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Antifungal Activity of the Cyanobacterium *Nostoc muscorum* Isolated from Al-Hassa, Saudi Arabia

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The cyanobacterium*Nostocmuscorum* was isolated from Al-Hassa, Saudi Arabia. Present study has undertaken effectiveness of *Nostocmuscorum*in different solvent (Petroleum ether, Chloroform, petroleum ether, Methanol) extract against five plant pathogenic fungi namely *Alternaria alternate, Aspergillusflavus, Fusariumoxysporum, Rhizoctoniasolani* and *Rhizopusstolonifer*. Different organic extracts were prepared at a concentration of 1mg/disc.The data obtained here showed that high efficiency of the algal culture filtrates in suppressing the fungal mycelial growth. The best results were obtained by culture filterateextract, chloroformic and methanolic extracts of the algal cell pellet. The antifungal activity of the algal culture filtrates and extracts of algal cell pellets may contribute to the presence of bioactive compounds. This study can provide potential candidates for the development of biocontrol agents against these fungi.

Key words: Al-Hassa**;** Antifungal; Biological control; *Nostocmuscorum;* Exudates Extracts.

Microalgae display a diversity of primary and secondary metabolites, and release several of these substances to their environment actively or passively living or dead after decomposition and lysis. Algal secondary metabolites have a diverse antagonistic activity that lead to disintegration of microbial growth. The screening of extracts or isolated compounds from different natural sources is a common way to discover biologically active metabolites. Algae excrete various organic compounds into their environment. So, some biologically active compounds were identified among these exo-metabolites e.g. some antibacterial di-terpenoids in *Nostoc commune*[1-2] or antifungal peptides in *Tolypothrix byssoides*[3] and algicidal phenolic compounds[4]. The antimicrobial substances involved may target various kinds of microorganisms, prokaryotes as well as eukaryotes. The properties of secondary metabolites in nature are not completely understood[5]. Secondary metabolites influence other organisms in the vicinity and are thought to be of phylogenetic importance.

Cyanobacteria or the blue green algae possess a diverse structure and have a wide distribution throughout the globe. They are considered to be a rich source of products having pharmaceutical and toxicological potential, which primarily include metabolites such as proteins, fatty acids, antioxidants [6] and vitamins and pigments[7]. Cyanobacteria have the capability to produce metabolites both intracellular and extracellular. Secondary metabolites from cyanobacteria are associated with toxic, hormonal, antineoplastic and antimicrobial effects [8-10]. Cyanobacterial strains belonging to the genera *Microcystis, Anabaena, Nostoc, Oscillatoria, Nodularia, Aphanizo menon* and *Cylindro spermum* are known to produce a number of cyclic peptide hepatotoxins and alkaloid neurotoxins exhibiting algicidal, fungicidal, pesticidal, cytotoxic, immunosuppressive and enzyme-inhibiting activity[11-13]. Certain free fatty acids produced by algae exert inhibitory effects

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on a variety of aquatic organisms [14-15]. The inhibition is based on the fact that fatty acids primarily affect the plasma membranes [16].

Fungal pathogens cause many diseases in plants leading to destruction of resources in agriculture. Traditional methods to circumvent fungal pathogens involve the application of synthetic fungicides and growing resistant cultivars. The fungicides are harmful to the environment and expensive., In addition, there is gradual increase in resistant pathogens. Biological control is a better choice and Cyanobacteria are wonderful organisms with diverse range of potentials[17]. The present study focuses on the activity of cyanobacteria-*Nostocmuscorum* as the antifungal agent.

MATERIALS AND METHODS

Isolation and Purification of Cyanobacterial strains

Nostoc muscorum was isolated was isolated from Al-Hassa, Saudi Arabia. Rippka and Herdman[18] modified medium was used for isolation and cultivation of the cyanobacterium. Pure cultures of living specimen was prepared using subculture with agar plate method [19]. Preserved specimen was prepared and the living specimen was incubated in 500 ml conical flasks. The flasks were incubated at 26 °C + 1.0 under continues illumination of (250 mmol $m^{-2}s^{-1}$) provided by cool white fluorescent lamps set on 14:10 h photoperiod. All cultures were shaken twice daily to prevent cells from clumping. Sterile technique was used at all times. The resulted culture were identified based on morphology following taxonomic keys of Desikachary[20].

Pathogenic fungal strains

The fungal strains were isolated from diseased plants. They were grown in the laboratory, identified and characterized on the basis of their morphological properties[21]. Five fungal strains were selected for these study namely *Alternaria alternate, Aspergillus flavus, Fusarium oxysporum, Rhizoctonia solani* and *Rhizopus stolonifer.* Fungal strains were kept and cultured on potato dextrose agar medium slants and plates.

Preparation of supernatant and cell extracts of the cyanobacterium *Nostoc muscorum*

Algal culture was harvested after 25 days by centrifugation at 500 rpm for 15 min. Culture supernatant was collected and the algal biomass was air-dried and further subjected to extraction by mixing well in the organic solvent. Liquid–liquid extraction technique [22] was employed for the extraction of algal Extracellular metabolites. 0.5g of dried algal material was extracted in 20ml of an organic solvent (Methanol, chloroform, ethyl acetate and petroleum ether),kept in an orbital shaker for overnight as described by Sivasubramanian *et al.*,[23]. The obtained extracts were filtered with Whatman no.1 filter paper and the filtrate was collected. The solvents were removed under reduced pressure at 50°C using rotary evaporator (Buchi, B-480).

Antifungal bioassay test

As mention above five fungal species were selected as test organisms such as, *Alternaria alternate, Aspergillus flavus, Fusarium oxysporum, Rhizoctonia solani* and *Rhizopus stolonifer*. Dried extracts and supernatants were dissolved in 1 ml of their extraction solvents to yield a stock solution of 50 mg/ ml and the antimicrobial activity was determined by the disk diffusion method [24]. Filter paper discs (6 mm) were saturated with 20 µl of test solution (1 mg/ disc), dried under Laminar Air Flow and placed on PDA plate which had been inoculated with a mycelial disk of a fungus. The plates were incubated at 28 ºC for 5 days. Discs treated with 20 µl solvent were used as negative controls. The extracts and supernatants containing antifungal components produce distinct clear zones around filter paper discs. Diameter of clear zones were determined and used as an indication of antifungal activity [24].

RESULTS AND DISCUSSION

Nowadays, there is a large availability of clinically useful antibiotics and also a continuous search for new anti-infective agents which remain indispensable. Some of the major antibiotics have indeed considerable drawbacks in terms of limited antimicrobial spectrum or serious side effects [4]

The results of antifungal activity of *Nostoc muscorum* extracts against selected plant pathogenic fungi at a concentration of 1 mg of extract/ disc are shown in Tables 1 and Plates 1-5. It is clear from the study that the diameter of the inhibition zone depends mainly on type of the

Table 1. Antifungal activity of *Nostocmuscorum* extracts as presented by inhibition zone diameter (in Cm)

Fungal species	Extracellular filterate	Diameter of inhibition zone (cm) Solvent			
		Ethylacetate	Chloroform	Methanol	Petroleumether
Rhizoctoniasolani	2. 5± 0.1	2.0 ± 0.3	2.1 ± 0.4	2.4 ± 0.3	0.9 ± 0.2
Rhizopusstolonifer	2.2 ± 0.2	0.7 ± 0.0	2.1 ± 0.2	2.2 ± 0.3	0.7 ± 0.0
Aspergillusflavus	1.1 ± 0.1	1.3 ± 0.1	1.8 ± 0.2	0.9 ± 0.2	1.0 ± 0.2
Fusariumoxysporum	1.7 ± 0.1	1.2 ± 0.2	1.6 ± 0.3	1.7 ± 0.2	1.3 ± 0.2
Alternariaalternata	2.5 ± 0.4	ND	3.3 ± 0.3	2.1 ± 0.1	ND

ND= not detected

extract and solvent used in the extraction process and the tested fungal organisms. Data reveal that the spent media extract was most effective compared to organic extracts of the cyanobacterium cell pellet extract. The diameters of inhibition zones measured showed that *Nostoc muscorum* was highly potent in terms of fungicidal activity (Table 1). For extracellular metabolites, the largest inhibition zones were recorded against *Rhizoctonia solani* and *Alternaria alternata* (2.5 cm) while the minimum was recorded for *Aspergillus flavus* (1.1 cm). For intracellular metabolites extracted from the cyanobacterium *Nostoc muscorum*, the data of Table 1 reveals that the best solvent for the extraction of endogenous metabolites was chloroform followed by methanol, ethyl acetate and finally petroleum ether. The maximum inhibition zone for chloroformic extract was 3.3 cm against *alternata* while the minimum 1.6 cm against *Fusarium oxysporium*. Methanolic extract of the

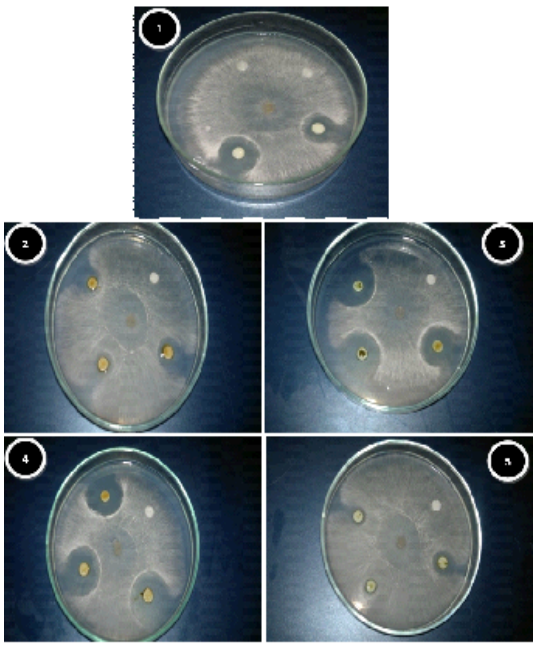

Plate 1. Antifungal activity of *Nostoc muscorum* extracts against *Rhizoctonia solani* at a concentration of 1 mg of extract/ disc. (1) Extracellular extract (2) ethyl acetate extract (3) Chloroform extract (4) Methanol extract (5) Petroleum ether extract

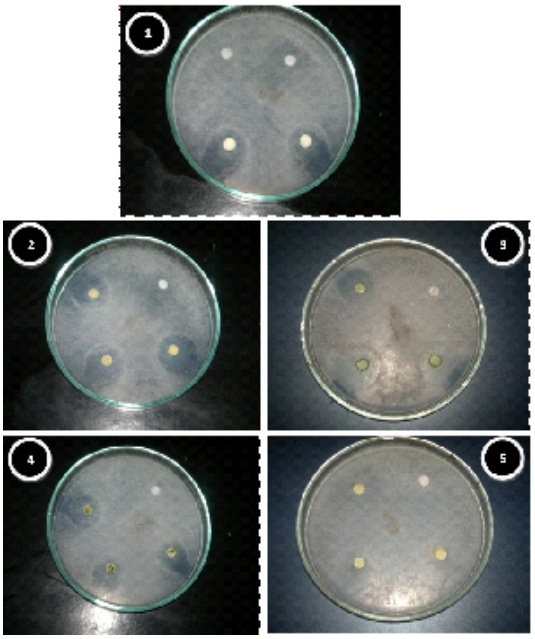

Plate 2. Antifungal activity of *Nostoc muscorum* extracts against *Rhizopus stolonifer*. at a concentration of 1 mg of extract/ disc. (1) Extracellular extract (2) ethyl acetate extract (3) Chloroform extract (4) Methanol extract (5) Petroleum ether extract

cyanobacterium cell pellet showed also potent inhibition zones of maximum 2.4 against *Rhizoctonia solani* and a minimum 0.9 against *Aspergillus flavus*. Ethyl acetate and petroleum ether extracts were the least potent against all tested fungi and no inhibition zone was detected for both solvents against *alternata*. The maximum inhibition zone of ethyl acetate extract was 2 cm against *Rhizoctonia solani* and that for petroleum ether was 1.3 cm against *Fusarium oxysporium*. It can be concluded from Table 1 that *Aspergillus flavus* was the less susceptible fungus to the cyanobacterium extracts as it showed the smallest inhibition zones.

A large number of microalgal extracts and extracellular products have been found to have antibacterial activity. The microalgae such as *Scytonema hofmannii*[25], *Hpalosiphon fontinalis*[26], *Chlamydomonas* sp. (Kellam and Walker, 1989), *Anabaena* sp. (Frankmolle *et al.*,1992), *Phormidium* sp. [27], *Microcystis aeruginosa*[30], *Nostoc* spp. [2], *Nostoc muscorum*[31], *Chlorella* sp., *Scenedesmus* sp. [32] and *Euglena viridis*[33] have been reported as the main groups of microalgae to produce antimicrobial agents. Screening efforts aimed to identify antimicrobial agents in cyanobacteria have revealed several promising lead compounds. Some of these substances identified including Nostocyclyne A [34], Nostofungicidine[35], Kawaguchipeptin B [30]and Nostocin A [36].

The ability to produce antimicrobial agents may be significant not only as defensive instrument for the algal strains but also as good source of new bioactive compounds from a pharmaceutical point- of- view[37] The recent investigations with Cyanobacteria have demonstrated the antimicrobial and antifungal effects of *Phormidium* sp.[4]. These reports are in agreement with our present study. Cyanobacteria are known to produce antitumor[38] antiviral [39], antibiotic [40] and antifungal compounds [41]. These compounds have been studied by various research

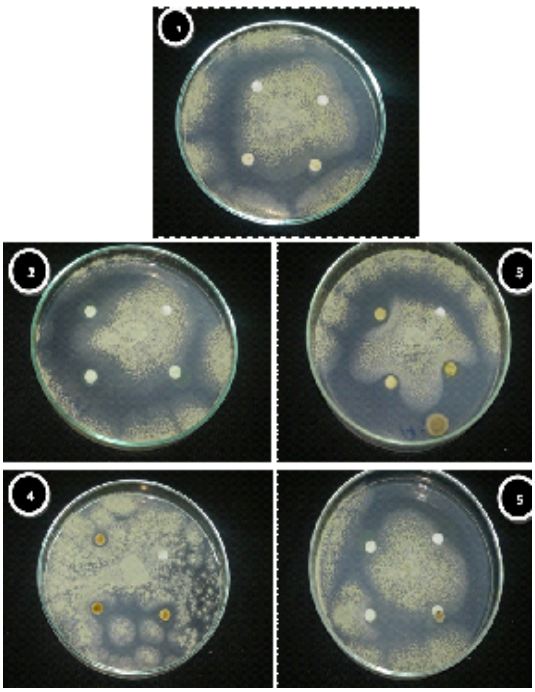

Plate 3. Antifungal activity of *Nostoc muscorum* extracts against *Aspergillus flavus* at a concenteration of 1 mg of extract/ disc. (1) Extracellular extract (2) ethyl acetate extract (3) Chloroform extract (4) Methanol extract (5) Petroleum ether extract

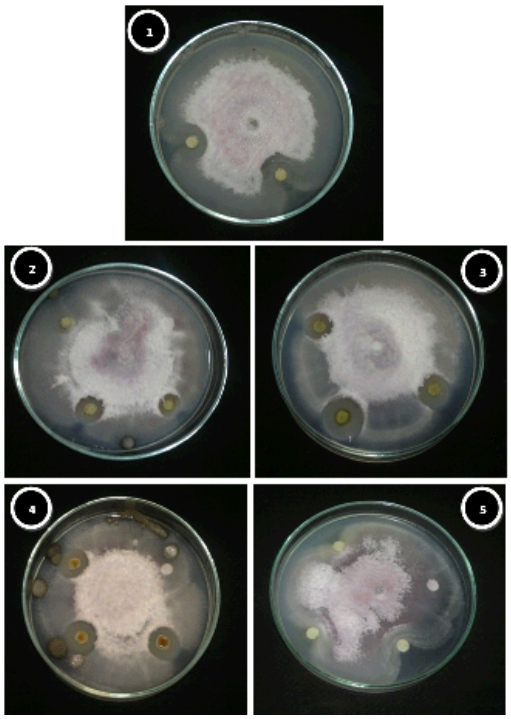

Plate 4. Antifungal activity of *Nostoc muscorum* extracts against *Fusarium oxysporium* at a concentration of 1 mg of extract/ disc. (1) Extracellular extract (2) ethyl acetate extract (3) Chloroform extract (4) Methanol extract (5) Petroleum ether extract

groups and have shown promising results in declining the growth of various microbes [42-43.] There are reports on the inhibition of growth in plant pathogenic fungi by cyanobacteria. Kim [44] reported that cyanobacterial strains *Oscillatoria, Anabaena, Nostoc, Nodularia*, and *Calothrix* exhibited antifungal activity against seven phytopathogenic fungi causing diseases in hot pepper. Mule [42] reported that extract of *N. commune* inhibited the growth of fungus *Candida albicans*. Cano [45] evaluated the antifungal activity of terrestrial cyanobacterium *N. muscorum* against the *Candida albicans* by 20.83.*Nostoc muscorum* reduced *Fusarium oxysporum* and *Rhizoctonia solani*. These results are in agreement with De Caire *et al.*,[46] who found that the growth of the plant pathogen *Rhizoctonia solani* was inhibited by extracellular products of *Nostoc muscorum*. A variety of solvents were used for the extraction of microalgal active metabolites and it had been found that the chance of getting antimicrobial activity was higher in culture supernatant and chloroformic and methanolic extracts of algal cell pellets.

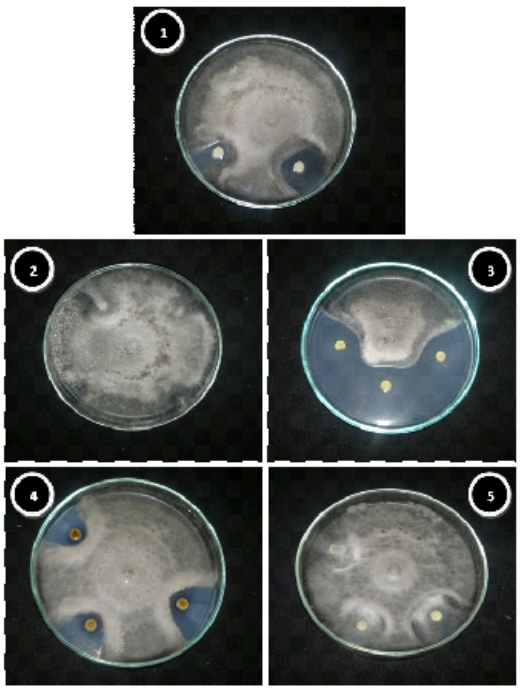

Plate 5. Antifungal activity of *Nostoc muscorum* extracts against *Alternaria alternate* at a concentration of 1 mg of extract/ disc. (1) Extracellular extract, (2) ethyl acetate extract, (3) Chloroform extract, (4) Methanol extract ,(5) Petroleum ether extract

It can be concluded that the *Nostoc* extracts obtained by various solvents used in this study had antifungal activities and that these extracts could be much more effective when compared with contemporary antibiotics and fungicides. This study can provide potential candidates for the development of biocontrol agents against these fungi. The results of this work indicate that *Nostoc muscorum* displays a potential that warrants further investigation.

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REFERENCES

1. Jaki, B., Orjala, J.. Sticher, O. A novel extracellular diterpenoid with antibacterial activity from the cyanobacterium*Nostoc commune*. *J. Nat. Prod*., 1999; **62**: 502-503.
2. Jaki, B., Orjala, J., Heilmann, J., Linden, A., Vogler, B. andSticher, O. Noval extracellular diterpenoids with biological activity from the cyanobacterium *Nostoc commune*. *J. Nat. Prod*., 2000; **63**: 339-343.
3. Jaki, B., Zerba, O., Heilmann, J, Sticher, O. Two novel cyclic peptides with antifungal activity from the cyanobacterium *Tolypothrix byssoides* (EAWAG 195). *J. Nat. Prod*., 2001; 64:154-158.
4. Kumar N.S., Sivasubramanian V., Mukund S. Antimicrobial and Antifungal activity of extracts of *Phormidium fragile* Gomont. *J. Algal Biomass Utln*., 2013; **4**(3): 66–71
5. Inderjit, K. M. M., Dakshini, K.M.M. Effect of cultivation on allelopathic interference success of *Pluchealanceolata*. *J. Chem..Ecol.,* 1994; **20**:1179-1188.
6. Archana, T., Shivani, M. Antioxidative Potential of Catalase in Bloom Forming Cyanobacteria-*Anabaena variabilis* and *Synechococcus elongates. Int. J. Pharm. Bio. Sci.*, 2012; **3**(3)(B): 956-966.
7. Borowitzka, M.A. Microalgae as sources of pharmaceuticals and other biologically active compounds. *J. Appl. Phycol*., 1995; **7**: 3-15
8. Carmichael, W.W. Cyanobacteria secondary metabolites the cyanotoxins. *J. Appl. Bacteriol*., 1992; **72**: 445- 459.
9. Patterson, G.M.L, Larsen L.K., Moore R.E.

Bioactive natural products from blue-green algae. *J. Appl. Phycol.*, 1994; **6**:151-157.

10. Shweta,Y., Sinha R. P., Tagiand A. K. Cyanobacterial secondary metabolites. *Int J Pharm Bio Sci.*, 2011; **2**(1): (B) 144-167.
11. Namikoshi, M., Rinehart, K.L. bioactive compounds produced by cyanobacteria. *J. Indus. Micrbiol. Biotechnol*., 1996; **17**:373-384.
12. Jaiswal, P., Prasanna, R., Singh, P.K. Cyanobacterialbioactive molecules : An overview of their " cidal" properities. *Can. J. Microbiol*., 2008; **54**:701-717.
13. Prasanna, R., Sood, A., Jaiswal, P., Nayak, S., Gupta, V., Chaudhary, V., Joshi, M., Natarajan, C.. Rediscovring cyanobacteria as valuable sources of bioactive compounds. *Appl. Biochem. Microbiol*., 2010b; 46:133-147.
14. Kamaya, Y., Kuragi, Y., Suzuki , K. Acute toxicity of fatty acids to the freshwater green alga *Selenastrum capriocornutum*. *Environ. Toxicol*., 2003; 18:289-294.
15. Mundt, S., Kreitlow, S., Jansen, R..Fatty acids with antibacterial activity from the cyanobacterium *Oscillatoria redekei* HUB 051. *J. Appl. Phycol*., 2003; **15**: 263-267.
16. Wu, J.T., Chiang, Y.R., Huang, W.Y., Jane, W.N. Cytotoxic effects of free fatty acids on phytoplankton algae and cyanobacteria. *Aquatic Toxicology*., 2006; **80**: 338-345.
17. Archana, T. d., Akshita, S. Antifungal activity of *Anabaena variabilis* against plant pathogens. *Int. J. Pharm. Bio. Sci.*, 2013; **4**(2): (B) 1030-1036.
18. Rippka, R., Herdman, M. Pasteur Culture Collection of Cyanobacterial Strains in Axenic Culture. Catalogue of strains. Institute Pasteur, Paris, France., 1993; pp 1-103.
19. Allen, M. M. Simple conditions for growth of unicellular blue-green algae on plates. *J. Phycol.*, 1968; 4: 1-4.
20. Desikachary, T. V.,Cyanophyta. I.C.A.R. Monographs on algae. Indian council of agriculture research publications. New Delhi, 1959; pp 686.
21. Samson, R.A., Houbraken J., Thrane U., Frisvad J.C., Andersen B. Food and Indoor Fungi. CBS Laboratory Manual Series 2. Centraalbureauvoor Schimmelcultures, Utrecht, The Netherlands. 2010; 125.
22. Sikdar, S.K., Cole, K.D., Stewart, R.M., Szlag, D.C., Todd, P., Cabezas, H. Jr. Aqueous Two-Phase Extractions in Bioseparations: *An Assessment. Biotechnology*., 1991; 9: 254.
23. Sivasubramanian V., Uma, R., and Niranjali, D.S. Evaluation of in vitro antioxidant activities and antiproliferative activity of green microalgae, *Desmococcus olivaceous* and *Chlorococcum humicola*. *J. Algal Biomass Uln*., 2011; **2**(3): 82– 93.
24. Ghasemi, Y., Tabatabaei,Y., M., Amini, A., Shokravi, M. S.,Zarrini, G. Parsiguine, Anovel antimicrobial substance from *Fischerella ambigua*. *Pharm. Biol*., 2004; 42: 318-322.
25. Pignatello, J.J., Porwol, J., Carlson, R.E., Xavier, A., Gleason, F.K.,Wood, J.M. Structure of the antibiotic cyanobacterian, a chlorine-containing γ-lactone from the fresh water cyanobacterium *Scytonemahofmannii*. *J. Org. Chem.*,1983; **48**: 4035-4038.
26. Moore, R. E., Cheuk,C., Yang, X. G., Patterson G.M.L. Hapalindoles, antibacterial and antimycotic alkaloids from the cyanophyte *Hapalosiphon fontinalis*. *J. Org.Chem*., 1987; **52**: 1063-1043.
27. Kellam, S.J., Walker, J.M. Antibacterial activity from marine microalgae in laboratory culture. *Br. Phycol. J*., 1989; **24**:191-194.
28. Frankmolle, W.P., Larsen, L.K., Caplan, F.R., Patterson G.M.L, Knubel G. Antifungal cyclic peptides from the terrestrial blue- green alga *Anabaena laxa*. Isolation and biological properties. *J. Antibiot*., 1992; **45**:1451-1457.
29. Fish, S.A., Codd, G.A. Bioactive compound production by thermophilic and thermotolerant Cyanobacteria (blue green algae). *World J. Microb. Biotech*., 1994; 10: 338-347.
30. Ishida, K., Matsuda, M., Kawaguchipeptin, H. M. B., Yamaguchi, K. An antibacterial cyclic peptide from the cyanobacterium *Microcystis aeruginosa*., *J. Nat. Prod.*, 1997; **60**:724-726.
31. Zaki, F. T., Fathi, A. A. Some factors affecting antimicrobial substance produced by the blue-green alga Nostocmuscorum. *Egypt. J. Biotechnol*., 2002; **12**(1): 14-25.
32. Ordog, V., Stirk,W.A., Lenobel R., Bancirova,M.,Stran, M.,StandenVan J.Screening microalgae for some potentially useful agricultural and pharmaceutical secondary metabolites. *J. Applied.Phycol*., 2004; **16**: 309-314.
33. Das, B.K., Pardhan, J, Pattnaik, P., Samantaray, B.R., Samal, S.K. Production of antibacterials from the freshwater alga *Euglena virids* (Ehren),*World J. Microb. Biotech.*, 2005; **21**: 45-50.
34. Ploutno, A., Carmeli, S. Nostocyclyne A, a novel antimicrobial cyclophan from the cyanobacterium *Nostoc* sp. *J. Nat. Prod.*, 1997; **63**: 1524-1526.
35. Kajiyama, S., Kanzaki, H., Kawazu, K., Kobayashi, A.. Nostifungicidine, anantifungal lipopeptide from the field grown terrestrial blue-

green alga *Nostoc commune*. *Tetrahedron Lett* ., 1998; **39**: 3737-3740.

36. Hirata, K., Takashina, J., Nakagami, H., Ueyama,S., Murakami, K., Kanamori, T. Miyamoto, K. Growth inhibition of various organisms by a violet pigment, Nostocin A, produced by *Nostoc spongiaeforme*. *Biosci. Biotech. Biochem*., 1996; **60**:1905-1906
37. Ghasemi, Y., Moradian, A., Mohagheghzadeh, A., Shokravi, S., Morowvat, M.H. Antifungal and antibacterial activity of the microalgae collected from paddy fields of Iran: Characterization of antimicrobial activity of *Chroococcus disperses., J. Boil. Sci.*, 2007; **7**: 904-910.
38. Moore R.E. Cyclic peptides and depsipeptides from cyanobacteria: are view, *J Ind. Microbio.*, 1996; **16**(2): 134-43.
39. Patterson G.M.L, Baker K.L., Baldwin C.L., Bolis C.M., Caplan F.R., Larsen L.K., Levine I.A., Moore R.E., Nelson C.S., Tschappat K.D., Tuang G.D., Boyd M.R., Cardellina J.H. II, Collins R.P., Gustafson K.R., Snader K.M., Weislow O.S., Lewin R.A. Antiviral activity of cultured blue-green algae (cyanophyta). *J. Phycol.*, 1993; **29**:125-130.
40. Bloor, S., England, R. R. Antibiotic production by the cyanobacteria *Nostoc muscorm*. *J. Appl. Phycology*,1989; **1**:367-372.
41. Kulik, M. M. The potential for using cyanobacteria (bluegreen algae) and algae in the biological control of plant pathogenic bacteria and fungi. *Eur. J. Plant Pathol*., 1995; **101**: 585-599.
42. Mule M. C. Z., Caire, G. Z., Cano, M. S., Halperin D. R. Bioactive compounds from *Nostoc muscorum* (cyanobacteria). *Cytobios*., 1995; **66**: 169-172.
43. El-Sheekh, M.M.,. Osman, M. E.H, Dyabb, M. A,. M. S. A. Production and characterization of antimicrobial active substance from the cyanobacterium *Nostoc muscorum.Environ. Toxic. and Pharm*., 2006; **21** : 42–50.
44. Kim J. D. Screening of Cyanobacteria (Blue-Green algae) from Rice Paddy Soil for Antifungal Activity against Plant Pathogenic Fungi. *Mycobiolog.*, 2006; **34**(3): 138-142.
45. Cano, M. M. S., Mule, M. C. Z., Cair, G. Z., Halperin, D. R. Inhibition of *Candida albicans* and *Staphylococcus aureus* by phenolic compound from the terrestrial cyanobacterium *Nostoc muscorum*. *J. Appl. Phycol.*, 1990; **2**: 29-81.
46. De Caire, G.Z., de Cano, M.S., de Mule, M.C.Z. and de Halperin, D.R. Antimycotic products from the cyanobacterium *Nostoc muscorum* against *Rhizoctonia solani*.*Phyton*.,1990; **51**: 1-4.