

Antimicrobial Activity of the Various Extracts of *Spirulina platensis* and GC-MS Analysis

Amal A. Al Hazzani, Afaf I. Shehata, Nadine M.S. Moubayed,
Hadeel Jawad Al Hourri and Gehan Elgaaly

Department of Botany and Microbiology, College of Science, King Saud University,
P.O. Box 22452, Riyadh, 11495, Kingdom of Saudia Arabia.

(Received: 18 April 2013; accepted: 25 May 2013)

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 antibacterial agents. In the attempt of extracting and producing newly active antimicrobial substances substituting the existing overgrowing antibiotic microbial resistance, *Spirulina platensis* cyanobacterium was extracted with five different volatile organic solvents, acetone, methanol, petroleum ether, chloroform and ethanol. Their antimicrobial effect was studied on gram positive such as *Staphylococcus aureus* ATCC 25923, *Staphylococcus xylois* obtained, *Bacillus subtilis* ATCC 6633, MRSA ATCC 12498, and gram negative bacteria *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 25966, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae* ATCC 700603, *Salmonella* sp. (clinical isolate) and *Candida albicans* ATCC 10231 and *Fusarium* sp. (clinical isolate) using the agar well diffusion technique. Larger inhibition zone was observed with methanol *S. platensis* extract mainly on MRSA. The GC-MS analysis of *S. platensis* methanolic extract revealed the major active fatty acids constituents such a tetradecanoic acid and octadecanoic acid assumed to provide the antimicrobial activity.

Key words: *Spirulina platensis*, Agar well diffusion Technique, Gas Chromatography and Mass Spectrophotometry (GC-MS) Analysis, Antimicrobial activity, Inhibition zone.

Cyanobacteria represent a large group within the prokaryotic kingdom revealing significant interest in terms of research work and promising new horizons in therapeutic pharmacology and bioremediations (Patterson G M L *et al.*, 1994). Cyanobacteria or blue green algae are the oldest oxygenic photosynthetic organisms known so far and they also serve as a rich source of novel bioactive metabolites, including many cytotoxic, antifungal and antiviral compounds that have received extensive studies for their potential as natural antimicrobial agents since they contain

a vast diversity of biologically active substrates (Radmer R.J. *et al.*, 1994). Screening of the blue green algae especially *Spirulina platensis* for the antimicrobial activity from decades proved that *S. platensis* produces a diverse range of bioactive molecules making them a rich source of different types of medicines (Kumar *et al.*, 2012) with different biological activities ranging from antibacterial and antifungal (Ghasemi *et al.*, 2003, 2007; Isnansetyo *et al.*, 2003; Jaki *et al.*, 1999; Kumdim *et al.*, 2003; Soltani *et al.*, 2005 and H. al Wathnani *et al.*, 2012) anti viral (Moore *et al.*, 1989) and even anti-algal activity (John *et al.*, 2003) alternating and substituting the use of the synthetic antibiotics used in the treatment of microbial infections (Kumar *et al.*, 2012) particularly

* To whom all correspondence should be addressed.
E-mail: afafsh@ksu.edu.sa

the pathogenic microorganisms, among which are the antibiotic resistant bacteria causing threats to humans .

Many studies have been established to prove the antimicrobial effect of metabolites extracted from algal species especially those derived from blue green algae (Zulpa *et al.*, 2003; Abedin *et al.*, 2008; Kulik, 1995).

Search for cyanobacteria with antimicrobial activity is extensively required (Borowitzka, 1995) due to the growing worldwide concern about increased percentage of infection by antibiotic resistant microorganisms, these developments ensured the discovery of new, promising, safer and more potent agents to treat serious bacterial and fungal infections (Kumar *et al.*, 2012). In the present study, the antimicrobial activity of the cyanobacterium *Spirulina platensis* was analyzed and identified using five different solvent acetone, methanol, ethanol, chloroform and petroleum ether against gram positive and gram negative human pathogenic bacteria among which methicillin resistant *Staphylococcus aureus* (MRSA) and *Candida albicans* giving new horizons in the treatment of the microbial infections. Scanning electron microscopy was applied to the processed cyanobacteria in addition GC-MS was performed in the aim of knowing its chemical composition giving the possibility for identifying promising chemical agents in the microbial treatment.

MATERIALS AND METHODS

Algal Cultivation

S. platensis (UTEX 2340) was obtained from the University of Texas Culture Collection. The algal culture was maintained on Zarrouk's medium at 30°C with 500 lux light intensity for 30 days. Samples were then shade dried (Gonzalez Del Val *et al.*, 2001) and grounded into powder. Subsequently the powdered samples stored in refrigerator.

Algal Extraction

10 g of the powdered *Spirulina platensis* were extracted with 100 ml of each of the solvents acetone, methanol, ethanol, chloroform and petroleum ether respectively. The extracts were incubated in 250-ml Erlenmeyer flasks in a rotating shaker for 3 days at 27°C and 100rpm (Spain model

Comecta, s.a). The extracts were then filtered through 0.45 µm membrane filter (Millipore Corporation, U.S.A.) the filtered extracts were kept at room temperature for evaporation for 2 to 3 days subsequently the crude extracts were aseptically transferred to sterile eppendorf tubes and stored at -4°C for later use.

Microbial isolates preparation

Bacterial isolates varying between gram positive such as *Staphylococcus aureus* ATCC 25923, *Staphylococcus xylois* obtained from the Microbiological Resources Center(Cairo, Egypt), *Bacillus subtilis* ATCC 6633, MRSA ATCC 12498 , and gram negative bacteria *Escherichia coli* ATCC 25922 , *Escherichia coli* ATCC 25966, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae* ATCC 700603, *Salmonella* sp. (clinical isolate) and *Candida albicans* ATCC 10231 and *Fusarium* sp. (clinical isolate), were obtained for the microbiology laboratory of king Khaled hospital, Riyadh, Saudi Arabia, isolates were inoculated on nutrient agar plates (N.A) and incubated at 37°C for 18-24 hrs. All isolates were kept in 15% glycerol at -4°C for further use.

Microbial Assay

Well – diffusion agar technique

Bacterial suspensions for each of the tested organisms were prepared in 9 ml sterile nutrient broth and were incubated at 37 °C for 18 hr to obtain a turbidity of 0.5 MacFarland. Each bacterial suspension correspondingly were spread on the surface of Mueller Hinton agar plates with a sterile cotton swab and kept to dry. The antimicrobial assay was achieved with the agar diffusion technique, consequently five equally distant 6 mm wells were made on the inoculated Mueller Hinton agar plates with the help of a sterile cork borer. Each well was loaded with 50µl of the different algal extract respectively using a micropipette, the extract was allowed to diffuse for 30 minutes at room temperature and the loaded plates were then incubated at 37°C for 18-24 hrs. Appearance of an inhibition zone indicated the presence of antibacterial and antifungal activity of the algal extract being tested against the bacterial and fungal isolates. All the experiments were carried out in triplicates.

Antimicrobial determination

Antibacterial and antifungal activity of

the algal extract was determined by the inhibition zone that was tabulated and indicated as (+) or (-) indicating the potent activity of the algal solvent extract respectively. (Table. 1)

The *Spirulina* antibacterial activity was compared with the three standard antibiotic discs against the organisms being tested respectively (Table 1).

AOXICILLIN\CLAV\ACID (AMC)

MEM 10

MXF 5

Scanning electron microscopy

Morphological characteristics of the selected crude cyanobacterium sample was studied using the scanning electron microscopy (SEM, JEOL, JSM, 3060LV) dried samples were soaked in a clean filter paper to be coated with gold by sputter gold coater and then attached on aluminium sample stub and then the sample was viewed with the scanning electron microscope (Ismet, 2003).

Chemical composition (GC-MS analysis)

GC/MS analysis for methanol *Spirulina* extracts were performed on a HP 5973 mass selective detector coupled with a HP 6890 gas chromatograph, equipped with a HP-1 capillary column. The column temperature was programmed

from an initial temperature of 70°C to a final temperature of 280 °C at 10 °C/min. The injector temperature was 150 °C (1 µL injection size), whereas the detector temperature was 250 °C. The carrier gas was helium (2mL/min). Identification of the individual components was performed by comparison of mass spectra with literature data and by a comparison of their retention indices (RI) relative to a C8-C32 *n*-alkanes mixture (Adams, 1995). A computerized search was carried out using the Wiley 275 L. GC/MS library and ARGEFAR GC/MS library created with authentic samples.

RESULTS

Antimicrobial activity

The antibacterial activity of *Spirulina platensis* extracts with different solvents tested on gram positive and gram negative bacteria were studied and tabulated indicating that the methanolic extract had the highest antibacterial activity (Ozdemir *et al.*, 2004; Kumar *et al.*, 2011) particularly against MRSA and *Staphylococcus xylois* (Fig. 1) followed by *Bacillus subtilis* and *Staphylococcus aureus* and showed no effect against *E.coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Salmonella* sp., *Klebsiella*

Table 1. Antimicrobial activity of different extracts of cyanobacteria *Spirulina platensis* using different solvents.

No	Bacterial Isolates	Acetone	Chloroform	Ethanol	Methanol	Petroleum ether	Antibiotic disc µg
1	<i>K. pneumonia</i>	-	+	-	-	-	MEM 10
2	<i>E. coli</i> (25966)	-	+	-	-	-	MEM 10
3	<i>E. coli</i> (25922)	-	-	-	-	-	MXF 5
4	<i>P. aeruginosa</i>	-	-	-	-	-	MEM 10
5	<i>Sal</i>	-	-	-	-	-	AMC 30
6	<i>E. faecalis</i>	-	-	-	++	-	AMC 30
7	MRSA	+	-	++	+++	-	MEM 10 AMC 30 MXF 5
8	<i>B. subtilis</i>	++	-	++	++	-	MEM 10
9	Staph A	-	-	-	++	+	MEM 10
10	Staph xylois	++	++	++	+++	++	MEM 10
	Yeast						
1	<i>C. albicans</i>	+	+	-	-	-	MEM 10
	Fungi						
1	<i>Fusarium</i>	-	-	-	-	-	-

(-) No activity, (+) low activity, (++) moderate activity, (+++) high activity.

All antibiotic discs used as positive control show higher antimicrobial activity, larger inhibition zone.



Fig. 1. (A) Effect of *Spirulina platensis* various organic extracts on *Staphylococcus xylosus* and MRSA methicillin resistant *Staphylococcus aureus* respectively; larger inhibition zone is observed with methanol solvent extract. A: acetone; M: methanol; E: ethanol; C: chloroform.

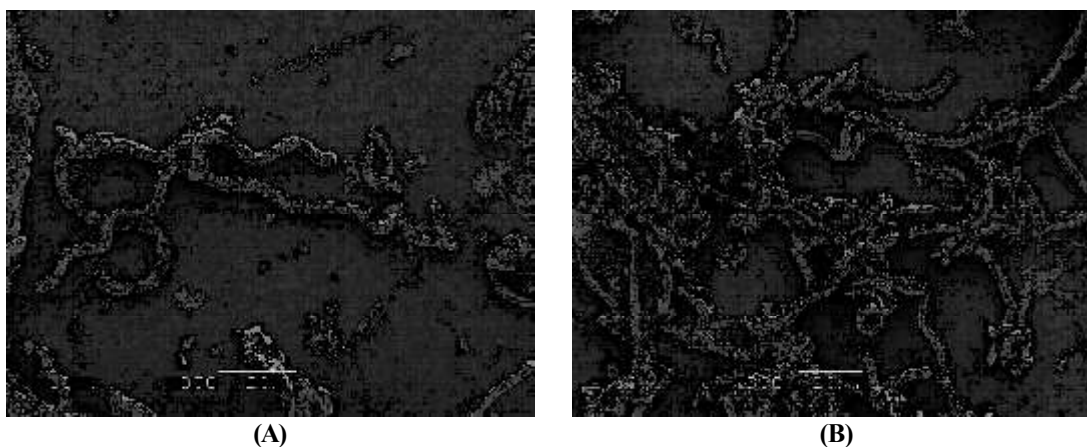


Fig. 2. Scanning electron microscopic revealing the morphological identification and examination of *S. platensis* (A, B)

pneumoniae and on *Candida albicans* and *fusarium* sp. Acetone, ethanol, chloroform and petroleum ether extracts showed minimal or no effect on the tested organisms. Acetone, ethanol and petroleum ether showed moderate activity on *S. xylosis*, *B. subtilis* and MRSA respectively; whereas chloroform had no activity against all the tested microorganisms. All positive controls with the antibiotic discs of different concentration showed larger inhibition zone on the Muller Hinton well diffusion agar plates indicating higher antimicrobial activity in comparison to *Spirulina* various extracts. (Table 1). This could be related to the season when *S. platensis* has been collected or could be related to the *S. platensis* state being fresh or dry.

Scanning Electron Microscopy

Crude spirulina sample was observed with the scanning electron microscopy to study and identify the morphological structure of the cyanobacterium being studied.

GC-MS Analysis

GC-MS analysis of *Spirulina platensis* methanol extracts were performed to determine the main chemical composition of *Spirulina* sample being studied. The major peaks obtained from the gas chromatogram determined the highest chemical constituent percentage of the cyanobacterium methanol extract. The most abundant chemical component was tetradecanoic acid followed by octadecanoic acid, heptadecanoic acid, pentadecanoic acid, hexadecanoic

acid, nanoic acid, nonadecanoic acid sulfurous acid respectively having antioxidant and antimicrobial activity (Lee *et al.*, 2007; Mishra and Sree, 2007).

DISCUSSION

The results obtained from the present study; the antimicrobial activity of *Spirulina platensis* extracts using different solvents indicated that the diameter of the inhibition zone depends mainly on the type of solvent being used and the method of extraction (Gonzalez *et al.*, 2001), the chemical composition of the cyanobacteria, and probably on the seasonal collection of *Spirulina platensis*, being freshly cultivated, as well as the type of the microorganisms being tested. (Kumar *et al.*, 2011) reported that the use of organic solvents in the algal extracts provides more consistent antimicrobial activity and does not negatively affect their bioactivity against antibacterial and antifungal species.

Many investigations suggested that the methanolic extract of the cyanobacteria revealed higher antimicrobial activity in comparison to other organic solvents. This could be related to the presence of antimicrobially active lipids and other active fatty acid compounds at high concentration in both *S. platensis* crude extracts and methanolic extract. Many studies (Kumar *et al.*, 2011; Demule *et al.*, 1996, Kumar *et al.*, 2006, Lee *et al.*, 2007; Lampe *et al.*, 1998; Xue *et al.*, 2002) indicated that methanolic extract of *S. platenis* had the most potent antimicrobial activity and showed more antibacterial effect on gram positive bacteria than on gram negative bacteria. This is mainly due to the disruption of the cellular membrane of bacteria, fungi and even yeasts (Lampe *et al.*, 1998). Lipids can penetrate the extensive peptidoglycan layer in the cell wall without any noticeable changes, reaching the bacterial membrane and causing its disintegration (Ramadan *et al.*, 2008). The difference in the susceptibility of gram negative bacteria to be killed by lipids is mainly due to the difference in their outer cell wall structure. Gram negative bacteria have a hydrophilic surface due to the side chains of lipo-polysaccharides preventing as such the hydrophobic molecules like lipids enter the bilayer (Bergsson, 2005).

In this preliminary work, *Spirulina platensis* methanol extract gave the highest

antimicrobial activity against some antibiotic resistant human pathogenic bacteria particularly gram positive bacteria among all other solvents being used in agreement with other studies indicating the highest microbial activity of the methanol extract (Ozdemir *et al.*, 2004, Lampe *et al.*, 1998, Bergsson, 2005), moreover, the GC-MS analysis for *S. platensis* methanolic extract showed that the major constituents of the cyanobacterium being studied were active fatty acids to which the antibacterial activity of *Spirulina platensis* could be related to (Lee *et al.*, 2007, Mishra & Sree, 2007), giving by this new promising horizons in the production of new, naturally bioactive agents, with an improved knowledge of the chemical composition of the algal extract secondary bioactive compounds and consequently more work to be done on the extraction of these different bioactive metabolites present at high concentrations in *Spirulina platensis* particularly bioactive fatty acids, and to study their individual microbial effect on gram negative and gram positive bacteria on the DNA basis.

ACKNOWLEDGMENTS

This research project was supported by a grant from the Research Center of the Center for Female Scientific and Medical Colleges Deanship of Scientific Research, King Saud University.

REFERENCES

1. Abedin RMA, Taha HM . Antibacterial and antifungal activity of cyanobacteria and green algae evaluation of medium components by Placket-Burman design for antimicrobial activity of *Spirulina platensis*. *Global J. Biotechnol. Biochem.* 2008; **3**: 22-31.
2. Adams Rp. Identification of essential oil components by gas chromatography/mass spectrometry. Allured Publishing: 1995, Carol Stream, IL.
3. Al Wathnani H., Ismet Ara, R.R. Tahmaz, T.H. Al Dayel and M.A. Bakir . Bioactivity of natural compounds isolated from cyanobacteria and green algae against human pathogenic bacteria and yeast. *Journal of Medicinal Plants.* 2012; **6**(18): 3425-3433.
4. Bergsson G., Antimicrobial polypeptides and lipids as a part of innate defence mechanism of fish and human fetus, M.Sc. dissertation,

- Karoliska Institute. Stockholm, 2005; 77.
5. Borowitzka MA .Microalgae as source of pharmaceuticals and other biologically active compounds. *J. Appl. Phycol.*, 1995; **7**: 3-15.
6. Demule MCZ, Decaire GZ, Decano MS . Bioactive substances from *Spirulina platensis*. *Int. J. Exp. Biol.*,1996; **58**: 93-96.
7. Ghasemi Y, Moradian A, Mohagheghzadeh A, Shokravi S, Morowvat MH . Antifungal and antibacterial activity of the microalgae collected from paddy fields of Iran: characterization of antimicrobial activity of *Chroococcus disperses*. *J. Biol. Sci.*,2007; **7**: 904-910.
8. Ghasemi Y, Tabatabaei Yazdi M, Shkravi S, Soltani N, Zarrini G .Antifungal and antibacterial activity of paddy-fields cyanobacteria from the north of Iran. *J.Sci. Islamic Repub. Iran*, 2003; **14**: 203-209.
9. Gonzalez Del Val. Platas G, Basilio A. Screening of antimicrobial activities in red, green and brown macroalgae from Gran Canaria (Canary Islands, Spain). *Int. Microbiol.*,2001; **4**: 35-40.
10. Ismet A (2003). Biological, molecular and chemical characterization of a selected antagonistic *Micromonospra sp.* isolated from mangrove rhizosphere. PhD. Thesis. Institute of Biological Sciences. Faculty of Sciences, University of Malaysia, Malaysia.
11. Isnansetyo A, Cui L, Hiramatsu K, Kamel Y . Antibacterial activity of 2,4- diacetylphloroglucinol produced by *Pseudomonas sp.* AMSN isolated from a marine algae, against vancomycin-resistant *Staphylococcus aureus*. *Int. J. Antimicrob. Agents*, 2003; **22**:545-547.
12. Jaki B, Orjala J, Sticher O . A novel extracellular diterpenoid with antibacterial activity from the cyanobacterium *Nostoc cmmume*, *J. Nat. Prod.*, 1999; **62**: 502-503.
13. John DM, Whitton BA, Brook AJ. The fresh water algal flora of the British isles, an identification guide to freshwater and terrestrial algae. Cambridge University Press, Cambridge, 2003; pp. 117-122.
14. Kulik MM . The potential for using cyanobacteria (blue green algae) and algae in the biological control of plant pathogenic bacteria and fungi. *Eur. J. Plant Pathol.* 1995; **101**(6): 585-599.
15. Kumar p., Angadi S, Vidyasagar G. Antimicrobial activity of blue green and green algae. *Ind.J.Pharm. Sci.*, 2006; **8**: 647-648.
16. Kumar Vinay, A.K. Bhatnagar and J.N Srivastava . Antibacterial activity of crude extracts of *Spirulina platensis* and its structural elucidation of bioactive compound. *Journal of Medicinal plants Research*, 2011; **5**(32): 7043-7048.
17. Kumar Vinay, A.K. Bhatnagar and J.N. Srivastava. Sensitization of solvent extract of *Spirulina platensis* (Geitler) against some Dermatophytes and related fungi. International conference on environment. *energy and biotechnology IPCBEE*, 2012; **33**.
18. Kundim BA, Itou Y, Skagami Y, Fudou R, Lizuka T, Yamnaka S, Ojika M. New hliangicin isomers, potent antifungal metabolites produced by a marine myxobacterium. *J. Antibiot.*, 2003; **56**: 630-638.
19. Lampe MF, Ballweber LM, Isaacs CE, Patton DL, Stamm WE . Killing of *Chlamydia trachomatis* by novel antimicrobial lipids adapted from compounds in human breast milk. *Antimicro. Agents Chemo.*, 1998; **45**: 1239-1244.
20. Lee YS, Kang MH, Cho YS, Jeong CS . Effects of constituents of *amomum xanthioides* on gastritis in rats and on growth of gastric cancer cells. *Arch. Pharm. Res.*, 2007; **30**: 436-443.
21. Mishra PM, Sree A. Antibacterial activity and GCMS analysis of the extract of leaves of *Finlaysonia obovata* (A Mangrove Plant). *Asi. J. PL. Sci.*, 2007; **6**: 168-172.
22. Moore RE, Cheuk C, Yang XG, Patterson GML. Hapalindoles antibacterial and antimycotic alkaloids from the cyanophyte *Hapalosiphon fontinalis*. *J. Org. Chem.*,1998; **52**:1036-1043.
23. Ozdemir G, Karabay N., Dolay M., Pazarbasi B. Antibacterial activity of volatile extracts of *Spirulina platensis*. *Phytother. Res.* 2004; **18**: 754-757.
24. Patterson G.M.L., Larsen L.K., Moore R.E. Bioactive natural products from blue-green algae. *Journal of Applied Phycology*, 1994; **6**: 151-157.
25. Radmer R.J. and Parker B.C. Commercial applications of algae opportunities and constraints. *Journal of Applied Phycology*, 1994; **6**: 93-98.
26. Ramadan M.F., Asker M.M.S., Ibrahim Z.K. Functional bioactive compounds and biological activities of *Spirulina platensis* lipids. *Czech J. Food. Sci.*,2008; **26**: 211-222.
27. Soltani N, Khavari-Nejad RA, Tabatabaei Yazdi M, Shokravi SH, Fernandez- Valiente E. Screening of soil cyanobacteria for antibacterial and antifungal activity. *Pharm. Biol.*,2005; **43**(5): 455-459.
28. Xue C, Hu Y, Saito H, Zhang Z, Li Z, Cai Y, Ou C, Lin H, Imbs A.B. Molecular species composition of glycolipids from *Spirulina platensis*. *Food chemistry*,2002; **77**: 9-13.
29. Zulpa G, Zaccaro MC, Boccazzi F, Parada JL, Storni M. Bioactivity of intra and extracellular substances from cyanobacteria and lactic acid bacteria on "wood blue stain" fungi. *Boil. Control*.2003; **27**: 345-348.