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

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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 antibacterial agents. In the attempt of extracting and producing newly active antimicrobial substances substituting the exisisting 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 xylosis obtained, Bacillus subtilis ATCC 6633, MRSA ATCC 12498 , and gram negative bacteria Escherishia coli ATCC 25922 , Escherichia coli ATCC 25966 Pseudomonas aeruginosa ATCC 27853, Enterococcus feacalis ATCC 29212, Klebsiella pnumoniae 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 octadececanoic acid assumed to provide the antimicrobial activity.

Key words: *Sprirulina 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 the pathogenic microorganisms, among which are the antibiotic resistant bacteria causing threats to humans .

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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 cynobacterium *Spirulina platensis* was analyzed and indentified 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 cynobacteria in addtition 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 xylosis* obtained from the Microbiological Resources Center(Cairo, Egypt), *Bacillus subtilis* ATCC 6633, MRSA ATCC 12498, and gram negative bacteria *Escherishia coli* ATCC 25922, *E. coli* ATCC 25966 *P. aeruginosa* ATCC 27853, *Enterococcus feacalis* ATCC 29212, *Klebsiella pnumoniae* 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, Ryiadh, 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 on 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 xylosis* (fig. 1) followed by *Bacillus subtilis* and *Staphylococcus aureus* and showed no effect against *E.coli, Pseudomonas aeruginosa, Enterococcus feacalis, Salmonella sp., Klebsiella pneumoniae and on Candida albicans and fusarium sp.* Acetone, ethanol, chloroform and

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

No	Pathogenic	*Spirulina platensis* Organic extractant					
	Bacterial Isolates	Acetone	Chloroform	Ethanol	methanol	Petroleum ether	Antibiotic disc µg
1	*K. pneumonia*	-	+	-	-	-	MEM 10
2	*E. coli (25966)*	-	+	-	-	-	MEM 10
3	*E. coli (25922)*	-	-	-	-	-	MXF 5
4	*P. aeruginosa*	-	-	-	-	-	MEM 10
5	*Sal*	-	-	-	-	-	AMC 30
6	*E. feacalis*	-	-	-	++	-	AMC 30
7	*MRSA*	+	-	++	+++	-	MEM 10 AMC 30 MXF 5
8	*B. subtilis*	+ +	-	+ +	++	-	MEM 10
9	*Staph A*	-	-	-	++	+	MEM 10
10	*Staph xylosus*	++	++	++	+++	++	MEM 10
	Yeast						
1	*C. albicans*	+	+	-	-	-	MEM 10
	Fungi						
1	*Fusarium*	-	-	-	-	-	-

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

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

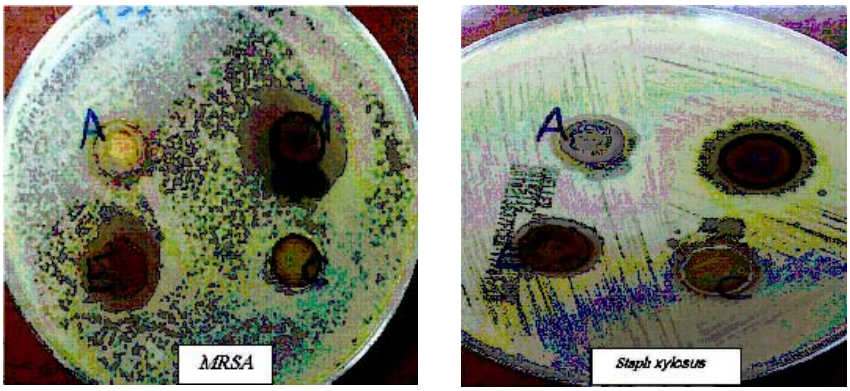
petroleum ether extracts showed minimal or no effect on the tested organisms. Acetone, ethanol and petroleum ether showed moderate activity on S. xylosis, B. subtilis and MRSA respectively; whereas chloroform had no activity against all the tested microorganisms. All positive controls with the antibiotic 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.

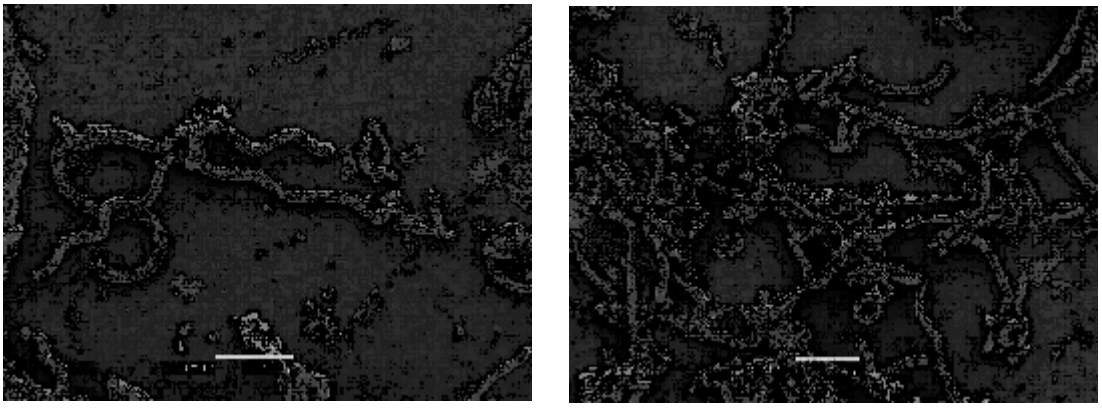
GC-MS Analysis

GC-MS anaylsis 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 cyanobacrerim methanol extract. The most abundant chemical component was tetradecanoic acid followed by octadececanoic acid, heptacoanoic acid, pentdecanoic acid, hexdecanoic acid, nanoic acid ,nonadecanoic acid sulfurous acid respectively having antioxidant and antimicrobial activity (Lee *et al*.,2007; Mishra and Sree, 2007).



A: acetone; M: methanol; E: ethanol; C: chloroform.

Fig. 1(A). Effect of *Spirulina platensis* various organic extracts on *Staphylococcus xylosus* and MRSA respectively; larger inhibition zone is observed with methanol solvent extract



(A) (B)

Fig. 2. Scanning electron microscopic revealing the morphological identification and examination of *S. platensis*

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.

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