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Production and characterization of antimicrobial active substance from *Spirulina platensis*

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ABSTRACT

Background and Objectives: The present work was carried out to investigate the ability of *Spirulina platensis* to produce antimicrobial substance against bacteria and fungi.

Materials and Methods: The cells of the cyanobacterium were subjected to different extractions and the purified antagonistic compound proved to be effective against broad spectrum of bacteria and fungi. The antagonistic compound was purified using thin layer chromatography.

Results: The results indicated that the IR spectrum showed bands at 1269 cm^{-1}, 1414 cm^{-1} (C-O-C), 1643 cm^{-1} (CO of amide),1563 cm^{-1} (C = C) and broad band 3441 cm^{-1} (of OH and NH)., ^{1}HNMR showed δ 0.8 (-CH3), δ 1.2 (-CH_2), δ 4.2(-OH), δ 7.2(-NH), δ 7.4 and δ 7.7 (aromatic CH)., Mass spectrum showed molecular ion beak at m/z = 341 (abundance (0.03%). Also, the elemental analysis gave molecular formula,$C_{15}H_{18}NO_8$.

Conclusion: The purified antimicrobial compound produced by *S. platensis* was more active against Gram positive, Gram negative bacteria and unicellular fungi, *C. albicans*. The highest biological activity was recorded against *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis* and *Aspergillus niger*. The results of this investigation proved that cyanobacteria could be a good source for production of antimicrobial agents which could be effective when compared with contemporary antimicrobial compounds.

Keywords: *Spirulina platensis*, antimicrobial activities, MIC, MCC

INTRODUCTION

Most species of cyanobacteria are known to produce intracellular and extracellular metabolites with diverse biological activities such as antialgal, antibacterial, antifungal and antiviral activity (1- 3). The genus *Spirulina* has an international demand in healthy foods, feed, therapeutics and diagnostics industries (4). Pharmacological activities of *Spirulina* sp. have been previously reviewed by several publishers, where it exhibits antiviral (5), antibacterial (6), antiplatelet (7), anticardiotoxic (8), hypocholesterolemic (9), antinephratoxic (10) and anti-hepatoxic effect (11). It also has a variety of health benefits and therapeutic properties (12) and may play a role in reducing the cadmium toxicity (13).

Several solvents such as methanol, dichloromethane, petroleum ether, ethyl acetate have been used to extract the bioactive materials of *Spirulina platensis* that has an antimicrobial activity against bacteria *in vitro* (6). This work aimed to clarify the production, extraction, purification and identification of the active metabolic product(s) produced by *Spirulina platensis* isolated from Egypt and to test their antimicrobial activities.

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MATERIALS AND METHODS

Organism. *Spirulina platensis* was isolated from freshwater channel of River Nile. The cyanobacterium was identified according to Prescott (14). The axenic culture was obtained using the method recommended by Bolch and Blackburn (15). The tested microorganisms were obtained from Hydrobiology Lab.; Microbiology, National institute of Oceanography and Fisheries, Cairo, Egypt.

Optimization of the medium constituents affecting *S. platensis* growth. The nitrogen source ($NaNO_3$; 2.5 gl^{-1}) in growth medium Aiba and Ogawa (16) replaced by Glycene (4.42 gl^{-1}), urea (1.824 gl^{-1}), ammonium sulphate (4.12 gl^{-1}) and ammonium chloride (3.146 gl^{-1}l), while phosphate (K_2HPO_4; 0.5 gl^{-1}) in the standard medium was replaced by Na_2HPO_4 (0.734 gl^{-1}), $Na_3PO_4.12H_2O$ (1.96 gl^{-1}), KH_2PO_4 (0.9 gl^{-1}) and NaH_2PO_4 (0.62 gl^{-1}). Flasks were incubated at the same conditions then dry weight of organism was determined.

Growth conditions. *Spirulina platensis* was grown in modified Aiba and Ogawa, medium (16). Each flask was aerated using air pump (150 bubbles /min.) through a sterilized plastic tube (17), this step also provided agitation. Flasks were incubated at 30 ± 2˚C and light intensity of 50 µ $Em^{-2}s^{-1}$ for 9 days at pH 9.0.

Antimicrobial activities of *S. platensis.* After 9 days growth, antimicrobial activity was tested using agar-well diffusion technique (18, 19). Gram +ve bacteria; *Bacillus subtilis* NCTC3610, *Staphylococcus aureus* ATCC13565, Gram -ve bacteria; *Pseudomonas aeruginosa* ATCC6939, *Escherichia coli* NCTC9132, yeast; *Candida albicans* ATCC10231, and fungi; *Aspergillus flavous* and *Aspergillus niger* were used as test organisms.

Four ml 0.15 M NaCl of fresh bacterial suspension (12 h. old) of each isolate with 10^6 CFU ml^{-1} for bacteria at OD 620 nm, and 10^4 cells ml^{-1} for yeast based on MacFerland's scale (20), were mixed well with 100 ml melted warm (≈45˚C) nutrient agar (NA). (1-140, Scharlau Chemie, S.A). The fungal suspension was prepared by six mm disk of the fungal growth (7 days old culture). in 0.15 M NaCl, 4 ml was mixed with 100ml potato dextrose agar (PDA) (1-483, Scharlau Chemie, S.A.) After the agar has set, 9 mm wells were cut in the agar with sterilized cork borer and filled with 100 µl of algal extract. Plates were kept for 6 hours in a refrigerator to allow the antimicrobial substance to diffuse through the inoculated medium, after 48-72 h incubation at 30 ± 2˚C, the clearance zones were recorded in (mm) of three replicates.

Production and extraction of the antimicrobial material. Twenty liters flask with 10L of sterilized medium inoculated with 3% of nine days old culture of *S. platensis*, and aerated using air pump (150 bubbles min^{-1}). through a sterilized plastic tube (Anaga & Abu, 1977) for 9 days at 30 ±2˚C and light intensity of 50 µ $Em^{-2}s^{-1}$, the biomass harvested using 20 µ nylon mesh and extracted using diethyl ether (1,1 v/v) shake overnight at room temperature (27-30˚C) at 100 rpm, collect the organic layer and recovery the solvent under reduced pressure using a rotate evaporator at 45˚C (21).

The residues (Crude extract, with a known weight) were re-dissolved in 95% dimethyl sulphoxide (DMSO), 20 µl of the extract was loaded to sterilized 6 mm discs of Whatmann filter paper No. 1 and allowed to dry at room temperature in laminar air flow bench (22).

Purification and identification of the antimicrobial material produced by *S. platensis*. Thin layer chromatography (TLC) plates were loaded by the crude extract and developed using chloroform, methanol 99:1 v/v solvent system. After migration (2 hours), the plate was removed and dried in a steam of warm air and detected by UV lamp (256 nm and 360 nm). Each spot was removed and re-eluted in diethyl ether, free from silica gel by washing several times by the same solvent on filter paper, 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 (1.0 × 25 cm). Five ml of the crude extract was loaded on the silica gel column and eluted using diethyl ether, chloroform, acetone and ethanol 95%. After 8 hours, eleven fractions were collected. Each fraction was free from solvent, re-dissolved in appropriate solvent and screened for its antimicrobial activity by disc diffusion method (22).Three trials for each were tested.

Structure determination of the antimicrobially active substance produced by *S. platensis*. In addition to classical color reactions that detect the

Table 1. MIC and MCC of purified antimicrobial substance produced by *Spirulina platensis* related to strptomycine and polymycin.

Microorganism	MIC µg ml^{-1}	MCC µg ml^{-1}	Streptomycin µg ml^{-1}	Polymyxin (IU)
B. subtilis NCTC 3610	60.0	80.0	2.0	ND
S. auerus ATCC 13565	65.0	90.0	2.5	ND
E. coli NCTC 9132	80.0	110.0	3.0	ND
P. aeruginosa ATCC 10145	85.0	120.0	3.50	ND
C. albicans ATCC 10231	30.0	45.0	1.50	20*
Aspergillus flavus	> 120	>200	ND	R
A. niger	> 120	>200	ND	R

ND, Not detected, R, Resistance, MIC, Minimum inhibition concentration.
MCC, Minimum cidal concentration.*300IU/300 µl

presence of certain groups in the molecule as the aromatic nature, reducing group, sugar moiety, phenolic group, diketon bond, protein peptide bond, guandine and indocile groups, (23) the spectroscopic analysis UV (200 –400 nm) spectrum, infra-red (500-4000nm) spectrum, Nuclear magnetic resonance (^{1}HNMR), C, N, H ratio and Mass spectrum were done at Cairo University Micro Analytical Center, Giza, Egypt.

Minimal inhibitory concentration (MICs) and Minimal cidal concentration (MCCs). MIC and MCC of the purified bioactive compound using bacteria, yeast and filamentous fungi were determined by using a well microplate technique (24). A concentration cover the range between 0.0 and 1000 µg ml^{-1} of the product was made. MIC level was evaluated optically, which exhibited the lowest growth at optical density λ620nm compared to the control tube (25). MCC the concentration that showed no growth (99.9 % inhibition). of each inoculum using viable plate count method on agar plates for bacteria (26), and Zapex Dox (27) the effect was observed as no growth appear on the plates. The assay results were compared with those obtained when challenging the bacterial isolates with commercial antibiotic as streptomycin (10 µg) and polymyxin (300 IU).

RESULTS

The highest biomass concentration of *Spirulina* was achieved in the 9th day of growth and accounted for 111.70±1.13 mg dry wt/100 ml (Fig. 1). Also sodium nitrate and dipotassium phosphate (standard nitrogen and phosphorus sources) gave the highest value of biomass 132.267 ± 0.902 mg dry wt./100 ml, while a significant decrease in biomass production was observed when urea was used (95.6 ± 1.1012 mg/ 100 ml) and no significant differences in biomass was recorded with Na_2HPO_4 or Na_3PO_4 (110.133 ± 0.4163 and 109.667 ± 0.7571 mg dry wt./100 ml, respectively). However the lowest biomass concentration was observed when K_2HPO_4 was replaced by NaH_2PO_4 (96.067 ± 0.5033 mg dry wt/100 ml).

It was found that the highest biological activity was recorded against *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis* and *Aspergillus niger*. The results revealed that diethyl ether and ethyl acetate exhibited antimicrobial activity against Gram +ve and Gram -ve bacteria, while petroleum ether exhibited antimicrobial activity against Gram -ve only and n-hexane had no activity against all test organisms. On the other hand, among the water-miscible solvents (acetone, methanol and ethanol) ethanol was the most effective solvent showed wide spectrum of antimicrobial activity against Gram +ve, Gram -ve bacteria and fungi as shown in Fig.2 and 3.

The test microorganisms differed in relation to their susceptibility to purified antibiotic produced by *S. platensis*. The Gram positive bacterium *Bacillus subtilis* was the most susceptibile bacterial species, while the Gram negative bacterium *P. aeruginosa* was the least susceptible, the purified antibiotic produced by *S. platensis* was observed to be more active against Gram positive, Gram negative bacteria and unicellular fungi, *C. albicans*. On the other hand most resistance species were the multicellular fungi as recorded data *C. albicans* was the most susceptible test organism with minimal inhibition concentration of 30.0µg ml^{-1}, while the Gram negative bacterium *P. aeruginosa* was the least susceptible one (MIC, 85µg ml^{-1}), while the most resistance isolates were the multicellular fungi *A. flavus* and *A. niger* (Table 1).

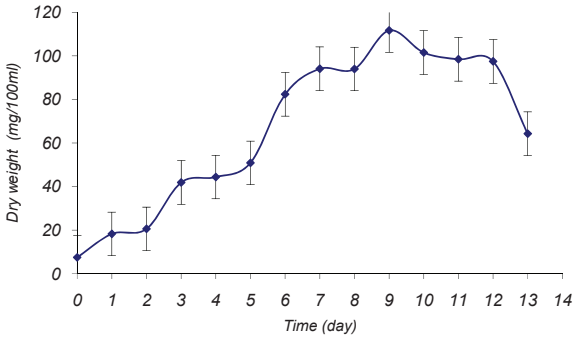

Fig. 1. Growth curve of *S. platensis* measured as mg dry wt./100 ml.

Characterization of the antimicrobial product produced by *S. platensis.* The purified compound was found to be yellowish green with no characteristic odor, soluble in methanol, diethyl ether, chloroform and dimethyl sulfoxide, but sparingly soluble in water and acetone with melting point 37-40°C (data not shown).

The spectroscopic analysis of the purified antimicrobial product indicated that the IR spectrum showed bands at 1269 cm^{-1}, 1414 cm^{-1} (C-O-C), 1643 cm^{-1} (CO of amide),1563 cm^{-1} (C = C) and broad band 3441 cm^{-1} (of OH and NH), (Fig. 4). 1HNMR showed δ 0.8 ($-CH_3$)., δ 1.2 ($-CH_2$)., δ 4.2(-OH)., δ 7.2(-NH)., δ 7.4 and δ 7.7 (aromatic CH)., (Fig. 5). Mass spectrum showed molecular ion beak at m/z = 341(abundance (0.03%) (Fig. 6). Also, the elemental analysis gave molecular formula, $C_{15}H_{18}NO_8$.

DISCUSSION

Spirulina sp. represents one of the most important commercial microalga for the production of biomass

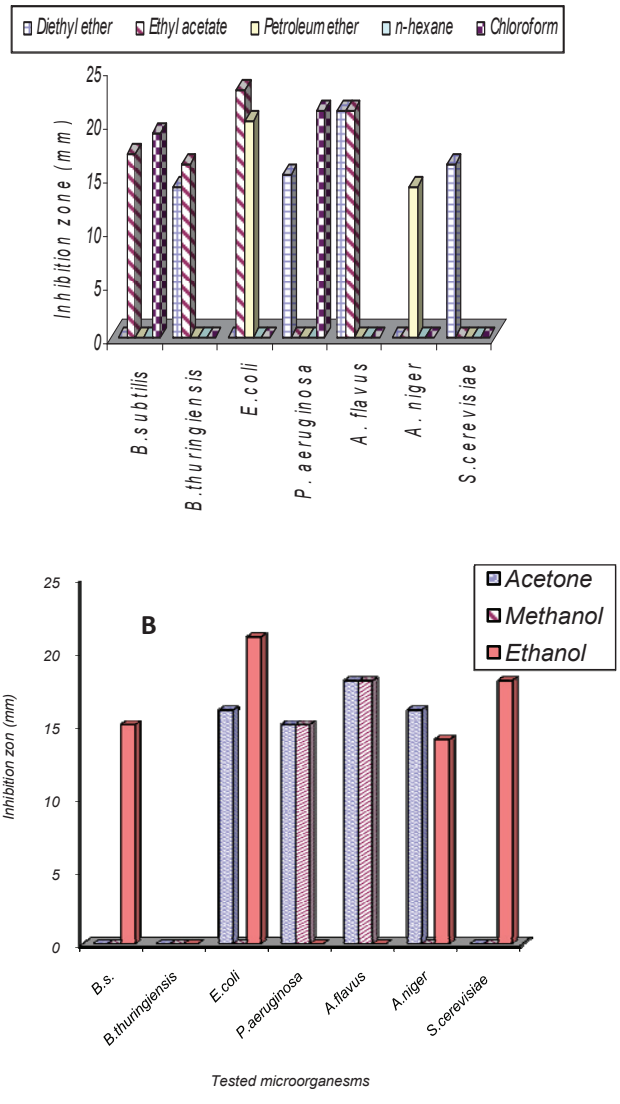

Fig. 2. Antimicrobial activities of different solvents extracts of *Spirulina platensis* (A). water immiscible solvents (B). water miscible solvents

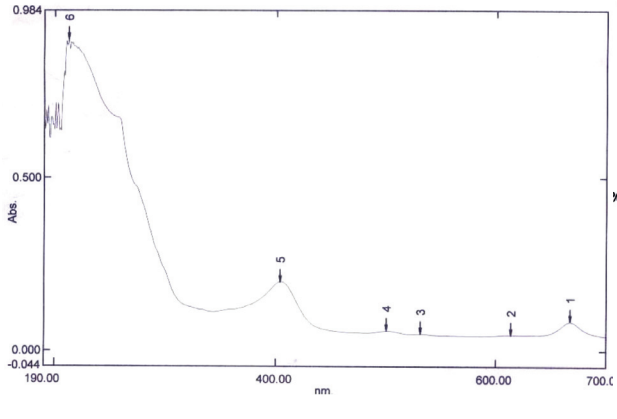

Fig. 3. UV Spectrum of the antimicribially active compound produced by *Spirulina platensis.*

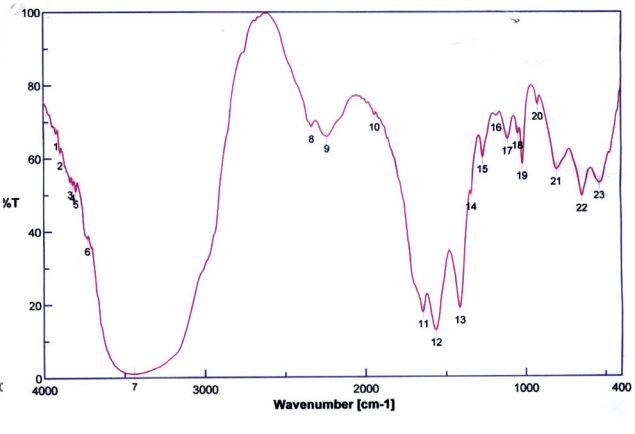

Fig. 4. IR Spectrum of the antimicribially active compound produced by *Spirulina platensis*.

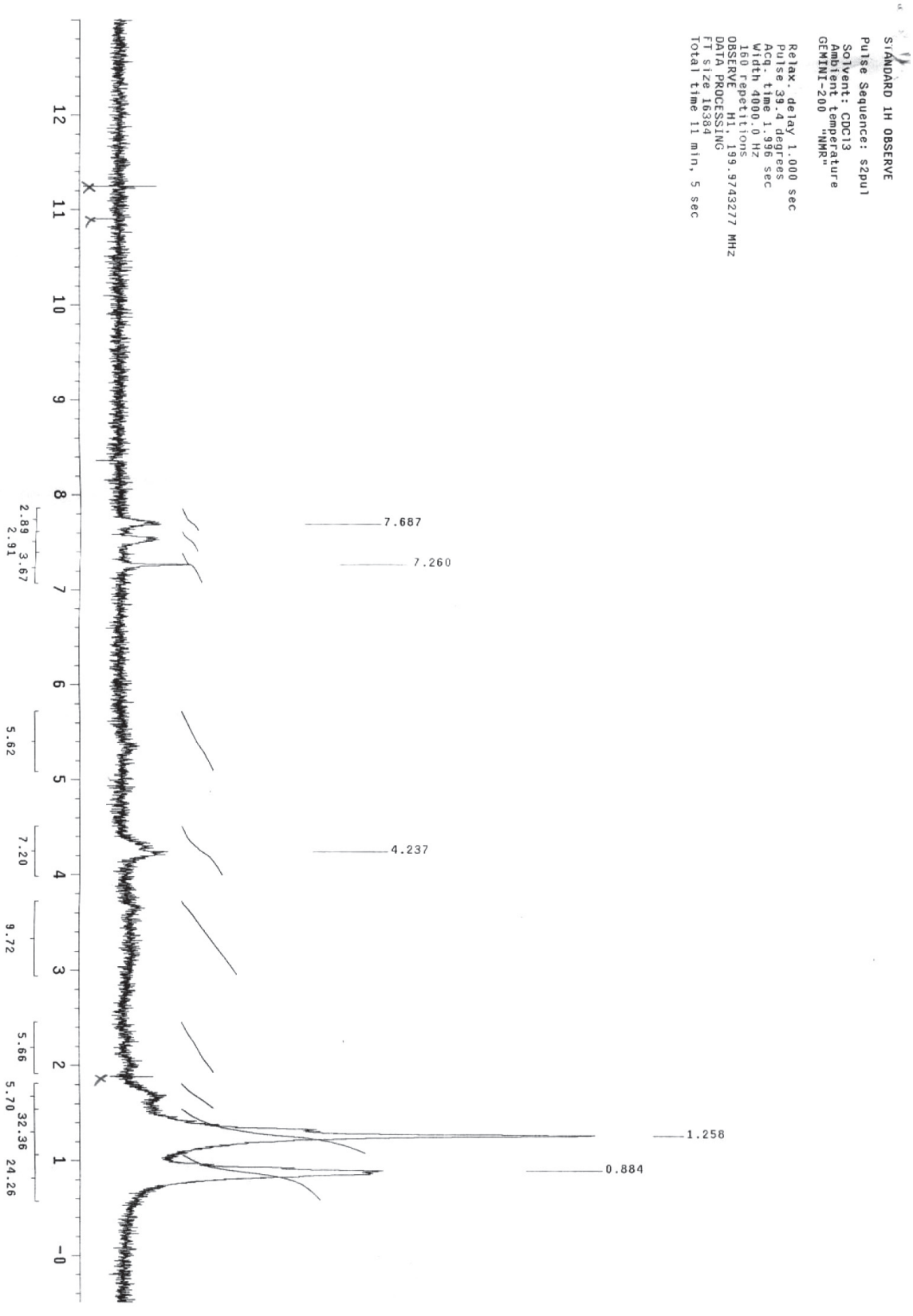

Fig. 5. [1]HNMR Spectrum of the antimicribially active compound produced by *Spirulina platensis.*

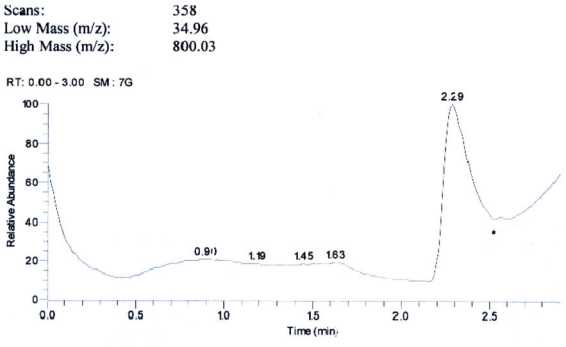

Fig. 6. Mass Spectrum of the antimicribially active compound produced by *Spirulina platensis.*

as healthy food and animal feed (28). A large number of microalgal extracts and extracellular products have been found to have antibacterial activity. The algal extracts showed antibacterial and antifungal activities, both culture filtrate and whole culture (cells and exometabolites) have been proved to have wide spectrum antimicrobial activity, where *Bacillus sublilis* and *Candida albicans* were the most sensitive species. The antimicrobial activity of microalgae could be explained by the presence of cyclic peptides, alkaloids and lipopolysaccharides (29). This activity may be due to the toxins produced by its cells like a number of blue green algae that produce toxins which have potential pharmaceutical applications. The present results go in harmony with those obtained by Volk and Furkert (30) who found that some microalgae had high biological activity against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida tropicalis*.

Organic solvents always provides a higher efficiency in extracting compounds for antimicrobial activities compared to water-based methods (31). The results showed that ethane and ethyl acetate and diethyl ether were the best solvents (water miscible and immiscible solvents) for extraction of the active substances. Many of the tested solvents were effective against all microorganisms; however, ethanol extract indicated an antimicrobial activity against several microorganisms. This could be related to the presence of bioactive metabolites which are soluble in ethanol but not diethyl ether (32). The present results are in agreement with Abedin & Taha (33) who reported that acetone and diethyl ether extracts of *Spirulina platensis* gave the highest antimicrobial activity against *Bacillus subtilis* and *Pseudomonas aeruginosa*. Also, Santoyo *et al*., (34) reported that hexane and petroleum ether extracts were slightly active than ethanolic extracts.

The antimicrobial activities of *S. platensis* could be attributed to different compounds belonging to a diverse range of chemical classes (6). The antimicrobial activity found in *S. platensis* extracts could be due to contain γ-linolenic acid (35), active fatty acid (36), synergetic effect of lauric and palmitoleric acid (37). The test microorganisms differ significantly in relation to their susceptibility to *S. platensis* antimicrobial substances, *Candida albicans* was the most sensitive microorganism (MIC µ50 g ml^{-1}) while Gram positive bacteria were more sensitive than the Gram negative bacteria. This may be attributed to the fact that cell wall in Gram positive bacteria consists of a single layer, whereas Gram negative bacterial cell wall is multilayered structure bounded by an outer cell membrane (38) and or due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism (39). On the other hand, some bacterial species did not respond to extracts of *S. platensis* where as the purified fractions showed broad-spectrum activity against different test organisms, this might be due to masking of antibacterial activity by the presence of some inhibitory compounds in the extract as observed by Sastry & Rao (40).

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