

Antibacterial activity of phenolic extract of *Spirulina platensis* and its structural elucidation of bioactive compound

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Abstract:

Spirulina as many other cyanobacteria species have the potential to produce a large number of antimicrobial substances, so they are considered as suitable organisms for exploitation as biocontrol agents of pathogenic bacteria. In the present study, antimicrobial activity of *Spirulina platensis* phenolic acid extract was investigated against pathogenic bacteria which cause sepsis in neonates. The algal extract was tested in vitro for their antibacterial effect against (*Bacillus cereus*, *Enterococcus casseliflavus*, *Kocuria kristinae*, *Staphylococcus aureus*, *Alloiooccus otitis*, *Roseomonas gilardii*, and *Aeromonas hydrophila*) using paper disc diffusion method and observed all these bacteria showed inhibition in growth by this extract. The bioactive components of *Spirulina platensis* have been assessed using GC-MS analysis. Eleven compounds in phenolic acid extract of the alga were identified. Bis(2-ethylhexyl) Phthtate (25.899%), Palmitic acid (14.263%), were the most prevailing compounds in phenolic acid extract.

Key words: *Spirulina platensis*, antibacterial activity, sepsis, paper-disc diffusion method, Gas chromatography-mass spectrometry (GC-MS) analysis.

Introduction

Neonatal sepsis continues to be a major cause of morbidity and mortality in the neonatal period. This is despite of improvement in antimicrobial therapy, advances in neonatal life support measures and the prompt recognition of perinatal risk factors for infection (**Feigin and Cherry, 1992**).

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Neonatal sepsis can be divided into early onset sepsis (EOS) and late onset sepsis (LOS) groups (**Karambin and Zarkesh, 2011**). There are different definitions for EOS, depending on the age at onset. Bacteremia and bacterial meningitis in less than 72 hour-old infants admitted to Neonatal Intensive Care Unit or in non-admitted infants aged less than 7 days are both grouped as EOS (**Schuchat, 2000; Hornik *et al.*, 2012; Edwards and Gonik, 2013**). Organisms that cause EOS are usually already colonized in mothers' rectovaginal area and can infect amniotic fluid, placenta, cervix or vagina. These pathogens are likely to ascend during amniotic sac rupture or before the onset of childbirth and cause intra-amniotic infection (**Rad *et al.*, 2016**).

Spirulina is used for health food, feed and for the biochemical products since 1980s. In fact, *Spirulina* is the most concentrated and nutritious whole food known to science, Moreover *Spirulina* has got no side effects and is non-toxic in nature (**Asghari *et al.*, 2016**). *Spirulina platensis* was also reported to present antimicrobial activity as well as to inhibit the replication of several viruses, such as *Herpes simplex* and HIV-1 (**Pyne *et al.*, 2017**).

Spirulina was recommended by both National Aeronautics and Space Administration (NASA) and the European Space Agency (ESA) as one of the primary foods during long-term space missions. *Spirulina* has been orally administered to patients as an anti-cancer and anti-viral agent although the molecular mechanism by which *Spirulina* acts on the immune system remains largely undefined (**Parages *et al.*, 2012; Alam *et al.*, 2013; Ismail *et al.*, 2015**). The present study aims to study *in vitro* antimicrobial activity of *Spirulina platensis* extract against pathogenic bacteria, which cause sepsis in neonates and separated and determined the most active component.

Materials and Methods

The study was a cross sectional one conducted on 50 neonates (age 0-28 days) with clinically suspected sepsis, who were admitted to the preterm unite in four hospitals in Sohag City, Egypt, namely, Sohag University Hospital (SUH), El helal Insurance Hospital (EIH), Life hospital (LH) and Lawfulness disassembly Hospital (LDH) and Some external intensive care units (ICU) in the centers, over a period of 18 months between March 2015 and August 2016.

The blue green alga (*Spirulina platensis*) as powder used in the present study was provided by Prof. Dr. Rawhya Abd El Latif Salah El Din, Prof. of Phycology, Faculty of Science, Al-Azhar University. Isolated strains of newborn babies' blood are *Bacillus cereus*, *Enterococcus casseliflavus*, *Kocuria kristinae*, *Staphylococcus aureus*, *Alloiococcus otitis* and other coagulase-negative *Staphylococci* such as *Staph. haemolyticus*, *Staph. Pseudintermedius*. Other negative strains such as *Roseomonas gilardii*, *Aeromonas hydrophila*.

Preparation of 24 hours pure culture

A loop full of each of the purified microorganisms was suspended on 5 ml of nutrient broth and incubated at 37°C for 24 hours (**Black, 1999**).

Preparation of extract

Phenolic compounds in *Spirulina platensis* were extracted as described by **Mattila et al. (2005)**.

Antimicrobial testing

Antibacterial activity of the extract was assayed using the disc diffusion method (**Bauer et al., 1996**). For inoculum preparation and assay of antibacterial activity, nutrient agar was used. The bacteria were sub cultured and routinely maintained on nutrient agar. Antimicrobial activity was evaluated using the disc diffusion technique in petri dishes. Discs loaded with the extracting agents were tested as controls (**Tuney et al., 2006**).

Chemical composition (GC-MS analysis)

The aliquots were directly injected into gas chromatography-mass spectrometry (GC-MS) for analysis. The method was based on **Hashimoto et al. (2001)**. The identification of the antimicrobial compounds was based on comparison of retention times and computer matching of the mass spectra with those of the National Institute of Standards and Technology (NIST) library and by direct comparison with published data.

Results and Discussion

Spirulina extract was tested against eight pathogens bacteria. The results are summarized in Table (1). The zone of inhibition of *Spirulina platensis* extract against bacteria was ranged between (7mm) to (14 mm). *Spirulina* extract showed antibacterial effect on wide range of bacteria and recorded highest zone of inhibition against Gram positive *Cocci Staph. Pseudintermedius* (14.0 mm) at concentration 100%, (11 mm) at conc. 75%, (10 mm) at conc. 50 % and (7mm) at conc. 25 % followed by *Bacillus cereus* (14.0 mm) at conc. 100% and (7.0 mm) at conc. 75 % followed by *Kocuria kristinae* (13 mm) at conc. 100% and (9.0 mm) at conc. 75 % then *Alloiococcus otitis* (11.0 mm) at conc. 100%, (10 mm) at conc. 75 %, (9.0 mm) at conc.50 % and (8.0 mm) at conc. 25 % then *Staph. aureus* (11.0 mm) at conc.100% and (8.0 mm)at conc. 75 % then *Enterococcus casseliflavus* (7.0 mm) at conc.100% .

For Gram negative bacteria, the maximum zone of inhibition was recorded in phenolic crude extract of *Spirulina platensis* against *Roseomonas gilardii* (10.0 mm) at conc. 100%, (7.5mm) at conc.75 %, 7.0 mm at conc. 50 % followed by *Aeromonas hydrophila* (7.5 mm) at conc. 100 only.

Bloor and England (1991) reported that extracellular metabolites produced by *Nostoc muscorum* inhibited the growth of *Bacillus circulans*. Also, **Austin (1992)** showed that the supernatants and extracts derived from a commercial heterotrophically grown spray-dried preparation of *Tetraselmis suecica* (Chlorophyceae) were observed to inhibit *Aeromonas hydrophila*, *A. salmonicida*, *Lactobacillus* sp., *Serratia liquefaciens*, *Staphylococcus epidermidis*, *Vibrio anguillarum*, *V. salmonicida* and *Yersinia ruckeri* type I.

Moreover, **Kaushik and Chauhan (2008)** reported that extracts of *Spirulina platensis* inhibited the growth of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Klebsiella pneumoniae*. They used hexane, ethyl acetate, dichloromethane and methanol to obtain the phenolic extracts and the methanolic extracts had the best results all this finding goes in harmony with the present investigation.

Table 1: Inhibition of Microorganisms by phenolic acid extract.

Test pathogen	Zone of inhibition (mm)			
	Conc.100%	Conc.75%	Conc.50%	Conc.25%
<i>Bacillus cereus</i>	14.0	7.0	----	----
<i>Kocuria kristinae</i>	13.0	9.0	----	----
<i>Staphylococcus pseudintermedius</i>	14.0	11.0	10.0	7.0
<i>Alloiococcus otitis</i>	11.0	10.0	9.0	8.0
<i>Roseomonas gilardii</i>	10.0	7.50	7.0	----
<i>Staphylococcus aureus</i>	11.0	8.0	----	----
<i>Aeromonas hydrophila</i>	7.50	----	----	----
<i>Enterococcus casseliflavus</i>	7.0	----	----	----

Although, the present investigation studied the antimicrobial activity of *Spirulina* against clinical isolates of *Bacillus cereus*, *Kocuria kristinae*, *Staphylococcus Pseudintermedius*, *Alloiococcus otitis*, *Roseomonas gilardii*, *Staphylococcus aureus*, *Aeromonas hydrophila*, *Enterococcus casseliflavus* and the finding from the present investigation is highly promising in view of the fact that the phenolic extract was successful inhibit the growth of the clinical isolates of the tested bacteria. Further research was carried out along this line may prove to be fruitful in bringing out a natural solution against these infection bacteria.

The present study reveals that the use of organic solvent such as ethyl acetate and methanol) in the preparation of *Spirulina* extraction and identified more compounds by GC-MS. From GC-MS analysis of solvent extracts of *Spirulina*, it was observed that ethyl acetate extract showed eleven components (Table 2 and Figure 1).

The most abundant compounds were Bis(2-ethylhexyl) Phthate (25.899%), Palmitic acid (14.263%), 3-Hydroxy-Butanoic acid (10.795%), Curan-17-oic acid, 19-(acetyloxy)-2,16-didehydro-20-hydroxy-,methyl ester (5.732%),

Hexadecanoic acid ethyl ester (5.720%), Phenol-d5 (1.661%), Methyl isoeugenol 2 (0.463 %), 3,5-di-tert-butyl- phenol (0.303%), 4-Tert-butyl-2,6,dinitro-phenol (0.131%), Clemastine (0.076%), Retalin (0.050%) were also obtained as well as some biological activities are shown in Table (2).

In present study, Bis(2-ethylhexyl) Phthate and Palmitic acid were identified in phenolic acid extract of *Spirulina* active compounds associated with the antibacterial properties. The maximum amount of Bis (2-ethylhexyl) Phthate compound present is (25.899%) which is responsible for antibacterial activity where exhibited strong cytotoxicity against human colon carcinoma cells. The DEHP is considered as pro inflammatory agent in other studies (**Oie *et al.*, 1997; Gourlay *et al.*, 2003**). It have antimicrobial activity against a number of Gram-positive bacteria; *Bacillus subtilis*, *Staphylococcus aureus* and *Streptococcus equosemens* but the inhibition of Gram negative bacteria was lower; *Escherichia coli*, *Pseudomonas aeruginosa* and *Closteridium perfringens*. On the other hand, the compound has strong effect against *C. albicans* (**Rowshanul and Rezaul, 2009**).

Hexadecanoic acid methyl ester is also known as palmitic acid ester. The fatty acid being effective as antioxidant, pesticide, anti-androgenic, nematocide, flavoring agent, hypocholesterolemic and lubricant. Similarly, **Gopalakrishnan and Vadivel (2011)** and **Vinay Kumar *et al.* (2011)** reported the presence of hexadecanoic acid methyl ester in the methanol extract of *Spirulina platensis* and the ethanolic extract of *Mussaenda frondosa*, respectively.

In contrast to the above study, the present investigation reported Hexadecanoic acid ethyl ester in the phenolic acid extract of entire alga of *Spirulina platensis*.

Table (2): GC-MS analysis of therapeutic activity and different compounds identified in phenolic acid and hydrolysate extract of *Spirulina platensis*.

No	R.T	Name of the Compound	Molecular Formula	Molecular Weight	Peak Area%	Bioactive/ Use
1	10.161	3-Hydroxy-Butanoic acid	C ₄ H ₈ O ₃	104.105	10.795	An energy source by the brain during fasting in humans. Also used to synthesis biodegradable plastics. (Cerone <i>et al.</i> , 2007)
2	11.507	Curan-17-oic acid,19-(aceryloxy)-2,16-didehydro-20-hydroxy, methyl ester	C ₂₀ H ₃₂ N ₂ O ₅	340.423	5.732	Anti-yeast activity. (Al-Marzoqi <i>et al.</i> , 2015)
3	12.469	Phenol-d5	C ₆ H ₆ O	99.144	1.661	Antioxidant civity and syntheses of anti-bacterial & anti-carcinogenic compounds. (Dehnen <i>et al.</i> , 1977)
4	14.626	Clemastine	C ₂₁ H ₂₆ ClN ₂ O	343.895	0.076	Antihistamine and anticholinergic (Kornhuber <i>et al.</i> , 2011)
5	14.649	Ritalin	C ₁₄ H ₁₉ NO ₂	233.311	0.050	Stimulants work by increasing dopamine levels in the brain. (Markowitz <i>et al.</i> , 2003)
6	20.857	Methyl isoeugenol 2	C ₁₁ H ₁₄ O ₂	178.231	0.463	Anticonvulsant, anxiolytic and antidepressant effects. (Paula <i>et al.</i> , 2012)
7	23.713	Palmitic acid	C ₁₆ H ₃₂ O ₂	256.4	14.263	Antibacterial activity (McGaw <i>et al.</i> , 2002)
8	23.759	Hexadecanoic acid ethyl ester	C ₁₈ H ₃₆ O ₂	284	5.720	Antioxidant, Hypocholesterolemic, Nematicide, Anti-Androgenic, Pesticide, Anti-androgenic, Flavor, Hemolytic, 5-Alpha-Reductase- inhibitor, Lubricant (Siddabathuni <i>et al.</i> , 2014)
9	32.595	Bis(2-ethylhexyl) Phthalate	C ₂₄ H ₃₈ O ₄	390.56	25.899	Antibacterial activity, cytotoxic activity, anticancer (Lee <i>et al.</i> , 2000; Gourlay <i>et al.</i> , 2003; Oie <i>et al.</i> , 1997)
10	40.464	4-Tert-butyl-2,6-dinitro-phenol	C ₁₀ H ₁₂ N ₂ O ₅	240.215	0.131	Herbicides, Pesticides, for aquatic weed control and for dodder control. (Lehn and Weinheim, 1995)
11	52.652	3,5-di-tert-butyl-phenol	C ₁₄ H ₂₂ O	206.329	0.303	Sedation for mechanically ventilated adults, and procedural sedation (Riezzo <i>et al.</i> , 2009)

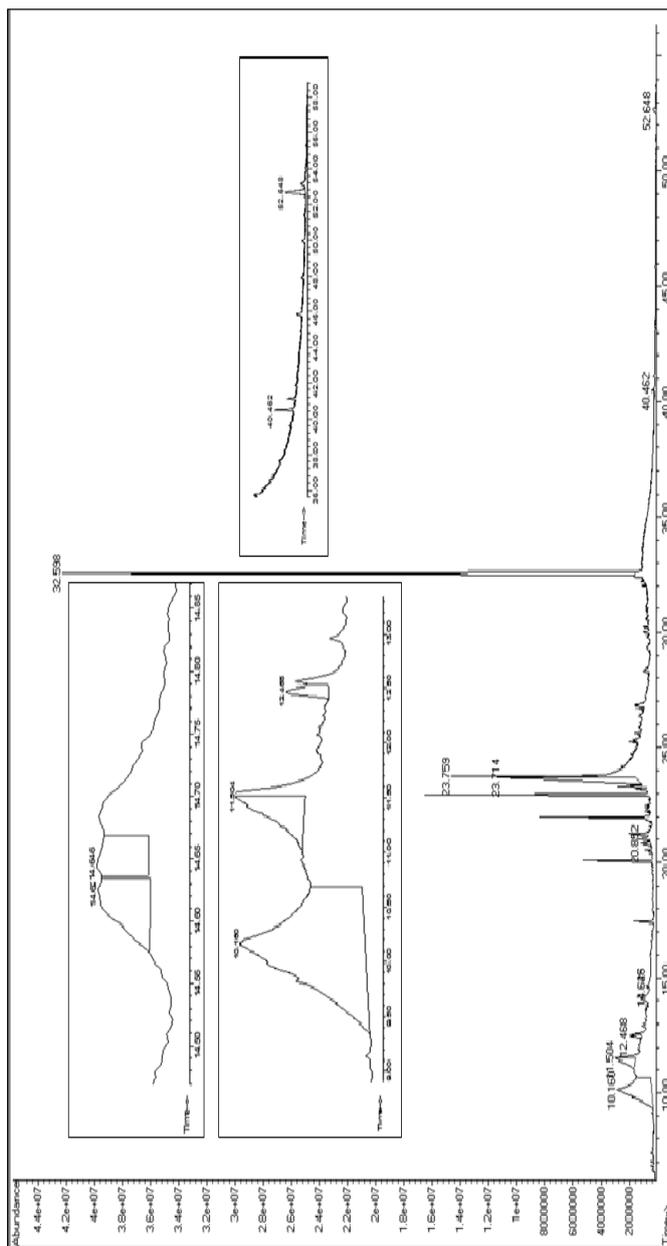


Figure (1): GC-MS Chromatogram of the phenolic acid and hydrolysate extract of *Spirulina platensis*

The antibacterial activity of algal compounds were extracted from algae depends upon the type of solvent used for extraction. The present study revealed that the use of organic solvents in the preparation of algal extracts provide more consistent antimicrobial activity. This observation clearly indicates that the polarity of antimicrobial compounds make them more readily extracted by organic solvents and using organic solvent does not negatively affect their bioactivity against antibacterial and antifungal species. Many investigations mentioned that the methanol extracts of

Nostoc muscorum revealed antibacterial activity on *Sclerotinia sclerotiorum* by **Ishida et al. (1997)**. Also the methanolic extract of a blue green alga has been investigated by **Kumar et al. (2006)** for in vitro antimicrobial activity against *Proteus vulgaris*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Aspergillus niger*, *Aspergillus flavus* and *Rhizopus nigricans* using agar cup diffusion method. The antimicrobial activity of methanolic extract of *S. platensis* was also explained by **Demule et al. (1996)** due to the presence of γ -Linolenic acid and compound was also present in the methanol extract. In the present study in phenolic acid extract found Bis(2-ethylhexyl) Phthate, Palmitic acid, Phenol-d5, Clemastine and Hexadecanoic acid ethyl ester which have antimicrobial and cytotoxic activity, anti-inflammatory, anti-cancer, anti-histaminic, antioxidant.

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النشاط المضاد للبكتيريا لمستخلص الفينول لطحلب *Spirulina platensis* وفصل المركبات الفعالة بيولوجيا

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طحلب *Spirulina* والعديد من الطحالب الخضراء المزرققة لديهم القدرة على إنتاج عدد كبير من المواد الفعالة المضادة للميكروبات، لذلك تعتبر تلك الكائنات مناسبة لاستخدامها كعوامل للمكافحة البيولوجية للبكتيريا المسببة للأمراض. وعلى ضوء تلك المعلومات تم استخدام مستخلص حمض الفينول من طحلب *Spirulina platensis* كمضاد للبكتيريا المسببة لأمراض التسمم الدموي للأطفال حديثي الولادة في هذه الدراسة. وعليه تم اختبار التأثير الضد بكتيري لمستخلص الطحلب على الأنواع الآتية الذكر (*Bacillus cereus*, *Enterococcus casseliflavus*, *Kocuria kristinae*, *Staphylococcus aureus*, *Alloiococcus otitis*, *Roseomonas gilardii*, and *Aeromonas hydrophila*) باستخدام طريقة الانتشار بالقرص الورقي، وقد لوحظ أن لمستخلص الطحلب تأثيرا مثبطا للنمو على جميع أنواع البكتيريا سالفة الذكر. وقد تم تقييم المواد الفعالة من الطحلب بواسطة استخدام تحليل GC-MS. أوضحت الدراسة وجود إحدى عشر مركب فعال من مستخلص حمض الفينول من الطحلب. وكانت أكثر المركبات السائدة في المستخلص هي (Bis (2-ethylhexyl) Phthate) ويحتوي على نسبة (25.89%) كما احتوى حمض بالميتيك على نسبة (14.263%)، وهي مركبات معروفة بقدرتها على التأثير الضد ميكروبي.