ORIGINAL ARTICLE

Anti-bacterial and Anti-tumoral Activities of *Spirulina Platensis* Extracellular Extract

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ABSTRACT

Introduction: Antibiotic resistance of pathogenic bacteria has become common due to the random use, as normally bacteria develop resistance mechanisms. Hence, alternative therapies are strongly needed such as algae extracts. Spirulina has recently acquired attention as potential pharmaceuticals because they lack toxicity and have antibacterial, antibiofilm and antitumor activities. **Methods:** About 25 isolates obtained from wound swabs [*Staphylococcus aureus* 4 (16%), *Pseudomonas aeruginosa* 7 (28%), *Pseudomonas stutzeri* 3 (12%), *Burkholderia cepatia* 4 (16%), *Acinetobacter spp* 5 (20%), and *Escherichia coli* 2 (8%)]. *Spirulina sp.* collected from ponds and the extract was prepared by soxhlet apparatus using 250 ml of methanol for 4 hours at 60˚C. **Results:**: Antibacterial activity of *Spirulina* extract (25µg/ml) showed inhibition zones (10, 8, 8 and 5) mm towards *Staphylococcus aureus*, *Pseudomonas stutzeri, Burkholderia cepatia* and *Escherichia coli,* respectively. While all tested bacteria inhibited at concentrations 50 and 100µg/ml, and *Burkholderia cepatia* has the greatest inhibition (15mm). Gas chromatography–mass spectrometry (GC-MS) analysis showed seven active compounds, and they were Hexadecanoic acid with peak area 93.92 %, Methanesulfonylacetic acid 3.93 %, Pentadecanoic acid 0.89 %, Octadecadienoic acid 0.71 %, Tridecanoic acid 0.52%, 2-Hydroxypropanoic acid 0.02%, and 2-pentanol, 3-chloro, 2-methyl 0.01%. Cytotoxic influence of methanol extract was evaluated in two adenocarcinoma cell lines derived from breast cancer tissue, MCF7 and MDA-MB231, and both cell lines showed significant reduction of survival percentage compared to control. **Conclusion:** The study aimed to find out the antibacterial and antitumor effects of the *Spirulin*a methanol extracellular extract, and determine the active compounds in it.

Keywords: *Spirulina* extract, GC-MS analysis, Antibacterial, Antitumor activity.

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INTRODUCTION

The developed resistance of infectious microbes was the major problem, and bacteria which cause hospital-acquired infections were resistant to at least one common germicide. Therefore, alternative antimicrobic approaches have been anticipated (1). So, to contending the risk of bacterial infection and preventing wound sepsis, nobel antimicrobial agents were used, and it has the most prevalent of bactericidal effect (2). Some kinds of bacteria resist antibiotics, especially if they are usually exposed to the same antibiotic via developing resistance mechanism(s) which prevents being susceptible to antibiotics and unable to treat burn patients with wound infection (3). The most common pathogens are *Pseudomonas aeruginosa, Klebsiella sp.*, *Acinetobacter sp.* and *Staphylococcus aureus*, because they can produce number of virulence factors important in pathogenesis of invasion infection, and have multi druge resistance (4).

Recently, algae extracts have many applications in medicine, pharmacology, and environment. The medical applications of algae are extremely active, with a variety of commercially available products being used (5). Blue-green algae, like: *Nostoc, Spirulina,* and *Aphanizomenon* species have been tolerate environmental conditions, and used in food industry extensively (6). *Spirulina* is a blue, green algae, filamentous, expanded acceptance in many industries (including food) and considrable as important nutrient supplement to aquatic-culture foods. It can be collected and deal with easily and has great amounts of nutrients (7).

Spirulina platensis and its extracts have shown biological properties, such as preventing cancers, decreasing blood cholesterol levels, stimulating the immunological system, reducing toxic metals, toxicity of pharmaceuticals and the detrimental effects of radiation (8). These properties have been attributed to different compounds such as phenolics, carotenoids, organic acids, sulphated polysaccharides spirulan and polyunsaturated fatty acids. *Spirulina* likewise boost the nutritional abilities, consequently, extracts will be beneficial in therapeutic of hypertension, diabetes, and heart disease, etc. (4). The aim of this study is to investigate the antibacterial and antitumor effects of *Spirulina s*p. methanol extract on pathogenic bacterial species isolated from wound patients and cell lines derived from breast cancer tissue respectively, and to detecte the active compounds librated from the extract by using GC-MS analysis.

MATERIALS AND METHODS

Pathogenic bacteria

Wound swabs were obtained from patients attending Al- Kindey hospital in (Baghdad/ Iraq) by sterile swabs, using Amies transport medium (Himedia-India) and then, transported to the lab.

Isolation of Bacteria

Samples of bacteria isolated on MacConkey and Blood agar (Himedia-India), then other biochemical tested on Mannitol salt and Nutrient agar (Himedia-India). The plates incubated for 48hr at 37°C (9) .

Characterizations of isolates

Bacteria characterized by cultural, microscopical, and biochemical tests conferring to (9), then the identification was confirmed by apparatus of Vitek 2 (Biomerieux).

Isolation and identification of algae samples

Spirulina sp. was collected from the ponds located near political sciences college at Baghdad University. Algae isolation was conducted using Serial dilution method (10) . Samples were cultured using the BG-11 medium for cyanobacteria algae growth. The samples were grown in 16:8 lights: dark condition and 268 µE/m/s, 25±2Co. The samples were identified using a classical algae classification. The main morphological feature of Spirulina platensis is non-heterocystous multicellular cylindrical trichome arranged in an open helix shape with evident cross-walls (10). Spirulina platensis was collected at the 28th day by using centrifuge (for 15 min, at 4000 rpm) (11).

Methanol extraction

Conferring to (12), two grams of dried weight algae was put in specific cylinders of soxhlet. Then 250 ml of methanol was mixed with powder at 60˚C for 4 hours until the mixture becomes colorless. Rotary evaporator was used to dry the extract at 40°C, then the extract port in container at 25°C overnight. After that extract weighed and storaged (13).

Spirulina sp. extract as antibacterial agent

Agar well diffusion method was used to examine the antibacterial activity of Spirulina sp. Extract (14). Tested bacterial isolates (1-2 colonies) mixed with normal saline (2 ml) to prepare the bacterial suspension and attuned to 0.5 McFarland tube which is equal to 1.5Ч108 CFU/ml. Bacterial suspension (0.1 ml) was spread as carpet method on Muller Hinton agar plates, and wells (6 mm) have been made in the medium. About 100g of dried Spirulina extract was added to 1L of distill water as stock solution which is used to prepare working concentrations (25, 50, and 100 µg/ml). Following that, extract dilutions were dispersed in relevant wells, then incubated at 37°C for 24hr. Inhibition zones diameter was determined per each well.

Analysis of active compounds by GC-MS

Methanol extract of Spirulina sp. was analysized using Agilent GC-Mass in Ministry of Science and Technology. Temperatures of injector and detector were established at 280 °C, whereas the temperature of column established at 100 °C. Sample (5µl) injected to the column by using split (1:10) mode of the run. Identification of the compounds done by comparison with library of NIST (15).

Cell culture

Two human cell lines (MCF7 and MDA-MB231) derived originally from the mammary glands of the breast cancer tissue were utilized. MCF7 is an estrogen receptor positive cells (ER+ve), however, MDA-MB231 cells lack expression of this receptor (ER-ve) (16, 17). These cells were obtained from VACSERA Co. (Cairo, Egypt). Cells were maintained on RPMI 1640 culture medium (EuroClone, Italy) supplemented with 10% fetal bovine serum (Biowest, South America) in a humid chamber at 37˚C and 5% CO_2.

Cytotoxicity assay

The cytotoxic effects of methanol extract was evaluated in vitro by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT powder (Macklin, Shanghai, China) was dissolved in PBS to prepare the MTT solution (5mg/ml). Prior to the exposure of cells to methanol extract and to guarantee cells adherence, ten thousand cells were seeded in each well and incubated overnight in a 96 well plate. Increasing concentrations (1µg/ml, 10µg/ml, 100µg/ml and 250µg/ml) of the algal extract in addition to the control vehicle (CV) were incubated for 24 hours after added to the cells. For all treatments, 3 replicate wells were utilized. After incubation, growth media was aspirated from the plate and all wells were washed in PBS. Into each well, 20 µl of serum free media was added in addition to a similar volume of MTT solution (5mg/ml) per well before incubation for further 3 hours in dark at 37˚C. Fifty microliter of dimethyl sulfoxide (DMSO) was then

added accompanied with shaking for 10 minutes to dissolve MTT. Microplate reader (Expert Plus reader; Asys Hitech GmbH, Eugendorf, Austria) was then used to measure absorbance at 620 nm wavelength. The formula below was used to measure the percentage of viability from raw absorbance data.

$$Viability\ \% = \frac{(A\ test - A\ blank)}{(A\ control - A\ blank)} \times 100$$

where A represents absorbance. GraphPad prism software was utilized to plot viability curve and to determine the growth inhibitory concentration that decreases 50% of viability (GI_{50}) from the same curve (18).

RESULTS

Wound bacteria Isolation and characterization

About 25 of bacterial spp. isolated from wound infections and identified by Viteck system. About 21 isolates were Gram negative bacteria belong to 5 types: (*Pseudomonas aeruginosa*, *Ps.stutzeri*, *Burkholderia cepatia*, *Acinetobacter spp.*, and *E.coli*) and *Pseudomonas aeruginosa* was the highest percentage (28%). Only 4 isolates were Gram positive and belong to *Staphylococcus aureus*. All isolates showed resistance to Ampicillin, Cefazolin and Cetriaxone in 100% percentage (Table I).

Table I : Resistance to Ampicillin, Cefazolin and Cetriaxone in 100% percentage

Antibiotic	Bacterial isolates				
	P.aeroginosa	*A.baumannii*	*E.coli*	*Burkholderia cepatia*	*P.stutzae*
Ampicillin	-	-	R	-	R
Ampicillin/Sulbactam	-	R	R	-	R
Cefazolin,	R	R	R	I	R
Ceftriaxone	-	R	R	R	R
Cefepime,	R	R	R	R	S
Aztreonam	-	-	R	R	R
Ertapenem	-	-	S	-	-
Imipenem	R	R	S	I	S
Meropenem	R	R	S	S	S
Amikacin	R		S	R	S
Gentamicin	R	R	R	R	R
Tobramycin	R	R	R	R	S
Ciprofloxacin	R	R	R	I	S
Moxifloxacine	-	-	R	-	-
Tigecycline	R	S	S	-	R
Nitrofurantoin	-	-	I	-	-
Trimethoprime/ Sulfamethoxazole	-	R	R	R	-

CONTINUE

Table I : Resistance to Ampicillin, Cefazolin and Cetriaxone in 100% percentage (CONT.)

Antibiotic	Bacterial isolates				
	P.aeroginosa	*A.baumannii*	*E.coli*	*Burkholderia cepatia*	*P.stutzae*
Chloramphenicol	-	-	-	S	-
Levofloxacin	R	R	R	I	S
Piperacillin	R	-	-	R	S
Kanamysin	R	R	-	-	R
Streptomycin	R	-	-	-	R

S: susceptible; I: intermediate; R: resistant; -: Not tested

Spirulina platensis

Spirulina platensis isolated and characterized according to morphological variation and taxonomical approaches (19), and exhibited blue –green, spirals with broad (3-4.0) µm and with distant (3-5.0) µm . Dried methanol extract was used in another tests.

Antibacterial activity of Spirulina platensis extract

Inhibition zones were determined to detect the effect of *Spirulina sp.* extract on the bacterial isolates from wound patients.

Methanol extract (conc.=25µg/ml) inhibited *Staphylococcus aureus, Ps. stutzeri, Burkholderia cepatia* and *E.coli,* and inhibition zones were (10, 8, 8 and 5) mm, respectively. While methanol extract inhibited all tested bacteria at doses 50 and 100µg/ml. Interestingly, 100µg/ml concentration showed the best effect, and *Burkholderia cepatia* exhibited the highest inhibition (15 mm) among remaining doses and bacteria respectively (Table II).

Table II: Antibacterial effect of *Spirulina* extract against wound isolates

Bacterial isolates	Inhibition zones (mm) at conc.		
	100 µg/ml	50 µg/ml	25 µg/ml
Staphylococcus aureus	13	11	10
Pseudomonas aeruginosa	12	11	-
Ps. stutzeri	14	9	8
Burchaderia cepatia	15	10	8
Acinetobacter spp.	11	9	-
E.coli	11	10	5

GC- mass analysis for *Spirulina* methanol extract

As shown in Table III, seven active compounds were detected in the methanol extract *Spirulina* sp and associated with diverse biological activities like antibacterial, anticancer effects. The antibacterial and cytotoxic activities by Spirulina extract may be due to the presence of Hexadecanoic acid with peak area 93.92 %, Methanesulfonylacetic acid 3.93 %, Pentadecanoic

Table III: Active compounds of methanol extract of *Spirulina spp*

Compound	Retention time	Area%	Height%
Hexadecanoic acid	6.324	93.92	42.86
Methanesulfonylacetic acid	6.486	3.93	36.63
2-Hydroxypropanoic acid	8.008	0.02	0.29
2-pentanol, 3-chloro, 2-methyl	12.187	0.01	0.07
Octadecadienoic acid	18.926	0.71	7.72
Pentadecanoic acid	20.998	0.89	8.81
Tridecanoic acid	28.032	0.52	3.36

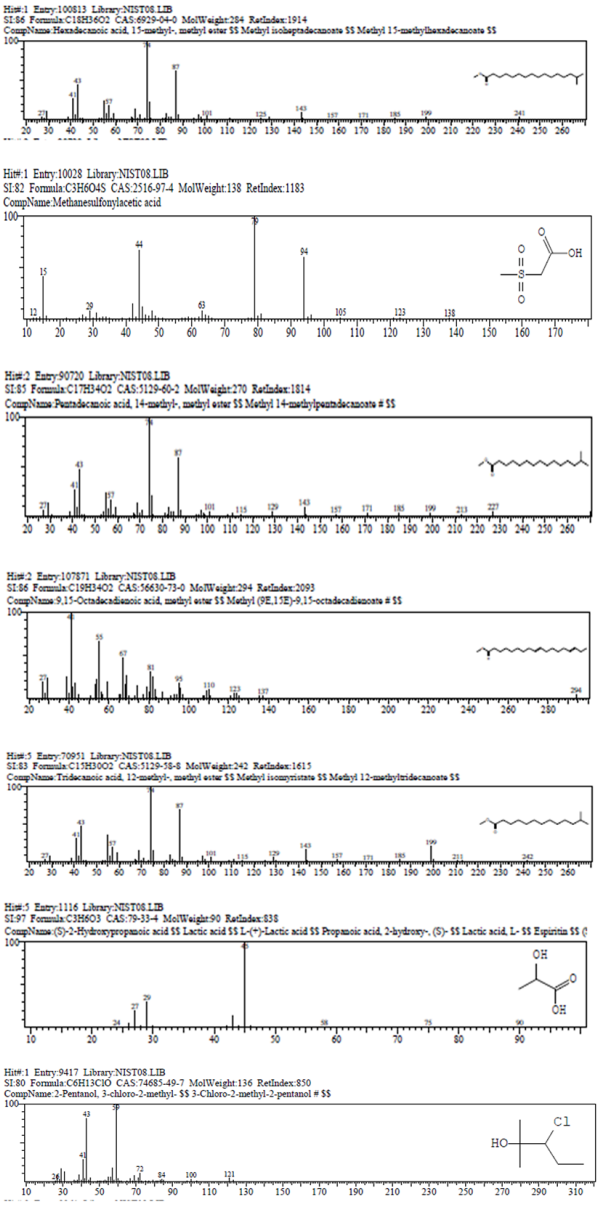

Figure 1: Chromatogram of active compounds of Spirulina extract detected by GC-MS analysis which indicated the presence of Hexadecanoic acid with peak area 93.92 %, Methane sulfonyl acetic acid 3.93 %, Pentadecanoic acid 0.89 %, Octadecadienoic acid 0.71 %, Tridecanoic acid 0.52%, 2-Hydroxypropanoic acid 0.02%, and 2-pentanol, 3-chloro, 2-methyl 0.01%.

acid 0.89 %, Octadecadienoic acid 0.71 %, Tridecanoic acid 0.52%, 2-Hydroxypropanoic acid 0.02%, and 2-pentanol, 3-chloro, 2-methyl 0.01% (Figure 1).

The cytotoxic influence of methanol extract on breast cancer cell lines

The cytotoxic influence of methanol extract was evaluated in two adenocarcinoma cell lines derived from breast cancer tissue, MCF7 (ER^{+ve}) and MDA-MB231 (ER^{-ve}) (Figure 2). These cells were incubated with escalating doses of methanol extract for 24 hours prior to assessment of viability by MTT assay (Figure 2A, C).

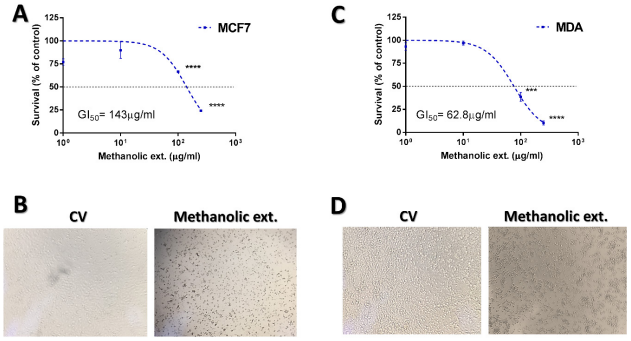

Figure 2: Cytotoxic effect of methanol extract of Spirulina spp. significantly reduces survival of Breast cancer cell lines. MCF7 (A) and MDA-MB231 (C) cells were incubated for 24 hours with increasing concentrations of methanolic extract followed by assessment of viability by MTT assay. Curve points denote the percentage of survival ± SEM of 2 or 3 replicate wells. GraphPad prism software was used to plot the nonlinear regression curve fit. The pictures B and D show the effect of co-treating MCF7 (B) and MDA-MB231 (D) cells with CV (left) or with 250µg/ml of methanol extract (right) for 24 hours, magnification 100x.

The reduction in the survival rate of MCF7 cells was apparent (10-20%) after exposure to 1µg/ml and 10µg/ml, however, no significant reduction in cells proliferation was measured in MDA-MB231 cells co-cultured with the same concentrations. Interestingly, MDA-MB231 cells treated with 100µg/ml and 250µg/ml showed greater cytotoxicity (Viability mean ± SEM: 38.6 ± 4.8%; 10.3 ± 2.1% respectively) than MCF7 cells treated with the same doses (Viability mean ± SEM: 66.5 ± 1.1%; 23.4 ± 0.85% respectively) (Table IV). Therefore, the GI50 value of MDA-MB231 (62.8µg/ml)

Table IV: Flow cytometric analysis of A549 and WRL68 cells after treated with ZnO-NPs.

Concentration of *Spirulina* extract (µg/ml)	Viability (%)	
	MDA	MCF 7
250	10.3± 2.1	23.4± 0.85
100	38.6 ± 4.8	66.5± 1.1
10	93.88± 0.9	76.75± 6.2
1	96.2± 1.8	83.68± 5.2

Values are expressed as mean ±SD of three experiments.

cells was about one half the GI_{50} of MCF7 cells (143µg/ml) (Figure 2 B, D).

Pictures taken to cells treated with the top concentration of Spirulina methanol extract for 24 hours showed significant reduction in cells number and sizes of both ER^{+ve} and ER^{-ve} cells in comparison with cells treated with control vehicle (Figure 5B, D). Hence, MDA-MB231 (ER^{-ve}) was more sensitive than MCF7 cells (ER^{+ve}) to methanol extract as GI_{50} values suggested (Figure 3).

MCF7 (A) and MDA-MB231 (C) cells were incubated for 24 hours with increasing concentrations of methanolic extract followed by assessment of viability by MTT assay. The pictures B and D were taken by light microscope showing the effect of co-treating MCF7 (B) and MDA-MB231 (D) cells with CV (left) or with 250µg/ml of methanol extract (right) for 24 hours, magnification 100x. Curve points denote the mean percentage of survival ± SEM of 2 or 3 replicate wells. GraphPad prism software was used to plot the nonlinear regression curve fit, p values were measured by t-Test (***=p<0.001; ****=p<0.0001).

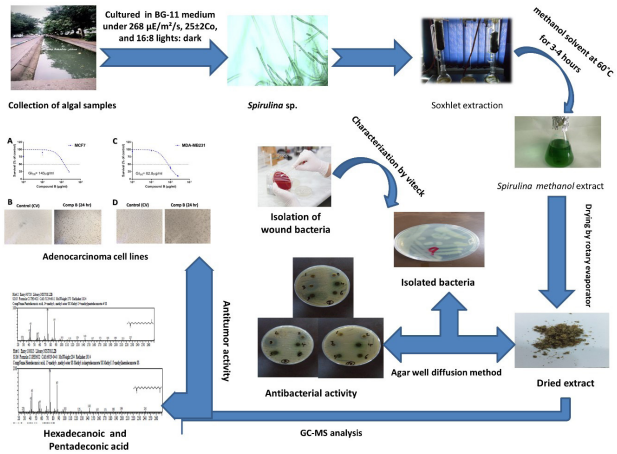

Figure 3: Methanol extract of Spirulina spp. significantly reduces survival of breast cancer cell lines. MCF7 (A) and MDA-MB231 (C) cells were incubated for 24 hours with increasing concentrations of methanolic extract followed by assessment of viability by MTT assay. The pictures B and D were taken by light microscope showing the effect of co-treating MCF7 (B) and MDA-MB231 (D) cells with CV (left) or with 250µg/ml of methanol extract (right) for 24 hours, magnification 100x. Curve points denote the mean percentage of survival ± SEM of 2 or 3 replicate wells. GraphPad prism software was used to plot the nonlinear regression curve fit, p values were measured by t-Test (*=p<0.001; ****=p<0.0001).**

DISCUSSION

Wound infection bacterial pathogens include: *Proteus spp., Streptococcus spp, Staph. aureus, E.coli, Pseudomonas aeruginosa, Klebsiella pneumoniae,*. and *Enterococcus spp.* They showed resistance to numerous antibiotics and disinfectants, and have the capability to colonize skin and they grow in minimal nutrients (20).

Cell wall structure, cell physiology, metabolism or degree of contact may affect the prevalence of higher susceptibility of Gram-positive bacteria in comparison with Gram-negative bacteria by *Spirulina sp* extract (21). The main antibacterial mechanism of *Spirulina sp.* extract was the formation of reactive oxygen specie (ROS) as mentioned in previous studies (22).

Previous reports showed that the compounds such as Hexadeconic, Octadecane and Heptadecane were found in both algae and plant species and presented potent antioxidant, anticancer and antimicrobial effects (23). GC-MS analysis of methanol and acetone extracts of *Spirulina platensis* found hexadecane, heptadecane, Eicosane, octadecane, phytol and pentadecane compounds and ,interestingly, these compounds showed antibacterial activity against *Staphylococcus aureus* and *Salmonella typhimurium* (4).

These results agreed with (24 Mofeed et al., 2018), which reported that human breast adenocarcinoma cell line growth was inhibited by using crude extract of *Spirulina platensis*. Moreover, several studies indicated anti-tumor effect of *Spirulina platensis* crude extract against several human cell lines, as stated in (25) who used methanolic extract of *spirulina* as an anticancer agent to treat Ehrlich Ascites Carcinoma (EAC).

Blue-green algae manufactured an excessive amount of antitumor compounds as natural products. For the development of cancer therapy, additional studies must proposed to detect and purify specific antitumor compounds in such extracts. Seaweeds specific metabolites is also recommended for the detection of possible antiproliferative or antitumor compounds (26) .

CONCLUSION

Due to a diverse chemical ecology, algae have a great promise for production of powerful, cheap, and safe antitumor drugs, which brings in an intensive attention. Hence, this study explored antibacterial and antitumor effects of Spirulena spp. which exhibited broad-spectrum biocidal activity towards wound bacteria and cytotoxic effect against breast cancer cell lines and these effects were associated with various active compounds as GC-mass analysis depicted. Therefore, additional studies (*in vitro* and *in vivo*) are clearly needed to define precise mechanisms behind the effects observed throughout this study (Figure 3).

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