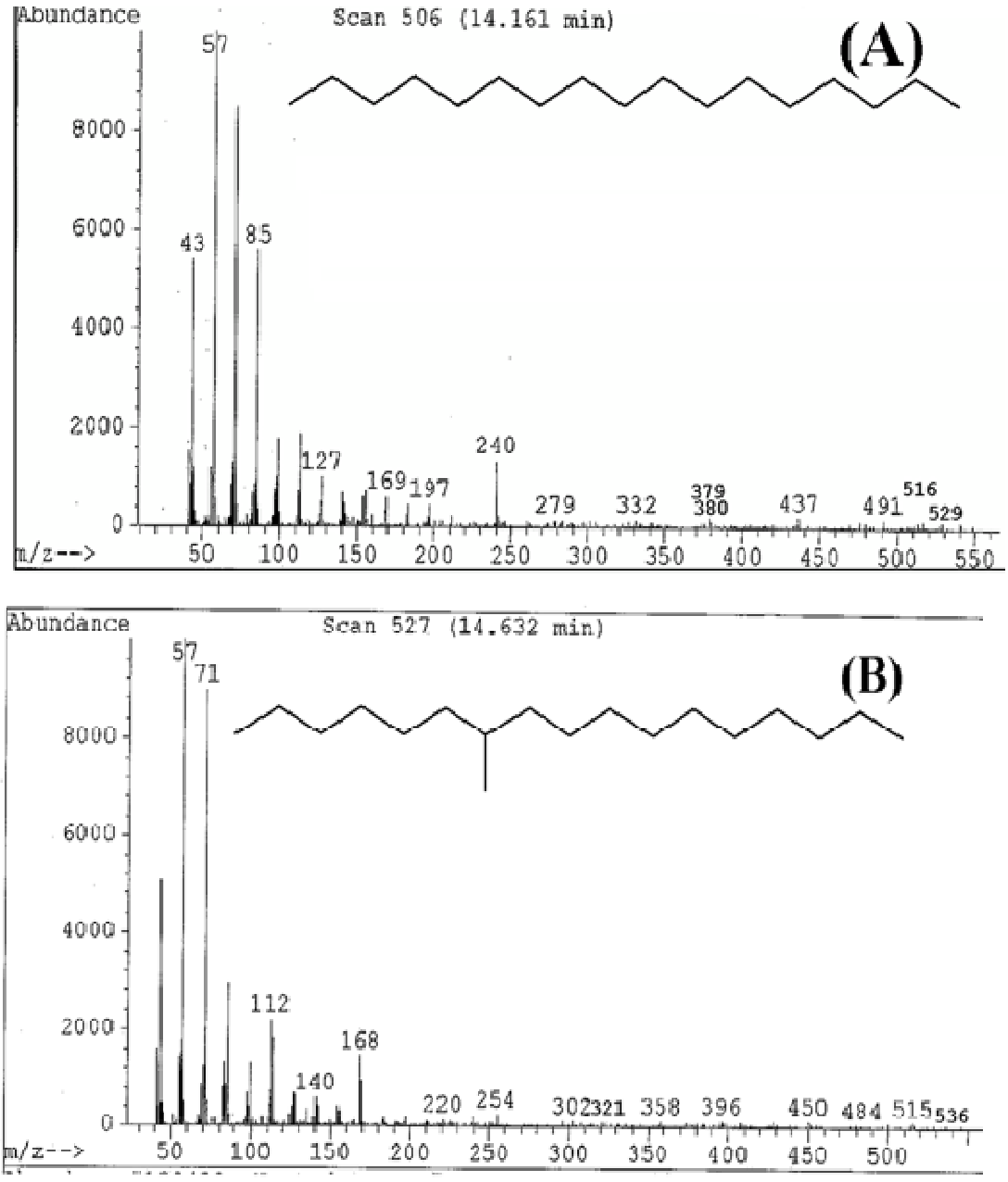

Figure 2. Mass spectrum of heptadecane (A) and heptadecane, 7 methyl, (B) in the ethyl acetate extract of *A. flos aquae*.

production is largely dependent on the algal species. Many researchers showed that, *Chrococcus* sp. Produced bioactive substances with antibiotic activity against *B. cereus, S. aureus* and *S. epidermidis* (Mian et al., 2003). Issa (1999) reported the antimicrobial activity of *O. angustissima* and *Calothrix parietina* against bacteria and fungi. He concluded that *B. cereus* and *S. aureus*, Gram-positive species were inhibited more than Gram-negative species (*E. coli* and *P. aeruginosa*) by the antibiotic applied. Bloor and England (1989) reported that the antibiotic produced by *N. muscorum* inhibits the growth of bacteria, notably multiple-resistant *S. aureus* and a biocide resistant *P. aeruginosa*. As estimated, it can be concluded that algal activity against Gram -ve bacteria was common as well as against Gram +ve and the tested fungi. These results go in harmony with those obtained by Fei et al. (2002), Walters et al. (2003) and Ghazala et al. (2004) in their studies against Gram +ve, Gram -ve bacteria and pathogenic fungi. The current study revealed that the number of spores produced by the two *Aspergilla* were significantly decreased with increasing concentration of ethyl acetate extracts of *A. flos aquae*,

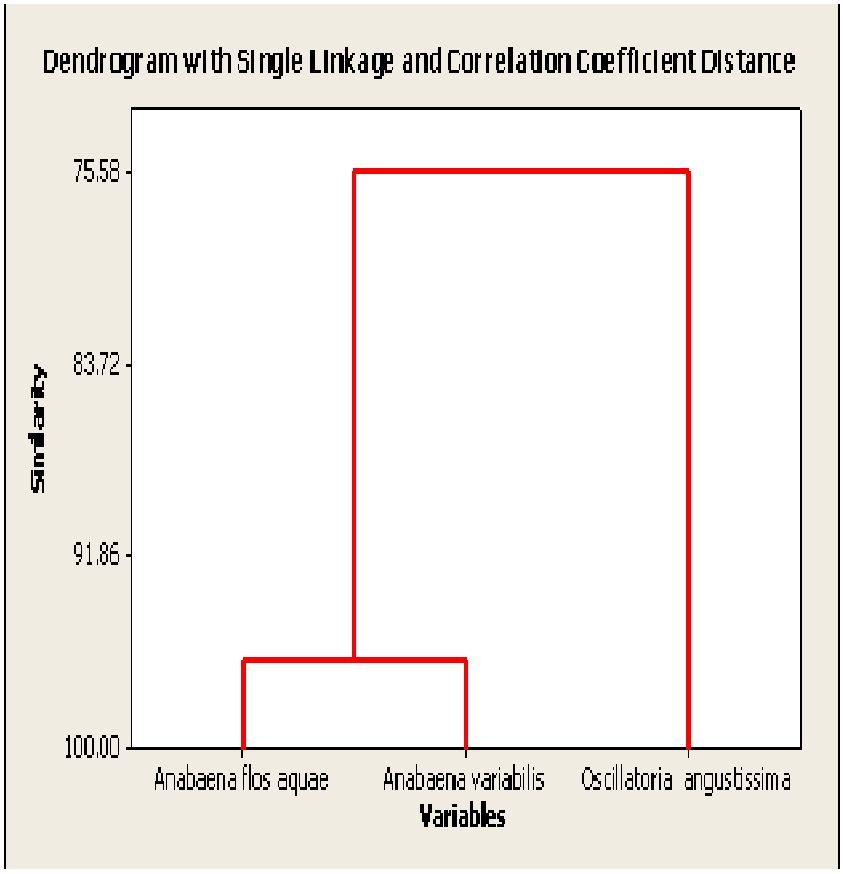

Figure 3. Cluster analysis showing the linkage between the different cyanobacterial ethyl acetate extracts.

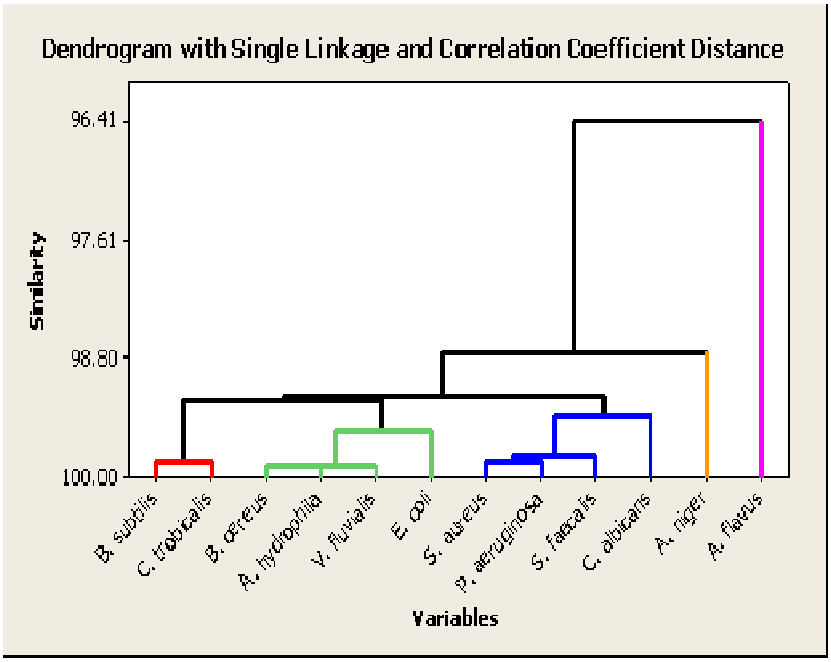

Figure 4. Cluster analysis showing the response of pathogenic microorganisms to the different cyanobacterial ethyl acetate extracts.

Table 5. Correlation matrix between different cyanobacterial ethyl acetate extracts and different pathogens.

Cyanobacterial species	*A. flos aquae*	*A. variabilis*	*O. angustissima*
A. flos aquae	1.000		
A. variabilis	0.925	1.000	
O. angustissima	0.512	0.449	1.000

Values more than 0.50 are significant (P ≥ 0.05).

Table 6. Correlation matrix between different pathogens affected by different cyanobacterial ethyl acetate extracts.

Pathogens	*B. subtilis*	*B. cereus*	*S. aureus*	*S. faecalis*	*E. coli*	*P. aeruginosa*	*A. hydrophila*	*V. fluvialis*	*C. albicans*	*C. trobicalis*	*A. niger*	*A. flavus*
B. subtilis	1.000											
B. cereus	0.966	1.000										
S. aureus	0.961	0.868	1.000									
S. faecalis	0.949	0.845	0.996	1.000								
E. coli	0.972	0.987	0.899	0.882	1.000							
P. aeruginosa	0.970	0.887	0.997	0.992	0.912	1.000						
A. hydrophila	0.949	0.998	0.837	0.812	0.984	0.857	1.000					
V. fluvialis	0.970	0.998	0.880	0.857	0.991	0.895	0.995	1.000				
C. albicans	0.984	0.919	0.982	0.980	0.946	0.988	0.896	0.923	1.000			
C. trobicalis	0.997	0.980	0.943	0.930	0.984	0.956	0.967	0.982	0.977	1.000		
A. niger	0.871	0.962	0.718	0.685	0.936	0.745	0.975	0.951	0.804	0.900	1.000	
A. flavus	0.686	0.834	0.495	0.453	0.808	0.515	0.868	0.833	0.585	0.724	0.928	1.000

Values more than 0.90 are significant (P ≥ 0.01).

A. variabilis and *O. angustissima*. These results are in agreement with that obtained with Moussa and Shanab (2001). The electron microscopic examinations of fungal growth treated with ethyl acetate extracts of *A. flos aquae* showed the presence of spores presenting morphological alterations. Gaspar et al. (2004) reported that light microscopy observations of the inhibition zone of fungal growth treated with curcuphenol showed the presence of non-germinated cells and spores presenting morphological alterations, such as reduced germ-tube and multiple germ-tube formation. The study also emphasizes the importance of exploring the bioactive metabolites and their antimicrobial activity of microorganisms for future biotechnological applications. Metabolites derived from cyanobacteria offer many advantages since cultures can be readily established and production can be optimized to produce sustainable yields on an industrial scale (Bloor and England, 1989). Although *A. flos aqua* in this study has been shown to produce antimicrobial and cytotoxic substances, the ethyl acetate extract of *A. flos aquae* had lethal effects to shrimp larvae with LC50 was 17 mg.ml-1 (Luesch et al., 2000). The ability to produce agents may be significant not only as a defensive instrument for the algal strains but also as a good source of the new bioactive compounds from a pharmaceutical point of view. In this work, ethyl acetate extract of the target species were found to be most active when compared to the other extracts. This extract was further selected for its GC-MS analysis. The result showed that the most bioactive compound produced by *A. flos aquae* was heptadecane and 7-methylheptadecane

representing 26.11, 18.65%, respectively. Heptadecane has been reported to be a common major volatile component in many other cyanobacterial species. Previous records reported that *S. platensis* consisted of heptadecane (39.70%) and tetradecane (34.61%) which can inhibit some Gram +ve and Gram –ve bacteria and *C. albicans* (Ozdemir et al., 2004) which is in good agreement with our results.

In conclusion, the results obtained in the present investigation clearly suggest that the production of pharmacologically products from *A. flos aquae, A. variabilis* and *O. angustissima* could represent a viable and environmentally friendly alternative to reduce the use of synthetic chemicals because of their unintended side effects for the control of pathogenic microorganisms.

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