Evaluation of *Spirulina Platensis* Crude Extract against some Pathogenic Microorganisms and Determination of Amino Acid Profile by HPLC, Erbil City

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Abstracts

The concept of biological controls and the use of algal extracts as antimicrobial substance has received widespread of attentions over the past few years. Therefore, the present study aimed to determine antimicrobial activities of *Spirulina platensis* and also Analysis of Amino acid profile by HPLC. For achieve this goal two different organic solvents have been used for extracts of *Spirulina platensis*, which were ethanol, and ethyl acetate. Crude extract of *Spirulina platensis* was tested in vitro against *Salmonella Typhi* (ATCC:14028), *Streptococcus pyogenes* (ATCC:19165) and *Candida albicans* (ATCC:10231) with Agar well diffusion method (1\10 W\V), additionally HPLC determined amino acid profile and the protein percentage per dry weight of extract. The results of present study declared that antimicrobial activity of crude extract of *Spirulina platensis* by ethanol was more effective than ethyl acetate, the highest inhibition zone was recorded against *Candida albicans* which was 19.5mm (ethanol solvent) and estimated protein percentage was 18.12 % in dry weight of *Spirulina platensis.*

Keywords: Amino acid, Antimicrobial, *Spirulina platensis*, Ethanol, and Ethyl acetate.

1. Introduction

Spirulina (*Spirulina platensis*), blue green algae, Family: Cyanophyta, order: Oscillatoriales that has a positive impact on human health and is considered as a healthy food worldwide. [1]. It is an edible, microscopic, multicellular, alkalophilic, photoautotrophic cyanobacterium belonging to Cyanophyta microalgae, known for its rich protein source, minerals, and nutrients [2]. This alga plays a role not only in food aspects but also in corrective properties against anemia, tumor growth and malnutrion. Moreover, extracts of *Spirulina platensis* documented medicinal properties, antimicrobial activities and ability to inhibit the replication of some viruses [3]. Additionally, biological properties and therapeutic properties of *Spirulina platensis* or it is extract reported, such as the ability to prevent cancers, decrease blood cholesterol level, reduce nephrotoxicity of pharmaceuticals and toxic metals and provide protection against the harmful effect of radiation [4] and [5].

Globally, more than 150.000 algae have been reported in fresh water and tested for their efficiency by many studies [6]. Many algal compounds have bacteriostatic and bactericidal activity, and several studies have examined them extensively [7], [8], [9], and [10]. Algal substances with bacteriostatic or bactericidal activity include amino acids, terpenoids, phlorotannins, hormones, phenolic compounds, halogenated ketones and alkanes, cyclic polysulphides, fatty acids and acrylic acids [11]. Among cyanobacteria members, *Spirulina platensis* is considered to be a good candidate for drug discovery [12]. In recent times, there is a growing interest in the area of research on the antimicrobial effects of *Spirulina platensis* on pathogenic microorganisms [13]. The major component in *Spirulina platensis* which has antimicrobial activity is heptade cane (39.7%), according to the previous study [13]. Previous investigation reported that methanol extract of *Spirulina platensis* has more potent activity than other organic extracts, spent medium of the culture exhibited little activity against E. coli [14].

The present study aims to determine antimicrobial activities of *Spirulina platensis* extracts with different solvents, additionally, estimate the protein percentage per dry weight of *Spirulina platensis* and list amino acid profile of mentioned algae.

2. Material and Methods

2.1 Culture and harvest of *Spirulina platensis*

The primary stock of *Spirulina platensis* was obtained from Degalla lake (60 Kilometer away from Erbil province) and standard key used for the identification [15] and , cultivated and harvested according to the standard method by [16]. The algae was cultured in a 250 ml conical flask containing 100ml of BG11 medium for *Spirulina sp.* Also pH has adjusted to around 7.1-7.4 by NaOH and HCL. The Growth of the algae culture was done in (1000 lux illuminated) growth room at 25±2◦C under 12/12 hour light- dark cycles for 16 days [17]. The algal mass is centrifuge at 10000 rpm for 15 minutes, then pellet was collected and dried by oven at 40 ◦C and finally preserved in dry plastic container at -4 ◦C for further analysis [18].

2.2 Preparation of extracts

Spirulina platensis is extract with two organic solvents: ethanol, and ethyl acetate. First, to 10 g of a sample, 100 mL of ethanol is add (10\100 W\V), and then the mixture is keep for forty- eight hours. at room temperature in dark glass bottle. The final extract

was filtered by Whatman filter paper and filtrate is concentrated in a rotary evaporator at room temperature (30°C) and the crude extract is kept in deep freeze(-20) use [19].

2.3 Antimicrobial activity

All microorganisms which have been used in the present study obtained from Media Diagnostic Center which is located in Iraq-Kurdistan Region, Erbil province, the crude extract of *Spirulina platensis* uses against *Salmonella Typhi* (ATCC:14028), *Streptococcus pyogenes* (ATCC:19165) and *Candida albicans* (ATCC:10231). The bacterial culture incubated on Muller Hinton agar (24 hr. at 37C) while yeast culture incubated on Potatoes Dextrose Agar (30 days at 37c). Agar well diffusion method used for checking the antimicrobial activity of extracts, for both bacteria and yeast the inhibition zones were measured with a ruler and compared with the negative control and positive control, erythromycin (15 µg) used as positive control for bacteria [18] while Itraconazole is used as a positive control for yeast [20].

2.4 Estimation of protein in *Spirulina platensis*

Biurette method is used for the estimation of proteins in *Spirulina platensis* depending on the previous study was done by [21], five grams of dried algae sample mixed with 1ml of distilled water and 4 ml of biurette reagent added which incubated in room temperature for thirty minutes then the mixture is centrifuged at 4000rpm for 10 minutes, the supernatant solution is collected and the optical density is measured in a spectrophotometer Shimadzu170,at 540 nm, finally, compared with standard curve of Total protein.

2.5 Analysis of Amino acid in *Spirulina platensis* by HPLC

The estimation of amino-acids in the present study is performed by HPLC system with a Spectra Physics (San Jose, CA, USA) chromatograph fitted with an intelligent LC-6A pumps (Shmadzu model, Tokyo, Japan), the injection loop sized 20-lL (Rheodyne, Cotati, CA, USA), the optical scanning detector forwarded by 6A UV-visible focus which linked to PC-1000 software for calculating data (Thermo Separation Products, Fremont, CA, USA), and temperature regulated at 27 ± 0.1 by an advance heater (Spectra-Physics model 8792). Methanol HPLC grade, standard solution of amino-acid 25µg/ml and phenyl isothocyanate reagent 50 Mm are obtained from (Aldrich chem. Co. Ltd).

Gradients are formed between two degassed solvents; Solvent A 5% methanol in 0.1 N sodium acetate buffer pH (7.0) and B methanol, linear gradients in from 0-20 minutes. In the present study the HPLC analysis is performed and detection is at 254nm of UV, while flow rate is 1ml per each minute and
injection is 20µl.

Derivatization procedure

Generally, the derivatization procedure was done according to [22], 10µl of aliquots of standard or unknown sample were mixed with 10µl of PTIC reagent after 1 minute, pH adjusted at 7.0 by using 50µl of 0.1 M sodium acetate. The whole sample is shake for ten minutes via ultrasonic bath, before injection of 20µl on HPLC column the

extract has been filtered using disposable filter paper 0.2um (Supelco company cat No 16534K). Finally, concentration for each compound were quantitatively determined by comparison the peak area of the standard with that of the samples, by the following equation then change to percentage:

Calculation:

$$\text{Concentration of sample } \mu g/ml = \frac{\text{Area of sample}}{\text{Area of standard}} * \text{conc. of standard} * \text{dilution factor}$$

3. Results and Discussion

Multidrug resistance *Streptococcus pyogenes* and *Salmonella Typhi* (Table 1) are selected for the antibacterial assay against ethanol crude extract of *Spirulina platensis.* However, *Candida albicans* is selected to test the extract efficiency against yeasts. The extracts show varying degrees of antibacterial activity against both pathogenic bacteria tested, the diameter of inhibition zone is 8.7mm, 19.3mm and 19.5mm for *Streptococcus pyogenes*, *Salmonella Typhi* and *Candida albicans*, respectively, as is shown in Table (2).

Algae are responsible to secret some types of specific components with a wide range of biological activities for instance antibiotics. Many investigations indicated that; the extracts of studied algae have impacts on the growth of some gram negative and gram positive species. These impacts are different upon the type of the extract and the species. Many studies have attempted to determine animicrobial activity of *Spirulina platensis* agansit many pathogenic microorganisms with different methods of extraction, also reported that antimicrobial activity of algal compounds depend upon the type of extraction solvent [23], [24] and [25].

Table 1: Resistance percentage rates of different antimicrobial agents against *Streptococcus pyogene* and *Salmonella typhi*.

Antimicrobial agent	*Streptococcus pyogenes*	*Salmonella typhi*
Ampicillin	85%	82%
Ampicillin/ Sulbactam	-	89%
Cefatizidime	92%	100%
Cefotixin	90%	100%

Table 2: Antimicrobial activity of crude extract *Spirulina platensis* by ethanol on pathogenic microorganisms

Microorganisms ,positive and negative controls	Crude extract of *Spirulina platensis* by ethanol(%70)
Streptococcus pyogenes	8.7mm
Salmonella Typhi	19.3mm
Candida albicans	19.5mm
Positive control for bacteria (Erythromycin)	5.3mm for *Streptococcus pyogenes* and 8mm for *Salmonella Typhi*
Positive control for yeast	12.7mm
Negative control	0mm for all micro organism

According to the present study, crude extracts inhibition zone of *Spirulina platensis* by ethanol are 8.7mm, 19.3mm and 19.5mm for *Streptococcus pyogenes* , *Salmonella Typhi* and *Candida albicans* , respectively, while inhibition zone of crude extract in case of ethyl acetate solvent were 3.5mm, 13.3mm and 7.2mm for *Streptococcus pyogenes* , *Salmonella Typhi* and *Candida albicans* , respectively. Generally, pathogenic microorganisms indicated various responses and sensitivity, The G +ve germs (in most cases) have responses more than G –ve germs; these differences is occurred because of genetic or chemical structure of microbes [29]; also may be because of cell membrane nature of the microorganisms which consisting of high level (90-95%) of peptidoglycan and also lipopolysaccharides and phospholipids (5-10%),in order to find a proper medium to reaction and also the entrance of antigerms factors (bactericidal and Bacteriostatic agents) to inside gram positive germs and destroying the cell membrane or protein biosynthesis units (DNA and RNA) by comparison with gram negative germs which their cell membrane consisting of double layers which is separated due to periplasmic space. The inner membrane contains peptidoglycan (5-10%), while the outer membrane contains phospholipids, lipoprotein and mucoploysaccharides. That means gram negative cell membrane consisting of high level of lipids which is (90-95%), which does not assist and not find a proper medium to make reaction and also entrance the antimicrobial agents and then decreases its effect on the pathogenic microorganisms [30].

A previous study by [3], were reported that *Spirulina platensis* extracts at concentration of 5.0 mg/ml inhibit the growth of bacteria with zone range between 10 mm to 20 mm, according to the same study, methanol crude extract of *Spirulina platensis* (5.0 mg/ml) record highest mean zone of inhibition (20±0.4 mm) against the Gram positive cocci *Streptococcus pyogenes* followed by *Staphylococcus aureus* (19±0.3 mm) and *Streptococcus epidermidis* (18±0.6 mm), while for gram negative are (19±0.8 mm), (19±0.5 mm) and (18±0.3 mm) for *Proteus mirabilis*, *Klebsiella pneumoniae* and *Escherichia coli* respectively. However, [14] disagreement with the present result which

concluded that hexane, ethyl acetate and dichloromethane extracts of *Spirulina platensis* (512 μg/ml) are found active only against S. aureus and had no effect against *E. coli, P. aeruginosa* and *S. typhi.*

Drug resistance isolates like *Streptococcus pyogenes* and *Salmonella Typhi* as mentioned previously in (Table 1) are selected for this antibacterial assay and *Candida albicans* are selected to represent yeast, the extracts show varying degrees of antimicrobial activity as shown in Table (3).

Table 3: Antimicrobial activity of crude extract of *Spirulina platensis* by ethyl acetate on pathogenic micro organisms

Microorganisms ,positive and negative controls	**Crude extract of *Spirulina platensis* by ethyl acetate**
Streptococcus pyogenes	3.5mm
Salmonella Typhi	13.3mm
Candida albicans	7.2mm
Positive control for bacteria(Erythromycin)	5.3mm for *Streptococcus pyogenes* and 8mm for *Salmonella Typhi*
Positive control for yeast	12.7mm
Negative control	0mm for all micro organism

The highest antibacterial activity of the mentioned extract is against *Salmonella Typhi* and the highest antifungal activity is against *Candida albicans* with the use of ethanol solvent. This finding agrees with the previous finding of [13], which compares methanol, ethanol, hexane, acetone and chloroform extracts of S. platensis which recorded the highest antimicrobial activity for methanol extracts followed by ethanol, hexane, acetone and chloroform extracts. Other study prefer acetone extracts of *Spirulina platensis* better than aqueous methanol and ethanol [26], also acetone extract of *Spirulina platensis* is reported with the inhibition zone of 17.0 mm, 11.0 mm and 10.0 mm for *Klebsiella pneumonia, Salmonella Typhi* and *Pseudomonas aeruginosa*, respectively.

Estimation of protein and Analysis of amino acids in *Spirulina platensis*

As previously mentioned, the Biurette method has been used for the estimation of proteins in *Spirulina platensis,* the percentage rate of protein is reported after measuring optical density and comparing the density to the standard curve of protein, the result was reported that protein percentage is 18.12 % in dry weight of *Spirulina platensis.* Amino acids profile of *Spirulina platensis* has been determined via HPLC method, and additionally the amount of each amino acid is detected as shown in Table (4). Notably, Tyrosine is the amino acid found in lowest content which is 6.49 μg/ml.

Table 4: Analysis of Amino acid in *Spirulina platensis* by standard HPLC method

Amino acid	Amount of amino acid by µg/ml	Percentage %
Aspartic acid	**41,83**	**4,52%**
Glutamic acid	**39,83**	**4,30%**
Glutamine	**59,13**	**6,38%**
Glysin	**26,60**	**2,87%**
Histadin	**37,81**	**4,08%**
Arginine	**42,64**	**4,60%**
Threonine	**37,48**	**4,05%**
Alanine	**42,07**	**4,54%**
Proline	**66,16**	**7,14%**
Tyrosine	**6.49**	**0,70%**
Tryptophan	**36,16**	**3,90%**
Methionine	**51,24**	**5,53%**
Cystine	**42,18**	**4,55%**
Valine	**60,93**	**6,58%**
Isoleucine	**52,34**	**5,65%**
Lucine	**59,43**	**6,42%**
Phenyle alanine	**46,72**	**5,04%**
Lysine	**176,33**	**19,05%**
Total		**97,03%**

The amino acid profile in the present study recordes higher content than those mentioned by [27], However, a study by [28] reportes that culture medium affects on protein and amino acid content in *Spirulina platensis* , and explains that the total protein content of platensis grown in salinated water is 48.59% by dry weight and 56.17% by desalinator wastewater. There are significant differences between these tests (p=0.001661).

The present study concluds that crude extracts of *Spirulina platensis* have antimicrobial activity against *Streptococcus pyogenes* , *Salmonella Typhi* and *Candida albicans* . Moreover, this study determined that antimicrobial activity of crude extract of algae by ethanol is more effective than extract by ethyl acetate, the highest inhibition zone was recorded against *Candida albicans* which is 19.5mm (ethanol solvent), estimated protein percentage is 18.12 % in dry weight of *Spirulina platensis* , and Lysine amino acid recorded highest amount among other amino acids which was 176,33 µg/ml, while Tyrosine found in lowest content which is 6.49 µg/ml.

Conflict of Interests.
There are non-conflicts of interest .

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الخلاصة

تلاقي فكرة استخدام مستخلصات الطحالب في السيطرة الحيوية ضد البكتريا و الفطريات المرضي رواجا كبيرا في السنوات الاخيرة الماضية ,لذلك كان الغرض من هذا البحث دراسة تاثير مستخلص طحلب *Spirulina Platensis* ضد بعض البكتريا و الفطريا ت الممرضة و دراسة نسبة الاحماض الامينية في هذا الطحلب

باستخدام تقنية HPLC.

لتحقيق هذا الهدف ، تم استخدام مذيبين عضويين مختلفين لمستخلصات سبيرولينا بلاتينسيس ، وهما الإيثانول ، واسيتات الإيثيل. تم اختبار المستخلص الخام من سبيرولينا بلاتنسيس في المختبر ضد السالمونيلا التيفية (ATCC: 14028) ، العقدية المقيحة (ATCC: 19165) والمبيضات البيضاء (ATCC: 10231) باستخدام طريقة نشر آجار بشكل جيد (10 \ 1 V \ W). أعلنت نتائج هذه الدراسة أن النشاط المضاد للميكروبات في المستخلص الخام من سبيرولينا بلاتنسيس بواسطة الإيثانول كان أكثر فعالية من أسيتات الإيثيل ، وسجلت أعلى نقطة تثبيط ضد *Candida albicans* التي كانت 19.5 ملم (مذيب الإيثانول) وكانت نسبة البروتين المقدرة 18.12 ٪ في وزن سبيرولينا بلاتنسيس.