Extraction and Characterization of Antimicrobial Active Substances from Green Alga *Chlorella vulgaris* and the Cyanobacterium *Pseudanabaena* sp.

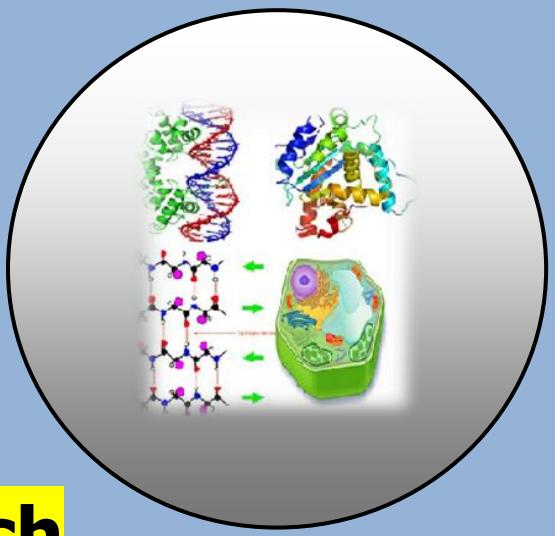
By

Mostafa M. El-Sheekh and Ghada W. Abou-El-Souod

ISSN 0970-4973 Print
ISSN 2319-3077 Online/Electronic

Global Impact factor of Journal: 0.756
Scientific Journals Impact Factor: 3.285
Index Copernicus International Value
IC Value of Journal 6.01 Poland, Europe

J. Biol. Chem. Research
Volume 32 (1) 2015 Pages No. 267-276



Journal of Biological and Chemical Research

An International Journal of Life Sciences and Chemistry

Indexed Abstracted and Cited in about 25 different Scientific Databases around the World

Published by Society for Advancement of Sciences®

J. Biol. Chem. Research. Vol. 32, No. 1: 267-276, 2015
(An International Journal of Life Sciences and Chemistry)

ISSN 0970-4973 (Print)
ISSN 2319-3077 (Online/Electronic)

Dr. G. W. Abou-El-Souod
http:// www.jbcr.in
jbiolchemres@gmail.com
info@jbcr.in

RESEARCH PAPER
Received: 14/01/2015 **Revised: 24/02/2015** **Accepted: 01/03/2015**

Extraction and Characterization of Antimicrobial Active Substances from Green Alga *Chlorella vulgaris* and the Cyanobacterium *Pseudanabaena* sp.

Mostafa M. El-Sheekh and *Ghada W. Abou-El-Souod
Botany Department, Faculty of Science, Tanta University, Tanta 31527, Egypt
***Botany Department, Faculty of Science, Menoufia University, Egypt**

ABSTRACT

The present study was aimed to analyze the existence of bioactive phytochemicals and antimicrobial role of the green alga Chlorella vulgaris and the cyanobacterium Pseudanabaena sp. Various organic solvents such as ethanol, methanol, chloroform and ethyl acetate were used for the preparation of the algal extracts which then subjected to chemical analysis and further used for antimicrobial studies. The intracellular and extra cellular active substances were used for the antimicrobial studies against the pathogens, Staphylococcus aureus and Escherichia coli. The results indicated that the active substance produced after 18 days of incubation and the chemical analysis showed the existence of bioactive compounds such as nonadecene, heptadecene, neophytadiene, thiosulfuric acid and others active compounds. Ethyl acetate was the best solvent for extracting the active material. The antagonistic material was purified using thin layer chromatography and chemically analyzed using spectroscopic techniques to elucidate the chemical nature of the antagonistic active compounds. The present study concludes that the green alga Chlorella vulgaris and cyanobacterium Pseudanabaena sp. are rich sources of pharmacologically active natural products.

Keywords: Antibacterial Activity, Chemical Characterization, Microalgae and Cyanobacteria.

INTRODUCTION

In last few years, products from microalgae and cyanobacteria have earned a higher attention of both researchers and manufactures. Considering the wide biodiversity of these microorganisms, they become stronger commercial sources of bioactive compounds.

In particular using antimicrobial features as objective function, microalgae and cyanobacteria have been under active scrutiny for their potential use toward food preservation (Mendiola et al., 2007). A large number of microalgal extracts have been found to have antibacterial activity. Pedersen and Dasilva (1973) have pointed out that the cyanobacterium *Calothrix brevissima* produce bromophenols, which possesses antibacterial activity. (Mundt et al., 2003) have proved that the cyanobacterium *Oscillatoria redekei* produce fatty acids which show antibacterial activity. Use of algae, especially the cyanobacteria (blue green algae), for antibiotics and pharmacologically active compounds has received ever increasing interest. There are a range of pharmaceutical products derived from algae. Chlorophytes were mainly distributed within the crust and the taxa of chlorophytes decrease obviously under the crust. In the developing stages of the biological soil crust, species diversity of chlorophytes changed a little, but species composition presented some differences. *Chlorococcum humicola*, *Chlorella vulgaris, Chlamydomonas ovalis* and *Chlamydomonas* sp., nearly existed in all developing stages of biological crusts (Zhao et al., 2005). Pratt et al., (1944) were the first to isolate an antibacterial substance from *Chlorella*. A mixture of fatty acids, named chlorellin, exhibited inhibitory activity against both gram positive and gram negative bacteria. Most species of cyanobacteria are known to produce intracellular and extracellular metabolites with diverse biological activities such as antialgal, antibacterial, antifungal and antiviral activity (Noaman et al., 2004; El-Sheekh et al., 2006; El-Sheekh et al., 2008; El-Sheekh et al., 2014). This study aims to reveal the biological production of bioactive compounds by the cyanobacterium *Pseudanabaena* sp. and further elucidate the structure of the antagonistic bioactive material and its antimicrobial activity against some microbes.

MATERIAL AND METHODS

Organisms

The green alga *Chlorella vulgaris* and the Cyanobacterium, *Pseudanabaena* sp. were obtained from Phycology laboratory, Faculty of Science, Menouifa University, Egypt. *Chlorella vulgaris* was earlier identified according to Prescott (1962) and the Cyanobacterium *Pseudanabaena* sp. was identified according to Lauterborn (1916). The axenic cultures were obtained using the method recommended by Bolch and Blackburn (1996). The tested microorganisms were obtained from Bacteriology Lab., Faculty of Science, Menouifa University, Egypt.

Growth conditions

Chlorella vulgaris was cultured in Kuhl medium (Kuhl 1962), flasks were incubated at 28 ±2°C and light intensity of 50 $\mu Em^{-2}s^{-1}$ for 18 days at pH 6.8. *Pseudanabaena* sp. was cultured in Allen medium (Allen and Stanier 1968) at 30 ±2°C under light intensity 30 $\mu Em^{-2}s^{-1}$ for 18 days at pH 7. The tested gram positive *Staphylococcus aureus* and Gram negative *Escherichiacoli* were cultivated in Nutrient agar (Washington, 1978).

Antimicrobial activities of *Chlorella vulgaris* and *Pseudanabaena* sp.

After 18 days of growth, the supernatant (the extracellular extract) was collected, lyophilized and was used as antimicrobial agent.

Thin layer chromatography

Thin layer chromatography containing silica gel 60 F 254, Merck KGaA was used in qualitative and quantitative separation of the antagonistic materials.

0.5 gram of the antagonistic material was dissolved in 10 ml of chloroform. The solution was passed through the column containing silica gel G. The material was eluted by chloroform: ethyl acetate 3:1, then the antimicrobial activity of the filtrate was assayed by diffusion disc technique. Also column chromatography was used for the same purpose using silica gel column. Each fraction was free from solvent, re-dissolved in appropriate solvent and screened for its antimicrobial activity by disc diffusion method (Ratnam and Raju, 2008).

Antimicrobial activity by Paper disc assay

Sterilized Petri dishes containing 10 ml semi solid medium were inoculated with 0.1 ml of bacterial suspension.Then,0.5 cm sterilized paper discs impregnated with 100 µl of air dried extracted bioactive material were placed on the top of the agar medium. Plates were kept in a refrigerator for 2 hours to allow the antimicrobial substance to diffuse through the inoculated medium. Plates were incubated for 24 h at 37 °C, and then examined for inhibition zones around the paper disc (El-Sheekh et al., 2006).

GC – MS analysis of the different components in ethyl acetate extract of *Pseudanabaena* sp.

The ethyl acetate extract of *Pseudanabaena* sp. was analyzed by GC-MS in the central laboratory of Pesticides in Dokki Street, Cario, Egypt. Identification of the chemical constituents of extract was made using Aligent 6890 gas chromatograph equipped with an aglient mass spectrometric detector, with a direct capillary interface and fused silica capillary column HP-5 ms (30 m × 0.32 mm × 0.25 µm film thickness). Extract of *Pseudanabaena* sp. was injected under the following conditions.

Helium was used as carrier gas at approximately 1.0 ml/ min., pulsed splitless mode. The solvent delay was 3 min. and the injection size was 1.0 µl. The mass spectrometric detector was operated in electron impact ionization mode with an ionizing energy of 70 e.v. scanning from m/z 50 to 500. The ion source temperature was 230 °C. The electron multiplier voltage (EM voltage) was maintained 1250 v above auto tune. The instrument was manually turned using perfluorotributyl amine (PFTBA). The GC temperature program was started at 60 °C (2 min) them elevated to 280 °C at rate if 8 °C/ min. the detector and injector temperature were set at 300 and 280 °C, respectively. Wiely and Wiely Nist mass spectral data base was used in the identification of the separated peaks.

RESULTS AND DISCUSSION

The results of this study reveals that ethyl acetate extract of green alga *Chlorella vulgaris* and the Cyanobacterium *Pseudanabaena* sp. have high activities against the tested bacterial microorganisms. The results in Table 1 show that, not all target strains tested were equally susceptible to antimicrobial materials produced from *Pseudanabaena* sp. These differences could be attributed to the strain of the bacteria used that could affect the results significantly (Philip et al., 2009).

The results also indicated that, *Pseudanabaena* sp. and *Chlorella vulgaris* produced the antagonistic material optimally at the stationary phase of growth when it cultured under shake conditions and reaches the maximum values at 18^{th} day of incubation.

Both active substances of extract of *Chlorella vulgaris* and *Pseudanabaena* sp. were separated into spots which migrated differently on TLC plate, therefore, TLC confirmed that the spots of active substance of *Pseudoanbena* sp. migrate faster than spots of active substance of *Chlorella vulgaris*.

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. Isaa (1999) reported the antimicrobial activity of *Oscillatoria angustissima* and *Calothrixparietina* against bacteria and fungi. He concluded that Gram positive bacteria, *Bacillus cereus* and *Staphylococcus aureus* were inhibited more than the Gram negative species *E. coli* and *Pseudomonas aeruginosa* by the antibiotic applied. Results in table1 also showed that gram positive bacteria were more susceptible than the gram negative bacteria. Prashant Kumar et al., (2006) stated that the difference of the fatty acid sensitivities between gram positive and Gram negative bacteria may result from the permeability of the outer membrane of Gram negative bacteria are more resistant to inactivation by medium and long chain fatty acids than Gram positive bacteria.

Table 1. The antimicrobial activities (diameter of inhibition zone measured as cm) formed by culture filtrate of *Chlorella vulgaris* and *Pseudanabaena* sp. dissolved in different organic solvents against different test organisms.

		organic solvent			**Target pathogen**			
		solvent chloroform			***Escherichia coli***		***Staphylococcus aureus***	
					I	E	I	E
species			Culture medium					
Chlorella vulgaris			Kuhl medium		1± 0.5	0.7± 0.2	1.8±0.4	1.2±0.0
Pseudanabaena sp.			Allen medium		1.5± 0.0	1.4± 0.0	2± 0.0	1.3 ± 0.0
					Target pathogen			
		solvent ethanol			*Escherichia coli*		*Staphylococcus aureus*	
					I	E	I	E
species			Culture medium					
Chlorella vulgaris			Kuhl medium		1.1± 0.3	1± 0.0	1.2±0.4	-
Pseudanabaena sp.			Allen medium		-	-	-	-
					Target pathogen			
		solvent methanol			*Escherichia coli*		*Staphylococcus aureus*	
					I	E	I	E
species			Culture medium					
Chlorella vulgaris			Kuhl medium		1.0± 0.2	-	1.0± 0.4	0.8± 0.3
Pseudoanabena sp			Allen medium		1.2± 0.0	-	1.5 ± 0.0	1.4± 0.0
					Target pathogen			
		solvent ethyl acetate			*Escherichia coli*		*Staphylococcus aureus*	
					I	E	I	E
species			Culture medium					
Chlorella vulgaris			Kuhl medium		1.0± 0.4	0.8± 0.3		1.9± 0.4	0.9± 0.05
Pseudoanabena sp			Allen medium		1.5± 0.2	1.5± 0.3		2.0± 0.2	1.5± 0.3

N.B: I: intracellular extracts, E: extracellular extracts

Table 1 show that intracellular extracts are more effective than their extracellular counterparts. Ethyl acetate was the best solvent to extract antimicrobial agent from *Pseudanabaena* sp which also induced the highest inhibition growth of bacteria. Table 1 show also that the biological activity of the antimicrobial agents produced by tested algae gave different results against different species of bacteria. It is clear that the diameter of the inhibition zone depends mainly on type of the algal species, type of the solvent used and the tested bacterial organism. Concerning the antibacterial effects, the results cleared that ethyl acetate of *Pseudanabaena* sp. gave the highest biological activities against *S. aureus* and *E. coli*.

Table 2. GC-MS analysis of the different components in ethyl acetate extracts of *Pseudanabaena* sp.

No	Compound	Retention time (min)	Area%
1	Phenol, bis(1,1 - dimethylethyl)	17.168	16.24
2	(Trans) -2-nonadecene	21.277	83.6
3	Neophytadiene, 2, 6,10 - trimethyl	21.923	91.51
4	Undecane	22.506	94.13
5	n- Hexadecanoic acid	23.746	87.88
6	Heptadecyl pentafluoropropionate	23.946	94.78
7	Thiosulfuric acid	26.232	87.09
8	Eicosene	26.369	87.51
9	Phenol, 2,2 -(1- methyl - 1,2- ethanediyl)	27.769	90.92
10	1- Heptadecene	28.61	99.32
11	2- methyl -7- phenylindole	29.553	42.44
12	Cyclotrisiloxane, hexamethyl	29.993	51.39
13	Di - (2- ethylhexyl)phthalate	30.33	32.86
14	18 - noramborx	30.679	56.26
15	Tetrasiloxane, decamethyl	31.845	55.17
16	Cyclotrisiloxane, hexamethyl	32.982	50.13
17	Trivinyl - s- Triazine-2,4,6 - (1H, 3H,5H) - Trione	33.565	72.25

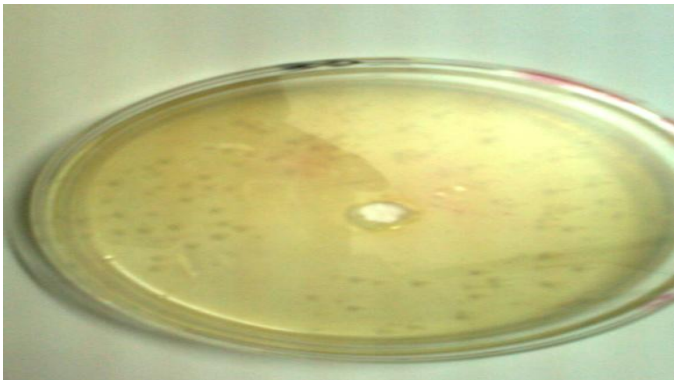


Fig 1. The plate shows antimicrobial activity of ethyl acetate extract from *Pseudoanabaena* sp. Zone of inhibition was measured in cm.

Fig 2. The plate shows no antimicrobial activity of ethanol solvent from *Pseudanabaena* sp. Zone of inhibition was measured in cm.

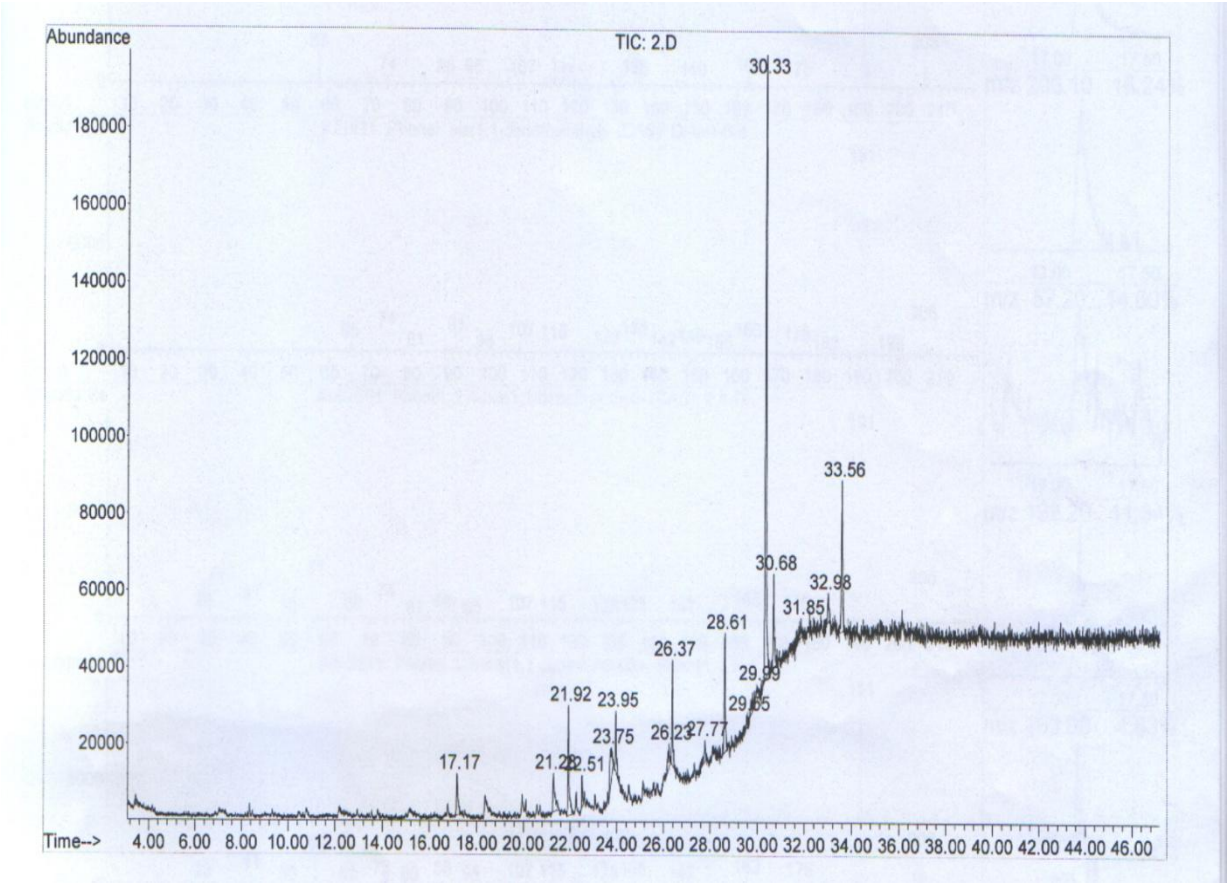
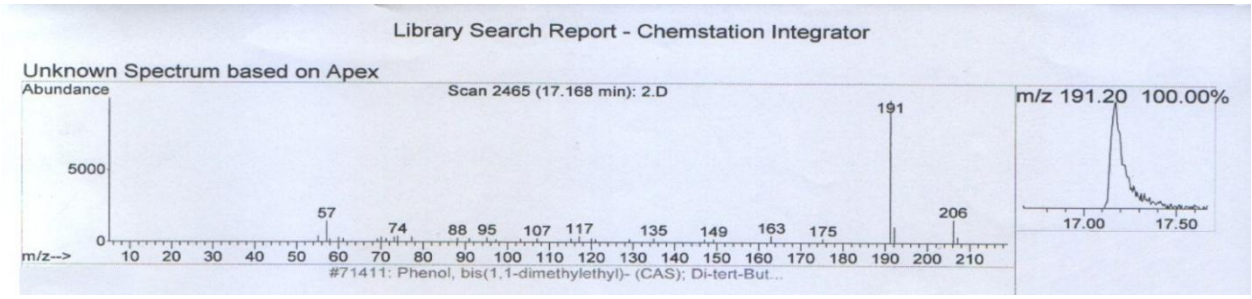
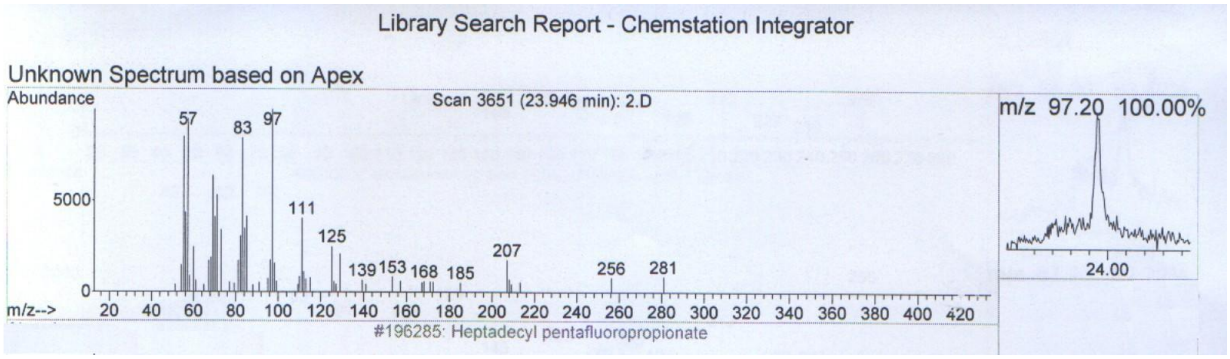


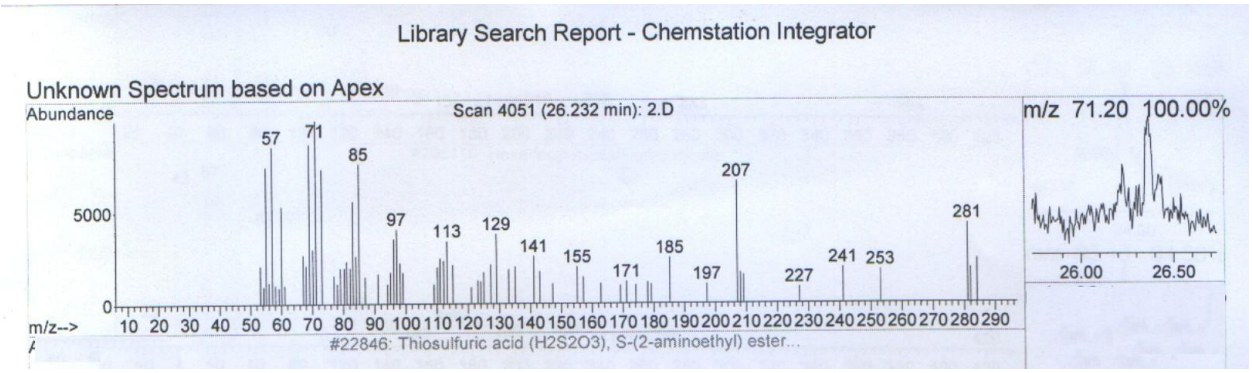
Fig 3. GC – MS analysis of the different components in ethyl acetate extracts of *Pseudanabaena* sp.


Library Search Report - Chemstation Integrator
Unknown Spectrum based on Apex
Abundance
Scan 2465 (17.168 min): 2.D
m/z 191.20 100.00%
#71411: Phenol, bis(1,1-dimethylethyl)- (CAS); Di-tert-But...

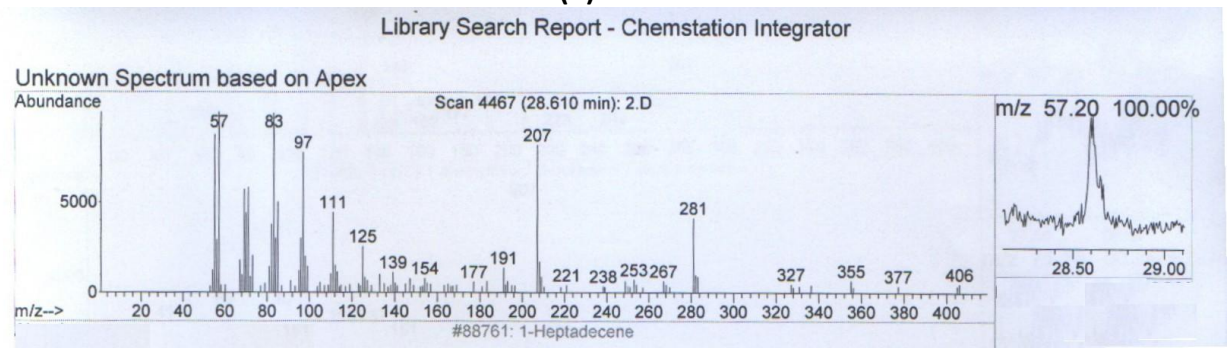
(A)


Library Search Report - Chemstation Integrator
Unknown Spectrum based on Apex
Abundance
Scan 3651 (23.946 min): 2.D
m/z 97.20 100.00%
#196285: Heptadecyl pentafluoropropionate

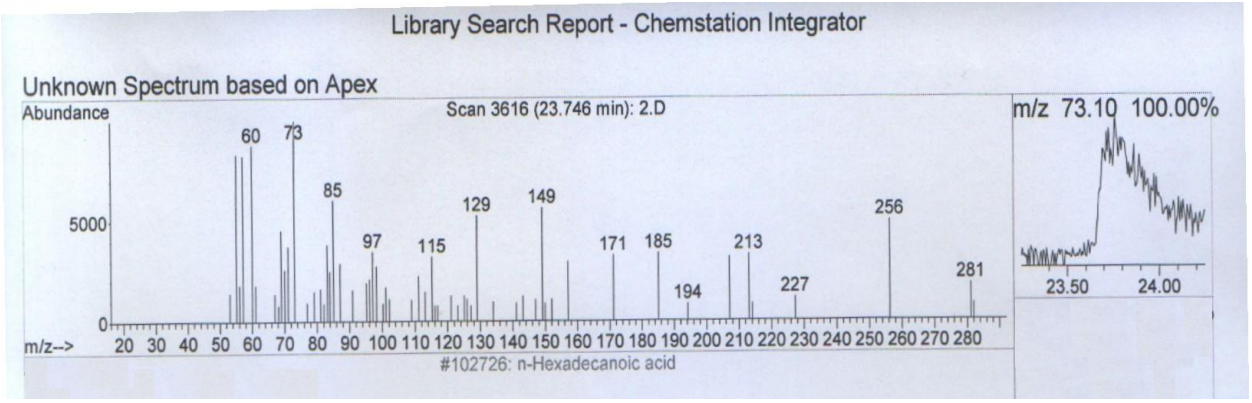
(B)


Library Search Report - Chemstation Integrator
Unknown Spectrum based on Apex
Abundance
Scan 4051 (26.232 min): 2.D
m/z 71.20 100.00%
#22846: Thiosulfuric acid (H2S2O3), S-(2-aminoethyl) ester...

(C)


Library Search Report - Chemstation Integrator
Unknown Spectrum based on Apex
Abundance
Scan 4467 (28.610 min): 2.D
m/z 57.20 100.00%
#88761: 1-Heptadecene

(D)



(E)

Fig 4. Mass spectrum of (A) phenol, bis (1,1- dimethylethyl) (B) Heptadecyl pentafluoropropoionate (C) Thiosulfuric acid (D) 1- Heptadecene (E) Hexadecanoic, palmitic acid in ethyl acetate extract of *Pseudanabaena* sp.

Ethyl acetate extract of the algae used was found to be more active when compared to the other extracts. This extract was further selected for its GC- MS analysis. The results in Table 2 and Fig 3 showed that the most bioactive compound produced by *Pseudanabaena* sp. was (1- Heptadecene, Heptadecyl pentafluoropropionate, Thiosulfuric acid representing 99.32 %, 94.78 %, 87.09%, respectively). Heptadecane has been reported to be a common major volatile component in many other cyanobacterial species. Previous work of Ozdemir et al., (2004) reported that *Spirulina platensis* consisted of heptadecane (39.70%) and tetradecane (34.61%) which can inhibit some Gram +ve and Gram –ve bacteria and the yeast *C. albicans*. These results are in agreement with the results of the present investigation.

The results in Table 2 also are in agreement with Vepritski et al., (1991) who reported that cyanobacterin LU-2, which produced by *Nostoc* sp., is a phenolic nature containing amino – sugar and effective against bacteria and fungi. These results are in agreement with De Cano et al., (1990) who observed inhibition of *C. albicans* and *S. aureus* by phenolic compounds from the terrestrial Cyanobacterium *Nostoc muscorum*.

CONCLUSION

The results obtained in the present investigation clearly suggest that the production of pharmacologically products from *Pseudanabaena* sp. 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. In general, cyanobacteria possess wider antibacterial spectra than microalgae, and intracellular extracts are more powerful than their extracellular counterparts.

ACKNOWLEDGEMENTS

I would like to thank students who are working in the Bacteriology Lab., Faculty of Science, Menouifa University, Egypt, for providing me with tested microorganisms.

REFERENCES

Allen, M.M. and Stanier, R.Y. 1968. Selective isolation of blue-green algae from water and soil. *J. Gen Microbiol*. 51(2), 203–209.

Bolch, C.J.S. and Blackburn, S.I. 1996. Isolation and purification of Australian isolates of the toxic cyanobacterium *Microcystis aeruginosa*. *Kutz. J. Appl Phycol*. (8), 5-13.

Washington, D.C. 1978. American Public Health Association, Standard Methods for the Examination of Dairy Products, 14th Ed.

Prescott, G.W. 1962. Algae of the western Great Lakes area WC Brown Co 977 pp Pls. (1), 136.

De Cano, M.M.S, De Mule, M.C.Z, De Caire, C.Z. and De Halperin, D.R. 1990. Inhibition of *Candida albicans* and *Staphylococcus aureus* by phenolic compounds from the terrestrial cyanobacterium *Nostoc muscorum*. *J. Appl. Phycol*. (2), 79 – 82.

El-Sheekh, M, Osman, M.E, Dyab, M. and Amer, M.S. 2006 . Production and characterization of antimicrobial active substance from the cyanobacterium *Nostoc muscorum*. *Environ Toxicol & Pharmacol*. (21), 42-50.

El-Sheekh, M.M, Dawah, A.M., Abd El-Rahman, A.M., El-Adel, H.M. and Abd El-Hay, R.A. 2008. Antimicrobial activity of the cyanobacteria *Anabaena wisconsinense* and *Oscillatoria curviceps* against pathogens of fish in aquaculture.*Annals Microbiol*. (58), 527-534.

El-Sheekh, M.M, Daboor, S.M., Swelim, M.A. and Mohamed, S. 2014. Production and Characterization of Antimicrobial Active Substance from *Arthrospira platensis*. *IranianJournal of Microbiology* 6 (2), 112-119.

Ghosh, A, Das, B.K., Roy, A., Mandal, B. and Chandra, G. 2008. Antibacterial activity of some medicinal plant extracts. *J. Nat. Med*. (62), 259 – 262.

Issa, A.A. 1999. Antibiotic production by Cyanobacteria *Oscillatoria angustissima* and *Calothrix parietina*. Environ. Toxicol. Pharmacol. (8), 33-37.

Kuhl, A. 1962. The physiology of inorganic condensed phosphates in *Chlorella* Volrong Bot. Hrsg. *Deut. Botan. Ges*. (1), 157-166.

Lauterborn, R. 1916. Die sapropelische Lebewelt. Ein Beitrag zur Biologie des Faulshlammes naturlicher Gewasser. Verh. Naturkundl. *Med. Ver. Heidelb*. (13), 395-481.

Mendiola, J.A, Torres, C.F. and Tore, A. 2007. Use of supercritical CO_2 to obtain extracts with antimicrobial activity from *Chaetoceros muelleri* microalgae. A correlation with their lipid content. *European food research and technology*: (224), 505- 510.

Mundt, S., Kreitlow, S. and Jansen, R. 2003. Fatty acids with antibacterial activity from the cyanobacterium *Oscillatoria redekei* HUP 051. *J. Appl. Phycol*. (15), 263-267.

Noaman, N.H, Khaleafa, A.M. and Zaky, S.H. 2004. Factors affecting antimicrobial activity of *Synechococcus leopoliensis*. *Microbiol Res*. (159), 395-402.

Ozgemir, G., Karabay, N.U., Dalay, M.C. and Pazarbasi, B. 2004. Antibacterial activity of Volatile Components and Various Extracts of *Spirulina platensis*. Phytother. Res. (8), 754 – 757.

Pedersen, M. and Dasilva, E.J. 1973. Simple brominated phenols in the blue green algae *Calothrix brevissima*. West. *Planta*. (11), 83- 96.

Philip, K., Sinniah, S.K. and Muniandy, S. 2009. Antimicrobial peptides in aqueous and ethanol extracts from microbial. Plant and fermented sources. *Biotechnology*, (1), 1-16.

Prashankumar, P., Angadi, S.B. and Vidyasagar, G.M. 2006. Antimicrobial activity of blue – green and green algae. *Ind J of Pharmi Sci*. 68(5), 647-648.

Pratt, R., Daniels, T.C., Eiler, J.B., Gunnison, J.B. and Kumler W.D. 1944. Chlorellin, an antibacterial substance from *Chlorella*. *Science*. (99), 351-352.

Prescott, G.W. 1962. Algae of the western Great lakes area WC Brown co.977pp, Pls 1136.

Ratnam, K. V. and Raju, R.R.V. 2008. *In vitro* antimicrobial screening of the fruit extracts of two *Syzygium* species (Myrtaceae). *Advan Biol*. (2), 17-20.

Veritski, A.A, Gromov, B.V., Titova, N.N. and Mamkaeva, K. A. 1991. Production of the antibiotic- algicide cyanobacterin Lu-2 by a filamentous cyanobacterium *Nostoc* sp. *Microbiologia*.(60), 21 – 25.

Washington, D.C. 1978. American Public Health Association, Standard Methods for the Examination of Dairy Products, 14th Ed.

Zhao, J., Zhang, B. and Zhang, Y. 2005. Chlorophytes of Biological soil crusts in gurbantunggut Desert, Xinjiang Autonomous Region. *Front Biol China*.23(2),189- 193.

Corresponding author: Dr. Ghada W. Abou-El-Souod, Botany Department, Faculty of Science, Menoufia University, Egypt
Email: gamalkoleap2003@yahoo.com