academicJournals

Vol. 12(18), pp. 2498-2509, 1 May, 2013
DOI: 10.5897/AJB12.1640
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

In vitro and *in vivo* preliminary results on *Spirulina platensis* for treatment of impetigo: Topical cream application

Saly F. Gheda[1], Maha A. Khalil[1]* and Shereen F. Gheida[2]

[1]Department of Botany, Faculty of Science, Tanta University, Egypt.
[2]Department of Dermatology and Venereology, Faculty of Medicine, Tanta University, Egypt.

Accepted 10 October, 2012

Impetigo is a highly infectious superficial bacterial disease, most common among pre-school children. Applying 11 antimicrobial agents to the *Staphylococcus aureus*, the most causative organism for impetigo, *S. aureus* isolates are resistant to all agents except of vancomycin and fusidic acid. Nevertheless, treatment of impetigo using antimicrobial agents may cause serious medical problems, such as destroying normal gut and skin flora and producing gastrointestinal irritations, dermatitis or serious hypersensitivity problems. Thus, the test of new microbial infection-fighting natural compounds is urgent. The *in vitro* measuring the antibacterial activity of *Spirulina platensis* extracts, following agar-well diffusion method, against methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) clinical isolates showed that the methanolic *S. platensis* extract is the most active. The *in vivo* efficacy of applied topical *S. platensis* creams, both methanolic extract and crude, in treatment of impetigo were compared. In general, clinical application of both active ingredients of *S. platensis* (Group 1-G1) and crude *S. platensis* form (Group 2-G2) gave promising and excellent response rates. However, the Group 1 application had the best efficacy, no side effects and no recurrence during the follow-up period. Gas chromatography-mass spectrometry (GC-MS) analysis of both crude alga and its methanolic extract concludes that the potential antimicrobial activity is attributed to synergic effect of some fatty acids. We propose that the higher percentage of linoleic and palmitic acids and the presence of squalane in methanolic extract of *Spirulina* most probably are the causes of its higher antimicrobial activity.

Key words: *Staphylococcus aureus,* impetigo*, Spirulina platensis*, extracts, topical cream, gas chromatography-mass spectrometry (GC-MS), antimicrobial activity.

INTRODUCTION

Bacterial resistance has been the main factor responsible for the increase of morbidity, mortality and health care costs of bacterial infections. The defense mechanism against antibiotics is widely present in bacteria and became a world health problem. The increasing prevalence of multidrug resistance strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the specter of untreatable bacterial

*Corresponding author. E-mail: mahakhalil90@yahoo.com. Tel: + 01110378557. Fax: +2 040 3305804.

Abbreviations: MRSA, Methicillin-resistant *S. aureus*; **MSSA,** methicillin-sensitive *S. aureus*; **SIRS,** skin infection rating scale; **GC-MS,** gas chromatography-mass spectrometry.

infections and adds urgency to the search for new infection-fighting strategies to control microbial infections (Mala et al., 2009).

The search for natural compounds with antimicrobial activity has gained importance in recent years due to growing worldwide concern about alarming increase in the rate of infection by antibiotic-resistance microorganisms (Kaushik and Chauhan, 2008). It is generally considered that compounds produced naturally, rather than synthetically, will be biodegraded more easily and will therefore be environmentally acceptable (Ozdemir et al., 2004).

Attention is now being focused on the natural components produced by cyanobacteria. Cyanobacteria are potential sources of high value chemicals and pharmaceuticals. *Spirulina* is one of the most potential cyanobacteria used in medicine. *Spirulina* is safe for human consumption as medicine, because it is free of microcystin toxin and the long term dietary supplementation of up to 5% of the *Spirulina* may be consumed without evident toxic side effects (Yang et al., 2011). It is known to produce intracellular and extracellular metabolites with diverse biological activities such as antifungal (Mac Millan et al., 2002), antiviral (Hayashi and Hayashi, 1996), and antibacterial activities (Kaushik and Chauhan, 2008). Several studies have shown that *Spirulina* or its extracts could prevent or inhibit cancer in humans and animals and recent works have indicated that this species has immune-promoting effects (Hirahashi et al., 2002; Subhashini et al., 2004). There is a growing interest in the area of research on the positive effects of *Spirulina platensis* (*Arthrospira platensis*) on human health. In spite of the fact that many studies focused on the antibacterial activity of *Spirulina*, there is a lack in its application on diseases caused by different bacterial strains.

Impetigo is a highly contagious bacterial infection of the skin, most common among pre-school children (Cole and Gaze wood, 2007). *Staphylococcus aureus* is the most important causative organism. *Streptococcus pyogenes* causes fewer cases, either alone or in combination with *S. aureu*s (Hirschmann, 2002). Treatment of impetigo includes either systemic or topical antibiotics. Systemic antibiotics generally yield good results, but can eliminate normal gut flora, produce gastrointestinal irritations or serious hypersensitivity problems. Topical antibiotics also yield good results, but may destroy normal skin flora and produce contact dermatitis (Capoluongo et al., 2001). Resist pathogens as methicillin-resistant *S. aureus* (MRSA) and cross resistance with antibiotics are also problems (Lewis and Steele, 2011). The incidence of strains of *S. aureus* resistant to the antimicrobial agents used in treating impetigo has been increasing. One important reason for this is the spread of MRSA outside hospitals and healthcare facilities (Dalager-Pedersen et al., 2011).

The aim of the study is to: (1) measure *in vitro* the antibacterial activity of different extracts of the laboratory grown culture of *S. platensis* against different clinical isolates of *S. aureus* collected from impetigo patients and (2) Evaluate the efficacy and the safety of topical *S. platensis* cream, both extract and crude in treatment of impetigo patients.

MATERIALS AND METHODS

Algal cultivation

S. platensis UMANS 11 was obtained from the culture collection of the Botany Department, Faculty of Science, Mansoura University, Egypt. Zarrouk medium (1966) was used for cultivation of *S. platensis*. Culture was illuminated at light intensity of 80 $\mu Em^{-2}s^{-1}$. Culture temperature was maintained at 30 ±1°C. *S. platensis* was grown until the late exponential phase of the growth of which the culture was harvested. The collected biomass was dried in a hot air oven at 60°C for 1 h.

Preparation of *S. platensis* extracts

Algal extracts were prepared according to Kaushik and Chanhan (2008) by mixing 10 g of dried *Spirulina* to 150 ml of solvents (methanol, ethanol, chloroform, acetone, dichloromethane and ethyl acetate), for 5 h at room temperature and sonicated for 5 min, and then centrifuged at 4000 rpm for 10 min. The obtained extract was freed from solvent by evaporation under reduced pressure and then resuspended in the appropriate solvent to make the solution of known concentration of 100 mg/ml. The extract was stored at -20°C in airtight glass bottle for the antibacterial assay.

Clinical microbiology

The microbiology testing of the clinical samples was performed by using culture swabs obtained from the target skin lesion for each patient where they were recruited from the Outpatient Clinic of Dermatology and Venereology Department of Tanta University Hospitals. The patient samples were transferred in 2 ml phosphate–buffered saline (PBS; NaCl, 8 g/L; KCl, 0.2 g/L; Na_2HPO_4, 1.15 g/L; KH_2PO_4, 0.2 g/L) and were forwarded to the Bacteriology Laboratory in Botany Department, Faculty of Science, Tanta University. All culture swabs were processed in the same day that they were collected. Each specimen was plated to a blood agar plate, MacConkey agar plate and a mannitol salt agar media. Culture plates were incubated up to 24 h at 37°C, then examined for colony morphology consistent with *S. aureus*. Identification of *S. aureus* colonies included test for catalase, slide coagulase and DNase (Koneman et al., 1992) as well as Api-Staph strip system (API System S.A., Montalïeu-Vercieu, France).

A positive bacterial growth (baseline) on nutrient agar, the diagnosis of skin lesions in clinical samples, was based on the presence of > 10^5 colony forming unit (CFU) of microorganisms/ml in the culture (Williams et al., 1990).

Antimicrobial susceptibility of the tested isolates

The susceptibility of the tested *S. aureus* isolates to 11 antimicrobial agents was performed by modified Kirby-Bauer single-disk diffusion technique on Muller Hinton agar (Robert et al., 2003). The used antimicrobials were penicillin, oxicillin, amoxicillin-clavulanic acid, tobramycin, gentamicin, ciprofloxacin, chloramphenicol, tetracycline, erythromycin, vancomycin and fusidic acid. The results of the susceptibility tests were interpreted according to the criteria established by the Clinical and Laboratory Standards Institute (CLSI, 2010). Methicillin-resistance was tested by using 1 mg oxacillin disk (Oxoid).

Antibacterial activity of algal extracts by agar-well diffusion

Antibacterial activity of the *S. platensis* extracts was determined by agar-well diffusion method (Shanmuga et al., 2002). Petri-plates containing sterilized Muller Hinton agar (Oxoid) were used. After solidification of medium, each plate was seeded with 0.1 ml of broth culture strains. The wells of 6 mm diameter were cut with sterile cork borer. Each well was filled with 0.1 ml of each algal solvent extract. Different solvents alone were used as control, for each test organism. For comparison, the standard drugs, 0.1 ml of ciprofloxacin (5 µg/ml), was used as positive control. After incubation, all plates were observed for zones of growth inhibition, and the diameters of these zones were measured in millimeters (mm). All tests were performed under sterile conditions in duplicate and were repeated three times, using tested solvents as negative control. After this experiment, we select the solvent that gave the largest inhibition zone, thus, the two topical creams were prepared from the crude alga (whole alga) and alga extracted with this solvent.

Topical cream preparation

Two topical creams were prepared from the crude *S. platensis* (whole alga) and its extract (using the best solvent which gives the largest inhibition zone). Preparation of topical creams was carried out according to Purushothamrao et al. (2010) in the Laboratory of Pharmaceutical Technology, Faculty of Pharmacy, Tanta University. Formulation of 50 g containing 0.5% of active ingredients was composed of two phases (oil phase: cetostearyl alcohol, 4.0 g; vaseline 7.5 g; liquid paraffin 3.75 ml; and aqueous phase: deionized water, 35 ml; sodium dodecyl sulphate (SDS), 425 mg; active ingredient 250 mg). Ingredients of oil phase were mixed together by melting in China dish on constant stirring. Components of aqueous phase were mixed together and warmed to about same temperature of oil phase. Aqueous phase was added to oil phase drop by drop on constant stirring until solidification. The preservative propyl paraben and methyl paraben were added after cooling.

Patients and clinical application

The clinical application of the topical cream was carried out on 30 patients (18 females and 12 males), aged below 12 years (2 to 11 years) with impetigo diagnosed on the basis of the typical appearance of the skin lesions. The patients were recruited from the Outpatient Clinic of Dermatology and Venereology Department, Tanta University Hospitals. The study and consent form were approved by the local ethical committee [Approval code: 427/03/11]. The studied patients were treated as a part of randomized, single blind placebo-comparative study and were divided into three groups: group 1 (G1) included 10 patients subjected to topical *S. platensis* cream made from active ingredients (extracted *Spirulina*); group 2 (G2) included 10 patients subjected to topical *S. platensis* cream made from crude algae (whole alga); and group 3 (G3) included 10 patients subjected to placebo (drug free) cream served as negative control.

Exclusion criteria

All patients that received concomitant antibiotic or other topical measures, such as wet dressings and patients had immunosuppressed state or other serious systemic disease.

Inclusion criteria

All types of impetigo (primary or secondary, bullous or non bullous) and positive baseline culture for *S. aureus* from a sample taken from the target lesion.

All the patients were subjected to complete history taking, general and dermatological examination. The cream formula was applied three times per day for seven days. Treatment was extended to 14 days if the 7th day clinical examination indicated that the infection was not cured. Clinical pictures and bacteriologic cultures were assessed before treatment and at seven days after treatment. Clinical follow up at 14 days after treatment for recurrence of any skin lesions.

Assessment of the efficacy of the treatment

Patients completed all seven days of treatment and were assessed at the end of treatment. Assessment was done on the basis of: (1) physician's evaluation, patient's opinion and digital image analysis of standardized colored photographs which were taken before and after treatment; (2) skin infection rating scale (SIRS); and (3) bacteriological culture examination after algal treatment.

Skin infection rating scale (SIRS)

Patients were evaluated by SIRS, which evaluated five signs and symptoms: exudate/pus, crusting, erythema/inflammation, itching, and pain on a scale (0 to 3): 0 = absent, 1 = mild, 2 = moderate, 3 = severe. Enrollment criteria were to include 2 to 12 years of age with a clinical diagnosis of impetigo with ≤10 impetigo lesions in total and a Gram stain of the target lesion showing Gram positive cocci. A total SIRS score of ≥ 4; at least three of five signs and symptoms are present at baseline and a score of ≥ 1 for exudate/pus. Subjects were screened, randomized, and began treatment all on the same day (Susan et al., 2011).

Clinical success was determined by sufficient resolution of signs and symptoms of infection of the target lesion as evidenced by the SIRS score of 0 each for exudate/pus, crusting and pain and 0 or 1 each for erythema/inflammation and itching. Clinical improvement was determined by a SIRS score of 0 for exudate/pus which does not meet all the criteria for clinical success. Clinical failure was determined by a SIRS score of 1 or greater for exudate/pus. Unevaluable was recorded when a valid clinical assessment could not be made. Clinical efficacy, a pretreatment and post-treatment SIRS score was determined (Susan et al., 2011). Adverse events were determined by the investigator and assessed.

Bacteriological culture examination after algal treatment

Bacteriological success was determined when the causative pathogen was isolated from the target lesion at baseline (*S. aureus*) was eliminated on culture. Bacteriological failure was determined by the non-eradication of the organism from the target lesion that was isolated at baseline.

Chemical composition of *S. platensis* by GC-MS

Gas chromatography-mass spectrometry (GC-MS) analysis was used to determine the compounds present in *S. platensis* and its methanolic extract, carried out in the Biochemistry Laboratory of the Faculty of Agriculture, Cairo University. The fatty acids sample was analyzed by an HP 6890 Series Gas Chromatograph System with an HP 5973 mass selective detector. The system was equipped with a TR-FAME (Thermo 260 M142 P) (30 m, 0.25 mm ID, 0.25 µm Film (70% Cyanopropyl Polysilphenylene-siloxane) capillary column, 200°C temperature injector and 250°C temperature transfer line). The oven temperature was programmed as follows: initial temperature; 80°C for 2 min, increase to 3°C min^{-1} up to 230°C,

Table 1. Comparison of antimicrobial susceptibilities of MRSA and MSSA.

Antimicrobial agent	Percentage of resistant *Staphylococcus aureus isolates**	
	MRSA (n = 6)	MSSA (n = 24)
Penicillin	100	95.8
Erythromycin	83.3	79.2
Clindamycin	83.3	70.8
Tetracycline	66·7	41·7
Ciprofloxacin	0	4.2
Chloramphenicol	16·7	12·5
Gentamicin	33·3	4·2
Oxacillin	100	0
Amoxicillin-Clavulanic acid	50	20
Vancomycin	0	0
Fusidic acid	0	0

*MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensitive *S. aureus.*

and then hold at 230°C for 5 min. The carrier gas was He_2 (1.5 ml/min). The amount of sample injected was about 1 µl (5 mg/2 ml) and the ionization energy was 70 eV. Qualitative identification of the different constituents was performed by comparison of their relative retention times and mass spectrum with those of authentic reference compounds (fatty acid methyl esters, purity 98% by GC). Also, probability merge search software and the NIST MS spectra search program were used.

Statistical analyses

The data were statistically processed to estimate the mean ± standard deviation (SD) of triplicates, and using the one way analysis of variance (ANOVA) to analyze the effect of different solvents of *Spirulina* on the antimicrobial activity. The SIRS data were statistically analysed and expressed as mean ± SD of triplicates. Also, the one way ANOVA and Student's t test were used. All data were analysed according to the Statistical Package for Social Sciences (SPSS) version 16.0 (SPSS Inc., Chicago, IL). A P value of < 0.05 was considered to be statistically significant.

RESULTS

Antimicrobial susceptibility of *S. aureus*

The antimicrobial susceptibilities of the MRSA isolates (n = 6) were compared with the methicillin-sensitive *S. aureus* (MSSA) isolates (n = 24) and are depicted in Table 1. The antibiotic susceptibility testing showed that 95.8% of the MSSA isolates were resistant to penicillin, 79.2% to erythromycin, 70.8% to clindamycin, 41.7% to tetracycline, 12·5% to chloramphenicol and 4.2% to gentamicin and ciprofloxacin.

The MRSA (3, 7, 11, 15, 19 and 24) isolates were multiresistant, with a high rate of resistance to clindamycin (83.3%) and tetracycline (66.7%), but all were susceptible to ciprofloxacin. The susceptibility profiles of MRSA to other antimicrobial agents were similar to those of MSSA. Only one of the 30 (3.3%) *S. aureus* isolates was sensitive to all tested antibiotics, whereas 22 of the 30 (73.3%) were resistant to at least three. Furthermore, no resistance against fusidic acid and vancomycin was observed.

Antibacterial activity of *S. platensis* extracts

Results of the antibacterial activity of *S. platensis* extracts against staphylococcal isolates are disseminated in Table 2. It is clear that the diameter of the inhibition zone depends mainly on the type of the solvent used and the tested antibacterial activity. The methanolic extract showed broad spectrum activity as the highest effective zone of inhibition was recorded against tested isolates, followed by dichloromethanolic extract. However, extracts prepared in acetone, chloroform, ethanol and ethyl acetate were found active mostly against MSSA isolates and no inhibitory effect was observed against all MRSA isolates (Figure 1). Interestingly, both methanolic and dichloromethanolic extracts of *S. platensis* were found active against MRSA.

It is observed that the antibacterial activity of all the extracts was significantly different ($P \leq 0.05$) from each other, when tested against the selected isolates, except MRSA. *S. aureus* (No. 30) showed maximum zone of inhibition (20.5 and 24.8 mm) to dichloromethanolic and methanolic extract of *Spirulina*, respectively. The negative control (solvent) showed no inhibition, whereas the positive control (antibiotic, ciprofloxacin) showed varied range of inhibition zone (13.5 to 23.8 mm). Generally, the methanol extract showed antibacterial activity against *S. aureus* isolates, but mostly nearest or less than that of the standard antibiotic (ciprofloxacin). The methanolic *Spirulina* extract gave the largest inhibition zone, thus, this methanolic extract (active ingredients) was used for the preparation of the topical cream.

Names of these components and their amounts are listed in Tables 6 and 7. In the methanolic extract of *S.*

Table 2. *In vitro* antibacterial activity of *S. platensis* extracts.

Number of *S. aureus* isolates	[#]Diameter of effective zone of inhibition (mm)						
	Acetone extract	Chloroform extract	Dichlorome-thane extract	Ethanol extract	Ethyl acetate extract	Methanol extract	Cip
1	13.3 ± 0.3	NA	11.6 ±0.3	10.7 ± 1.15	4.5 ±0.5	14.8 ± 0.3	16.7 ± 0.6
2	14.0 ± 0.5	NA	12.5 ± 0.5	11.3 ± 0.3	2.1 ± 0.3	18.2 ± 0.3	17.7 ± 0.6
3	NA	NA	11.3 ± 0.3	NA	NA	15 ± 0.5	15.7 ± 0.6
4	12.0 ± 0.5	8.2 ± 0.3	13.2 ± 0.3	11.3 ± 0.3	0.8 ± 0.2	17.3 ± 0.3	17.7 ± 0.6
5	19.3 ± 0.6	7 ± 0.5	12.2 ± 0.3	10.2 ± 0.3	NA	16.8± 0.3	17.7 ± 0.6
6	15.8 ± 0.8	8.3 ± 0.6	15.7 ± 0.3	11.5 ± 0.5	NA	22 ± 0.5	23.8 ± 0.8
7	NA	NA	12.5 ± 0.5	NA	NA	16.2 ± 0.3	16.3 ± 0.6
8	13.8 ± 0.6	5.5 ± 0.5	11.5 ± 0.5	9 ± 0.9	3.2 ± 0.3	14.2 ± 0.3	13.5 ± 0.5
9	12.8 ± 0.3	6.8 ± 0.8	13 ± 1.0	10± 0.5	2.2 ± 0.3	16.2 ± 0.3	15.5 ± 0.5
10	16.3 ± 0.3	7.2 ± 0.3	13.3 ± 0.6	NA	1.2 ± 0.3	15.2 ± 0.3	17.7 ± 0.6
11	NA	NA	12 ± 1.0	NA	NA	13.3 ± 0.6	17.3 ± 0.6
12	12.7 ± 0.3	6.8 ± 0.3	14.7 ± 0.8	8.7 ± 0.6	NA	16.7 ± 1.5	21.7 ± 1.5
13	14.2 ± 0.3	6.8 ± 0.3	13.8 ± 0.8	10.2 ± 0.3	1.5 ± 0.5	18.7 ± 0.6	20.3 ± 0.6
14	13.3 ± 0.3	7 ± 1.0	14.2 ± 0.7	11.2 ± 0.2	NA	21.5 ± 0.5	20.3 ± 1.5
15	NA	NA	13.2 ± 0.3	NA	NA	19 ± 0.5	20.7 ± 0.6
16	12.8 ± 0.7	6 ± 1.0	14.2 ± 0.3	11 ± 1.0	NA	20.8 ± 0.8	21 ± 1.0
17	13.2 ± 0.3	5.7 ± 0.3	11.8 ± 0.8	9.8 ± 0.8	NA	15.8 ± 0.8	19 ± 1.0
18	NA	4.5 ± 0.5	14.2 ± 1.0	10.5 ± 0.5	2.7 ± 0.3	18.2 ± 0.3	22 ± 1.7
19	NA	NA	12.8 ± 0.8	NA	NA	18.7 ± 0.6	23.2 ± 0.3
20	12.2 ± 0.3	NA	14.5 ± 0.5	NA	3.7 ± 0.6	20.2 ± 0.3	22.3 ± 1.5
21	11.2 ± 0.3	7.5 ± 0.5	15.2 ± 0.8	9.8 ± 0.8	NA	22.5 ± 0.5	22.7± 0.6
22	9.8 ± 0.8	6.5 ± 0.5	13.7 ± 0.6	11.2 ± 0.3	NA	21.2 ± 0.3	23.3 ± 0.6
23	11.2± 0.3	5.7 ± 0.6	12.2 ± 1.0	NA	NA	18.3 ± 0.6	22.2± 1.0
24	NA	NA	11.8 ± 0.3	NA	NA	17.3± 1.5	23 ± 1.0
25	14.2 ± 0.3	5.2 ± 0.3	12.5 ± 0.5	NA	3.5 ± 0.5	15.8 ± 0.8	16.7± 1.2
26	13.2 ± 0.3	4 ± 0.5	11.8 ± 0.8	8.3 ± 1.2	4 ± 0.9	16 ± 1.0	17.7 ± 0.6
27	NA	NA	13.2 ± 0.8	10.2 ± 0.3	5.7 ± 1.5	22.8 ± 0.8	22.3 ± 2.0
28	NA	NA	12.2 ± 0.3	NA	NA	18.8 ± 0.8	16.7 ± 0.6
29	16.3 ± 0.6	3.7 ± 0.6	13.5 ± 0.5	NA	NA	21.5 ± 0.5	24.2 ± 0.8
30	13.8 ± 0.3	5 ± 1.0	20.5 ± 0.5	11.2 ± 0.3	5 ± 1	24.8 ± 0.3	23.2 ± 0.8
F value	**895.183*** **	**140.028*** **	**20.809*** **	**330.464*** **	**53.159*** **	**59.051*** **	**30.690*** **

[#]Results are the means of diameter values ± standard deviation. NA, No activity. Effective zone of inhibition (total zone of inhibition-diameter of well). Cip, Ciprofloxacin (5 µg/ml). ***Highly significant at P ≤ 0.001 using one way analysis of variance (ANOVA).

platensis, the major component is squalane (37.9%). The algal extract also contains many antimicrobial fatty acids,

Clinical results

Overall, clinical response rate (success and improvement) in G1 and G2 was excellent. This response rate is higher than that for placebo group G3 (Table 3).

There was highly statistically significant difference in SIRS values before and after treatment (Table 4 and Figures 4 to 6) in G1 (P value = 0.001) and G2 (P value = 0.022), but there was no statistically significant difference in placebo group G3 (P value = 0.253). This is an indication for the high clinical efficacy of the treatment especially in G1. There was no recurrence of infection at the follow-up visits (day 14) in G1 and G2. No adverse effects were demonstrated in any group of the treatment.

Bacteriological results

Application of the *Spirulina* cream to impetigo patients for seven days decreased the number of *S. aureus* when compared with earlier treatment and with placebo cream. Bacteriologic cure rate after seven days treatment in G1 (active form) was higher than G2 (crude form). Treatment failure was noted in one crude form-treated patients group, but not in the active form-treated group (Table 5).

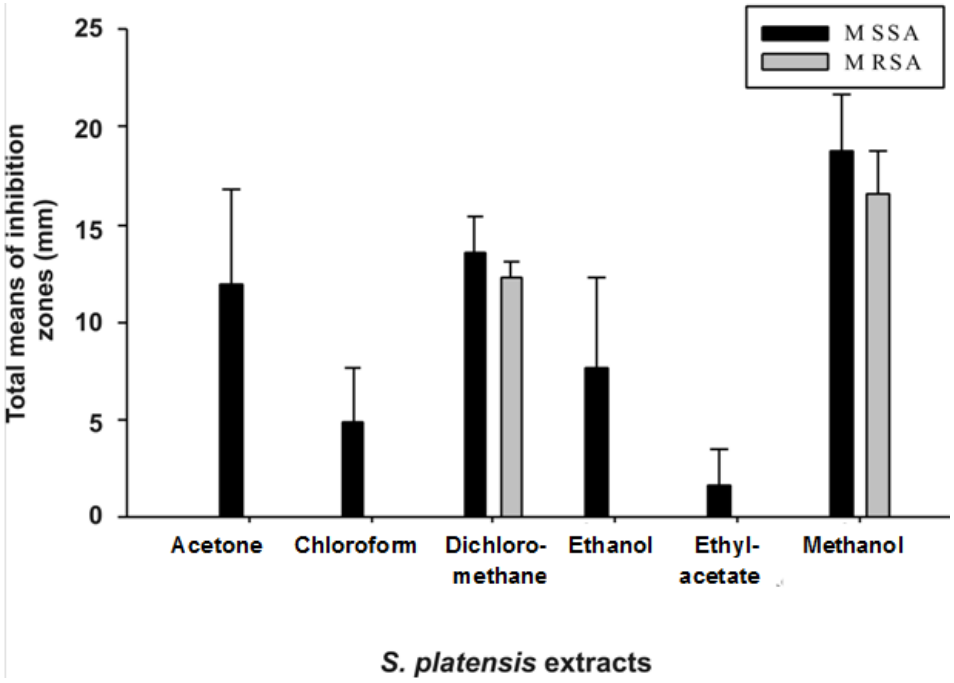

Figure 1. Antibacterial activity of *S. platensis* extracts against methicillin-sensitive *S. aureus (MSSA)* and methicillin-resistance *S. aureus* (MRSA).

Table 3. The clinical response to *S. platensis* cream treatment in the studied patients groups.

Group	Number of patient	Clinical response (%)		
		Success	Improvement	Failure
Methanolic extract of alga (active ingredients of *Spirulina*) (G1)	10	70	30	-
Whole alga (crude *Spirulina* form) (G2)	10	50	40	10
Placebo cream (G3)	10	-	20	80

Table 4. Comparison between SIRS score before and after *Spirulina* treatment in different studied groups.

SIRS Score	Group		
	G1 (n = 10)	G2 (n = 10)	G3 (n = 10)
Before treatment	4 - 10 6.35 ± 2.55	4 - 10 6.10 ± 2.07	4 - 11 7.52 ± 1.69
After treatment	0 - 3 1.88 ± 0.34	0 - 5 4.02 ± 0.58	3 - 10 6.36 ± 2.36
T test	6.362	3.595	0.655
P value	0.001**	0.022*	0.253

*Significant; **Highly significant; *Significant at P < 0.05; *Insignificant at P > 0.05; **Highly significant at P ≤ 0.001.

The GC-MS chromatograms show many compounds present in both crude alga and its methanolic extract like palmitic (12.5%), palmitoleic (0.85%), petroselinic (4.63%) and linoleic acids (4.11 %). While, in the crude *S. platensis*, the major components is the 4-Heptanol,4- ethyl-2,6-dimethyl-Thiazolone (39.79%). Subordinately, the crude alga contains many antimicrobial fatty acids such as palmitic (11.53%), stearic (1.42%), petroselinic (10.36%) and linoleic acids (1.58%). The amount of linoleic and palmitic acids in the algal extract is higher than that in algal crude

Table 5. Bacteriological response to *S. platensis* cream treatment in the studied groups.

Group	Bacteriological response		
	Success	Improvement	Failure
Methanolic extract of alga (active ingredients of *Spirulina*) (G1)	+	+/-	ND
Whole alga (crude *Spirulina* form) (G2)	+/-	+/-	-
Placebo cream (G3)	ND	-	-

+, Success (eradication of bacteria); +/-: mean of bacterial colony number were < 10^5 CFU/ml in the culture; -, failure (mean of bacterial colony number were > 10^5 CFU/ml in the culture); ND, patients were not present in these groups.

Table 6. GC-MS analysis of different compounds in methanolic extract of *S. platensis*.

Peak	Rt (min)	Area (%)	Name
1	15.11	21.56	Butylated hydroxytoluene
2	19.87	8.90	4-Heptanol,4-ethyl-2,6-dimethyl-2 (3H)Thiazolone, 4-methyl
3	21.19	2.58	Urea, trimethyl-1,1,3-trimethylurea, N,N-dimethyl-N-methylurea, 1,1,3-trimethyl-trimethylharnstoff
4	22.80	6.00	4,5-dimethyl-1,3-dioxolane. 1,3-dioxolane, 4,5-dimethyl-cis-4,5-dimethyl-1,3-dioxolane, 4-methyl-1,3-dioxane
5	24	12.50	Hexadecanoic acid, methyl ester (palmitic acid),
6	24.60	0.85	Cis-7-Hexadecenoic acid, methyl ester, cis-9-Hexadecenoic acid, methyl ester (palmitoleic acid)
7	25.97	0.97	Cis- 7, 10-Hexadecadienoic acid, methyl ester
8	29.34	4.63	Cis-6-octadecenoic acid, methyl ester (petroselinic acid)
9	30.66	4.11	Cis, cis-9,12-octadecadienoic acid, methyl ester (linoleic acid)
10	31.50	6.39	Squalane
11	31.81	24.37	Squalane
12	31.84	7.14	Squalane

Rt, Retention time.

Table 7. GC-MS analysis of different compounds in *S. platensis* crude.

Peak	Rt (min)	Area (%)	Name
1	19.89	39.79	4-Heptanol,4-ethyl-2,6-dimethyl-Thiazolone
2	21.25	15.34	Urea 1,1,3-trimethyl urea, N,N dimethyl N-methyl urea
3	22.82	12.97	2-methoxy carbonyl-1-cyclopropane
4	23.99	11.53	Hexadecanoic acid, methyl ester (palmitic acid)
5	28.71	1.42	Octadecanoic acid, methyl ester (stearic acid)
6	29.35	10.36	Cis-6-octadecenoic acid, methyl ester (petroselinic acid)
7	29.46	4.78	1,4-Dioxane
8	30.66	1.58	Cis, cis-9,12 octadecadienoic acid, methyl ester (linoleic acid)
9	38.75	2.24	Urea, N, N, dimethyl

(Figure 2 and 3).

DISCUSSION

The superficial bacterial infection of the skin (impetigo) is a global problem affecting mostly pre-school children. *S. aureus* and streptococci constitute a sizeable percentage of microorganisms detected from impetigo. Herein, results of antimicrobial susceptibility tests performed on MSSA isolates are entirely consistent with previously reported data by many researchers (Nagaraju et al., 2004; Liu et al., 2009). The reason for the high rate of resistance of MSSA to penicillin (95.8%) and erythromycin (79.2%) may be due to the overuse of topical penicillin or erythro-mycin, which is often prescribed for impetigo treatment. The incidence of MRSA isolates (20%) among the bacteria isolated from impetigo patients is consistent with many authors (Nishijima et al., 1995; Nishijima and Kurokawa, 2002).

The susceptibility of the MRSA isolates to the 11 used antimicrobial agents concludes that penicillin and erythromycin are no longer appropriate agents for treatment of impetigo. On the other hand, vancomycin or fusidic acid

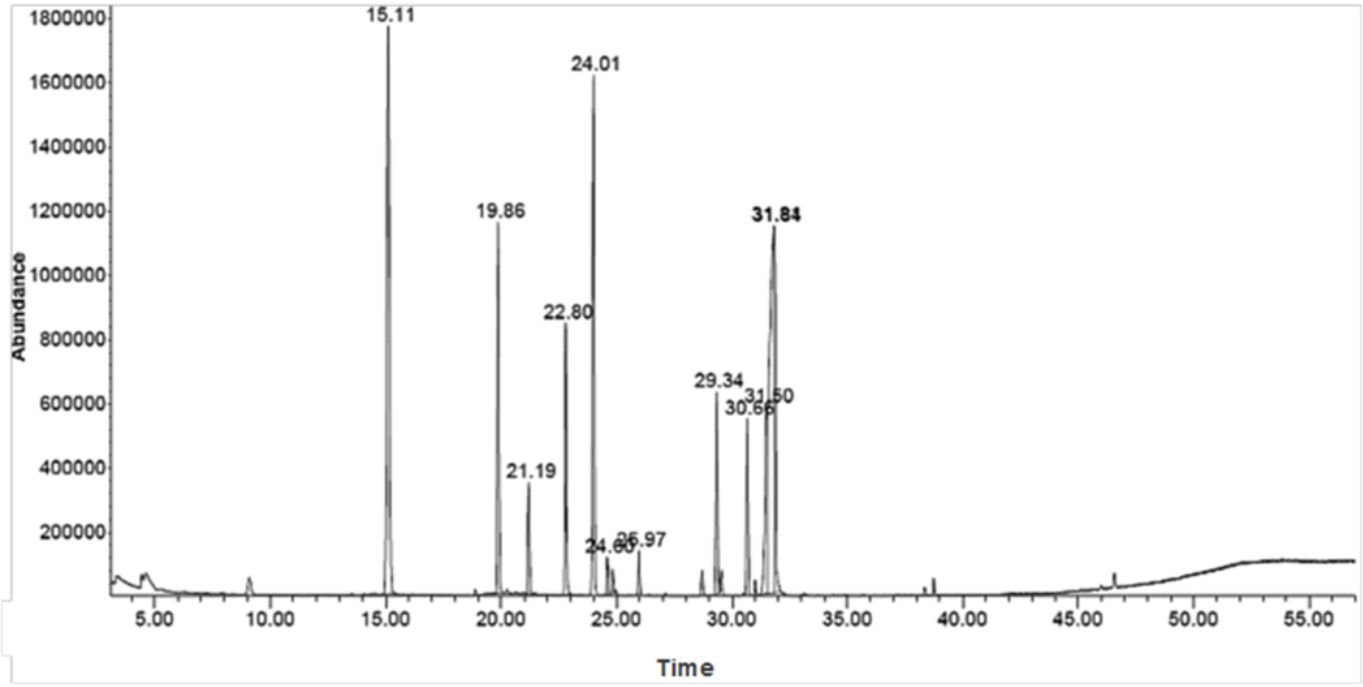

Figure 2. GC-MS chromatogram of methanolic extract of *S. platensis*.

may be effective antimicrobial agents for impetigo patients. These results support the finding by Nishijima et al. (2002). In addition, Liu et al. (2009) reported that the wiser choices for antibiotic drug therapy for patients with impetigo in China are mupirocin and fusidic acid. However, Dalager-Pedersen et al. (2011) found that about 50% of *S. aureus* isolates from impetigo patients were resistant to fusidic acid. This was explained as the *Staphylococcus* species quickly develop resistance to drugs and therefore can render agents such as mupirocin, fusidic acid, and erythromycin ineffective (Alsterholm et al., 2010; Rortveit et al., 2011).

Instead of the good results that can be obtained by some antibiotics in treatment of impetigo, many medical problems may be caused (Matsukura and Takahashi, 1998; Capoluongo et al., 2001). Hence, the need of natural active ingredients, such as *S. platensis* is increasing. The antibacterial activity of seven *S. platensis* extracts against *S. aureus* isolates from impetigo patients is significantly different. The methanolic extract showed the highest effective zone of inhibition against tested isolates. Similar results were also observed by Ozdemir et al. (2004) and Ranga Rao et al. (2010). More recently, Plazaa et al. (2010) reported that ethanol extract of *Spirulina* have higher antibacterial activity than other forms of the extracts. The differences may be due to the efficiency of the extraction methods to recover the active metabolites, solvents used (Tuney et al. 2006), susceptibility of strains (González del Val et al., 2001), assay methods and seasonal variation (Sasidharan et al., 2009).

The clinical application of topical cream made from both crude *S. platensis* and its methanolic extract revealed that patients treated with the algal extract cream (G1) had the best efficacy, no side effects and no recurrence during the follow-up period. These promising results for a topical algae formula could reduce the need for antibiotics therapy which is an expensive with more side effects. The results of this study showed that after a week of the topical *Spirulina* cream treatment, 12 of the 20 patients were clinically cured and seven patients had improved and these results were nearly similar to the results of Kuniyuki et al. (2005) used topical oxytetracycline hydrochloride (tetracycline) compared with a combination of topical tetracycline and oral antibiotics. After one week of topical tetracycline treatment, 22 of the 28 patients were clinically cured, and the remaining six patients had improvement. In the other treatment group, 14 patients out of 21 were clinically cured and seven patients improved by the combination of topical tetracycline and oral antibiotics. In our study, one patient in G2 (crude form) clinically failed. This result may be due to irregular use of the cream, two patients in G3 (placebo) were clinically improved, but not bacteriologically. This result may be due to the soothing effect of placebo cream. The current study provides a basis for the continued development of *Spirulina* cream for the treatment of impetigo.

Discussing the results of GC-MS analysis of the *S. platensis* and its methanolic extract conclude that the potential antimicrobial activity is attributed to different compounds belonging to a diverse range of chemical classes (Ozdemir et al., 2004). In both *S. platensis* and its extract, linoleic acid, an antibiotically active fatty acid, is present

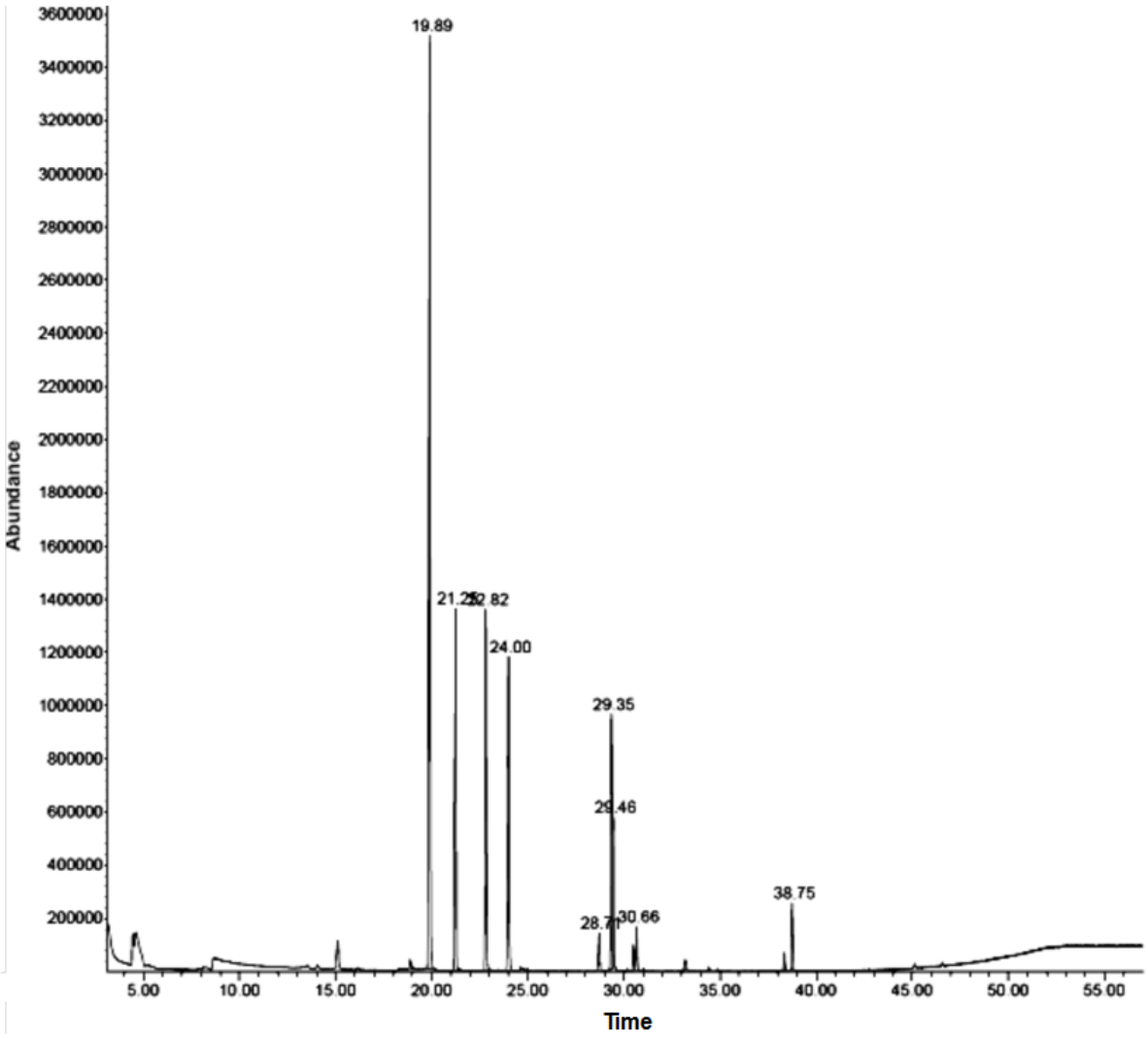

Figure 3. GC-MS chromatogram of *S. platensis* crude.

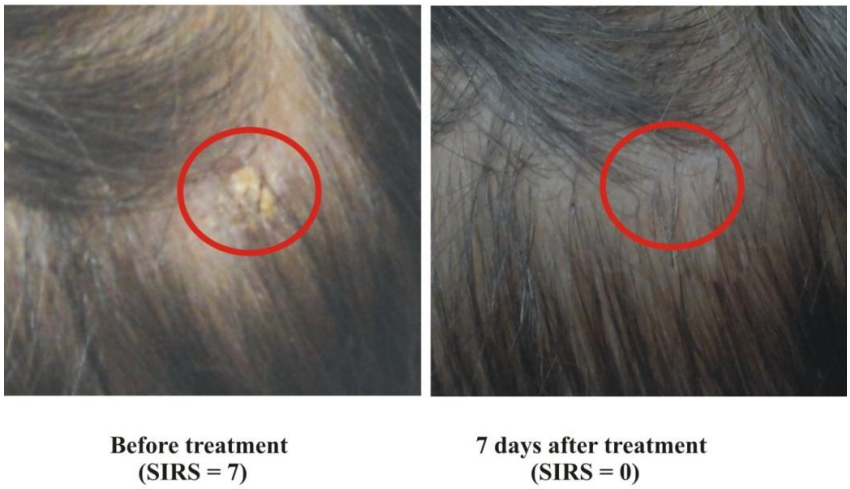

Figure 4. Impetigo of scalp of 5 years old female.

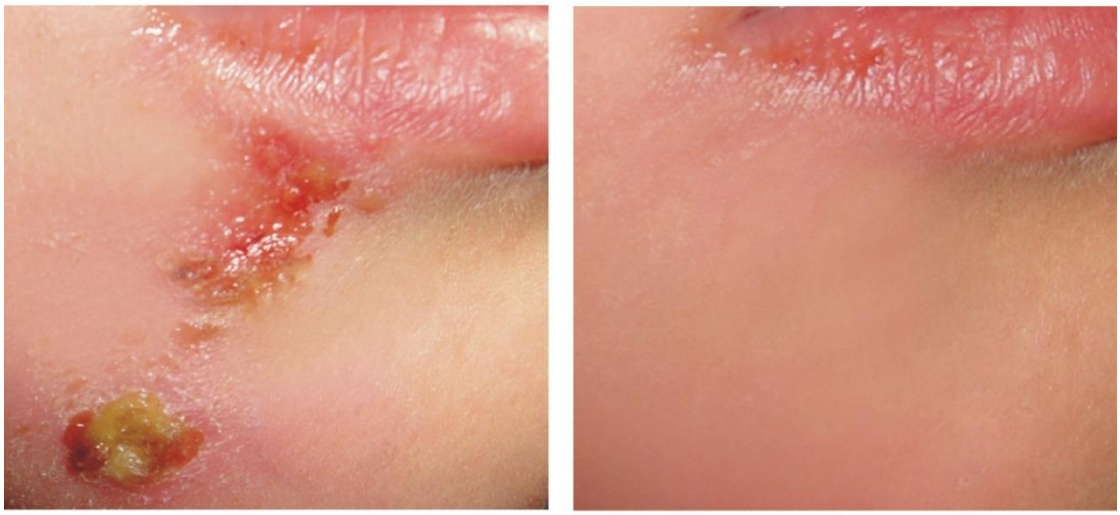

Figure 5. Impetigo of chin of 8 years old male.

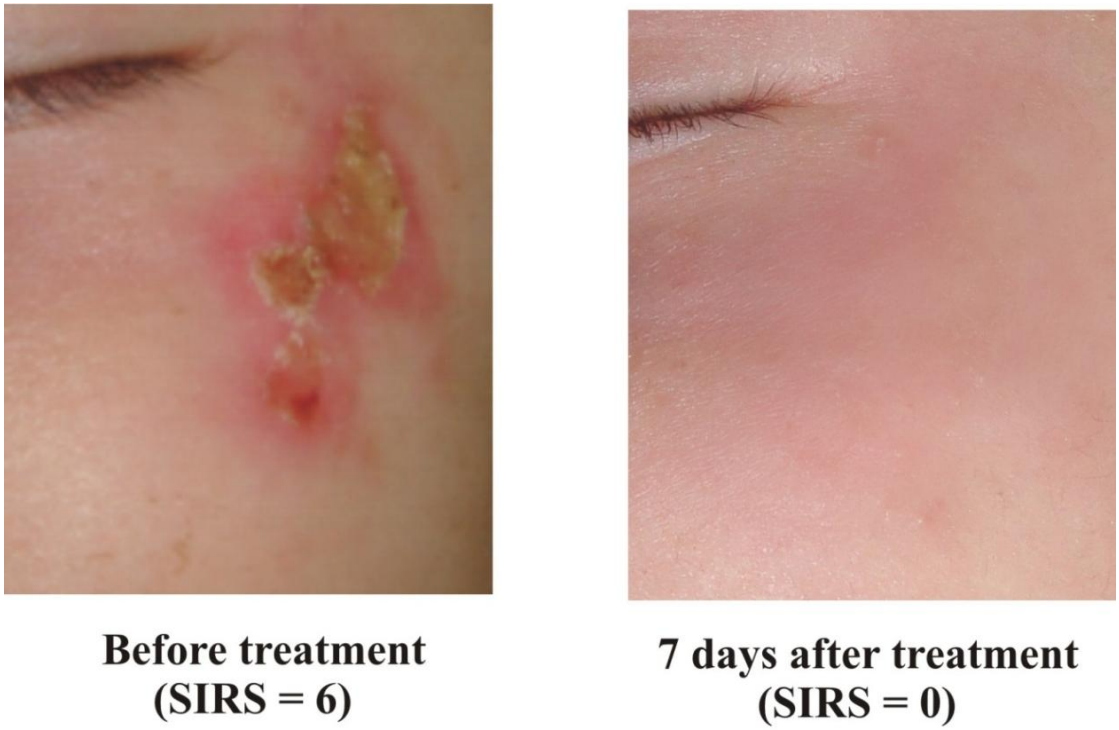

Figure 6. Impetigo of face of 2 years old male.

but with higher amount in the extract. However, linoleic acid amount is generally low in the tested samples (1.58 to 4.11%) compared to the other compounds. Thus, we propose that the linoleic acid is not only the antimicrobial agent in the tested *S. platensis* and its extract. The GC-MS analysis of the *S. platensis* methanolic extract shows other fatty acids that have also been reported to have some antimicrobial activity, especially palmitic, petroselinic, and palmitoleic acids. On the other hand, in crude alga, stearic, petroselinic and palmitic acids are present. The antimicrobial activity found in both *Spirulina* and its methanolic extract could be linked to a synergic effect of all these fatty acids. Our results agree with those reported by many authors (Benkendorff et al., 2005; Mendiola et al., 2007). It is assumed that the higher percentage of linoleic and palmitic acids in methanolic extracts of *Spirulina* compared to crude *Spirulina*, is most probably the cause of the higher antimicrobial activity of the methanolic *Spirulina* extract (active ingredients form treatment). Lipids kill microorganisms (Lampe et al., 1998) by leading to disruption of the cellular membrane as well as bacteria, because they can penetrate the extensive meshwork of peptidoglycan in the cell wall without visible changes and reach the bacterial membrane leading to its disintegration (Bergsson, 2005; Kumar et al., 2011).

In addition, the presence of squalane in methanolic extract of *Spirulina* as a major component (37.9%) may be an effective support in treatment of impetigo patients. There are many reports to explore the usefulness of squalane for treating skin. Squalane, the main component of skin surface polyunsaturated lipid shows some advantages for the skin as an emollient, antioxidant, for hydration and its antitumor activities (Rou-Huang et al., 2009).

Conclusions

Although, the *S. aureus* isolates collected from the impetigo patients are not resistant to some antibiotics, such as vancomycin and fusidic acid, treatment of impetigo using antimicrobial drug may cause serious medical problems. This adds urgency to the search for new infection-fighting natural compounds to control microbial infections. We summarize *in vitro* data of the antibacterial activity of the extracts of the laboratory grown culture of *S. platensis* against MRSA and MSSA isolates, which were collected from impetigo patients, and the *in vivo* efficacy and the safety of application of topical *S. platensis* creams (active ingredient and crude), in treatment of impetigo patients. The highest antibacterial activity of *S. platensis* extracts against *S. aureus* isolates was recorded with the methanolic one. Clinical application of active ingredients of *S. platensis* (G1) and crude *S. platensis* form (G2) gave an overall excellent response rates, with a high clinical efficacy of the treatment, especially in G1. Consequently, *S. platensis* methanolic extract, with its active antibiotically linoleic, palmitic, palmitoleic and petroselinic fatty acids, presents potential and safe alternative to synthetic antibiotics for the treatment of impetigo. However, further studies on large scale of population and multiple organisms to throw more light on the efficacy and safety of *Spirulina* cream in management of superficial bacterial infections especially impetigo are still required.

ACKNOWLEDGEMENTS

The authors thank Assistant Lecturer Lamiaa Mohamed, Microbiology Department, Faculty of Pharmacy, Tanta University, for helping with the preparation of topical creams from the crude alga and its methanolic extract. The manuscript benefited from the comments and suggestions of Prof. Mohamed Hamdy, Faculty of Science, Tanta University.

REFERENCES

Alsterholm M, Flytström I, Bergbrant IM, Faergemann J (2010). Fusidic acid-resistant *Staphylococcus aureus* in impetigo contagiosa and secondarily infected atopic dermatitis. Acta. Derm. Venereol. 90:52-57.

Benkendorff K, Davis AR, Rogers CN, Bremner JB (2005). Free fatty acids and sterols in the benthic spawn of aquatic mollusks and their associated antimicrobial properties. J. Exp. Mar. Biol. Ecol. 316:29-44.

Bergsson G (2005). Antimicrobial polypeptides and lipids as a part of innate defense mechanism of fish and human fetus. Thesis, Karoliska Institute, Stockholm.

Capoluongo E, Gilio A, Lavieri MM (2001). Genotypic and phenotypic characterization of *Staphylococcus aureus* strains isolated in subjects with atopic dermatitis. J. Dermatol. Sci. 26:145-155.

CLSI (2010). Performance standards for antimicrobial susceptibility testing. Twentieth informational supplement, document M100-S20, Clinical and Laboratory Standards Institute, Wayne, PA.

Cole C, Gaze wood J (2007). Diagnosis and treatment of impetigo. Am. Fan. Physician 75:859-864.

Dalager-Pedersen M, Søgaard M, Schønheyde, HC (2011). *Staphylococcus aureus* skin and soft tissue infections in primary healthcare in Denmark: a 12-year population-based study. Eur. J. Clin. Microbiol. Infect. Dis. 30:951-956.

González del Val A, Platas G, Basilio A, Cabello A, Gorrochategui J, Suay I, Vicente F, Portillo E, Jiménez del Río M, Reina GG, Peláez F (2001). Screening of antimicrobial activities in red, green and brown macroalgae from Gran Canaria (Canary Islands, Spain). Int. Microbiol. 4:35-40.

Hayashi T, Hayashi K (1996). Calcium spirulan, an inhibitor of enveloped virus replication, from a blue green alga *Spirulina platensis*. J. Nat. Prod. 59:83-87.

Hirahashi T, Matsumoto M, Hazeki K, Saeki Y, Ui M, Seya T (2002). Activation of the human innate immune system by *Spirulina* augmentation of interferon production and NK cytotoxicity by oral administration of hot water extract of *Spirulina platensis*. Int. Immunopharmacol. 2:423-434.

Hirschmann JV (2002). Impetigo: etiology and therapy. Curr. Clin. Top Infect. Dis. 22:42-51.

Kaushik P, Chauhan A (2008). *In vitro* antibacterial activity of laboratory grown culture of *Spirulina platensis*. Indian J. Microbiol. 48: 348-352.

Koneman EW, Allen SD, Janada WM, Schreckenberger PC, Winn WC (1992). The anaerobic bacteria. In: "Color Atlas and Text Book of Diagnostic Microbiology". 4th. Ed. J. B. Lippincott Company., Phildelphia. pp. 519-607.

Kumar V, Bhatnagar K, Srivastava JN (2011). Antibacterial activity of crude extracts of *Spirulina platensis* and its structural elucidation of bioactive compound. J. Med. Pl. Res. 5 (32):7043-7048.

Kuniyuki S, Nakano K, Maekawa N et al. (2005). Topical antibiotic treatment of impetigo with tetracycline. J. Dermatol. 32:788-792.
Lampe MF, Ballweber LM, Isaacs CE, Patton, DL, Stamm WE (1998). Killing of *Chlamydia trachomatis* by novel antimicrobial lipids adapted from compounds in human breast milk. Antimicrob. Agents Ch. 45:1239-1244.
Lewis LS, Steele RW (2011). Impetigo. Emedicine Specialist Dermatology (serial on line). Available from: http:www.emedicine.com
Liu Y, Kong F, Zhang X, Brown M, Ma L, Yang Y (2009). Antimicrobial susceptibility of *Staphylococcus aureus* isolated from children with impetigo in China from 2003 to 2007 shows community-associated methicillin-resistant *Staphylococcus aureus* to be uncommon and heterogeneous. Br. J. Dermatol. 16:1347-1350.
Mac Millan JB, Ernst-Russell MA, De Ropp JS, Molinski TF (2002). Lobocyclamides A-C, lipopeptides from a cryptic cyanobacterium mat containing *Lyngbya confervoides*. J. Org. Chem. 67:8210-8215.
Mala R, Sarojini M, Saravanababu S, Umadevi (2009). Screening for antimicrobial activity of crude extracts of *Spirulina Platensis*. J. Cell Tissue Res. 9(3):1951-1955.
Matsukura S, Takahashi K (1998). Two cases of allergic contact dermatitis due to a topical over the counter drug for sterilizing. Environ. Dermatol. 5:228-232.
Mendiola JA, Jaime L, Santoyo S, Reglero G, Cifuentes A, Ibanez E, Senoráns, FJ (2007). Screening of functional compounds in supercritical fluid extracts from *Spirulina platensis*. Food Chem. 102:1357-1367.
Nagaraju U, Bhat G, Kuruvila M, Pai GS, Jayalakshmi, Babu RP (2004). Methicillin-resistant *Staphylococcus aureus* in community-acquired pyoderma. Int. J. Dermatol. 43:412-414.
Nishijima S, Kurokawa I (2002). Antimicrobial resistance of *Staphylococcus aureus* isolated from skin infections. Int. J. Antimicrob. Agents 19: 241-243.
Nishijima S, Kurokawa I, Nakaya H (2002). Susceptibility change to antibiotics of *Staphylococcus aureus* strains isolated from skin infections between July 1994 and November 2000. J. Infect. Chemother 8:187-189.
Nishijima S, Nakagawa M, Sugiyama T, et al. (1995). Sensitivity of *Staphylococcus aureus* isolated from skin infections in 1994, to 19 antimicrobial agents. J. Int. Med. Res. 23:328-334.
Ozdemir G, Karabay NU, Dalay MC and Pazarbasi B (2004). Antibacterial activity of volatile component and various extracts of *Spirulina platensis*. Phytother. Res. 18:754-757.
Plazaa M, Santoyob S, Jaimeb L, García-Blairsy Reinac G, Herrerob M, Se˜noránsb FJ, Ibá˜neza E (2010). Screening for bioactive compounds from algae. J. Pharmaceut. Biomed. 51:450-455.
Purushothamrao K, Khaliq K, Sagare P, Patil SK, Kharat SS, Alpana K (2010). Formulation and evaluation of vanishing cream for scalp psoriasis. Int. J. Pharm. Sci. Tech. 4(1). 4(1): 32-41.
Ranga Rao A, Harshavardhan Reddy A, Aradhya SM (2010). Antibacterial properties of *Spirulina platensis*, *Haematococcus pluvialis*, *Botryococcus braunii* micro algal extracts. Curr.Trends Biotechnol. Pharm. 4:809-819.
Robert S, Anders RL, Niels F, Frabk E (2003). Evaluation of different disk diffusion/media for detection of methicillin resistance in *Staphylococcus aureus* and coagulase-negative staphylococci. APMIS 111:905-914.
Rørtveit S, Skutlaberg DH, Langeland N, Rortveit G (2011). Impetigo in a population over 8.5 years: incidence, fusidic acid resistance and molecular characteristics. J. Antimicrob. Chemother. 66:1360-1364.
Rou-Huang Z, Kulin Y, You Fang J (2009). Biological and pharmacological activities of squalene and related compounds: Potential uses in cosmetic dermatology. Molecules 14:540-554.
Sasidharan S, Darah I, Noordin MKMJ (2009). Screening antimicrobial activity of various extracts of *Gracilaria changii.* Pharm. Biol. 47: 72-76.
Shanmuga PK, Gnanamani A, Radhakrishnan N, Babu M (2002). Antimicrobial activity of *Datura alba.* Indian Drugs. 39:113-116.
Sieradzki K, Roberts RB, Haber SW, Tomaez A (1999). The development of vancomycin resistance in a patient with methicillin-resistance *Staphylococcus aureus* infection. N. Engl. J. Med. 340:517-523.
Subhashini J, Mahipal SVK, Reddy MC, Reddy MM, Rachamallu A, Reddanna P (2004). Molecular mechanisms in C-Phycocyanin induced apoptosis in human chronic myeloid leukemia cell line-K562. Biochem. Pharmacol. 68:453-462.
Susan MI, Kenneth DK, Daisy MB, Josefina AF, Naomi O, Azar N, Bahram N,Chris C, Dmitri D, Behzad K, Mark A (2011). NVC-422 topical gel for the treatment of impetigo. Int. J. Clin. Exp. Pathol. 4:587-595.
Tuney I, Cadirci BH, Unal D, Sukatar A (2006). Antimicrobial activities of the extracts of marine algae from the coast of Ural (izmir, Turkey). Turkish J. Biol. 30:171-175.
Williams REA, Gibson AG, Aitchison TCAG, Lever R, Mackie RM (1990). Assessment of contact-plate sampling technique and subsequent quantitative bacterial studies in atopic dermatitis. Br. J. Dermatol. 123:493-501.
Yang Y, Park Y, Cassada DA, Snow DD, Rogers DG, Lee J (2011). *In vitro* and *in vivo* safety assessment of edible blue-green algae, *Nostoc commune* var. *sphaeroides kützing* and *Spirulina platensis*. Food Chem. Toxicol. 49:1560-1564.
Zarrouk C (1966). Contribution a l´etude d´une cyanophycée. Influence de divers facteurs physiques et chimiques sur la croissance et la photosynthèse de *Spirulina maxima* (SETCH et GARDNER) GEITL. PhD Thesis, Université de Paris.