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Phytochemical screening and antibacterial activity of crude extracts of *Spirulina* species isolated from Lonar Lake

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Abstract
Spirulina plank tonic blue green algae are gaining increasing attention because of its nutritional and medicinal properties. The attempt of this work was to compare the antibacterial activity of *Spirulina* sp. isolated from Lonar Lake commercially available in Market. Organic solvents and aqueous extracts of *Spirulina* were prepared and Phytochemical were identified and the extracts were evaluated for their antibacterial activities against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, *Proteus vulgaris*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* by disc diffusion method. Methanol, ethanol, acetone and aqueous extracts of *Spirulina platensis* showed good antibacterial activity against *Staphylococcus aureus*, *Salmonella typhi* and *Escherichia coli* by both diffusion methods. Preliminary Phytochemical analysis reveals that the presence of alkaloids, carbohydrates, glycosides, sterols, terpenoids, proteins, tannins, saponins, and flavonoids whereas proteins and tannins were not present in methanol extract. Ethanol extract showed the presence of terpenoids and flavonoids while including these glycosides are absent in acetone extract. Saponins present in all the extracts.

Keywords: antibacterial activity, Lonar Lake, *Spirulina* sp., phytochemicals

Introduction
Spirulina, a cyanobacteria is a photoautrophic microorganism, widely distributed in nature and is consumed as human food supplement for centuries because of its best known nutritional value. It contains 78% proteins, vitamins, 4-7% lipids, minerals, carbohydrates and some natural pigments but average may vary due to growing condition (Anbarasan, 2010) [1]. Due to the presence of these phytonutrients, it has corrective properties against several diseases like cancer, hypertension, hypercholesterolemia, diabetes, anaemia etc. (Bhowmik *et al*., 2009) [4].

Regarding the origin of salinity and alkalinity of Lonar lake water it is argued that the evaporation of the lake water in the absence of the drain was responsible for the alkalify of the lake waters (Blandford, 1868) [5]. Lake Alkalinity is also due the conversion of sulphate ion to carbonate through the intermediate formation of sulphide. All these characteristics of lake resulted into an extreme alkaline ecosystem with all different microbial type prevailing in and around the lake (Jhingran and Rao 1954) [8]. Microorganisms like *Spirulina* and other micro algae are predominant as primary producer are present along with alkaline bacteria and fungi (Vonshak, 1997) [13]. *Spirulina* is symbiotic, multicellular and filamentous blue-green algae which fix the nitrogen from air. *Spirulina* are found naturally in saline lakes and rivers in tropical regions (Dillon *et al*., 1995) [6]. *Spirulina* has been studied under various scientific researchers for more than 20 years and consumed in more than 70 countries worldwide. It contains an unusually high amount of protein, between the ranges of 55% to 77% by dry weight, depending upon the source (WHO, 1976). This algal species is a potentially source of nutrition for human health, as well as an important resource for fighting bacterial infections in human (Sánchez *et al.*, 2003) [12]. Presently it is very useful nutritious for prevention and symptomatic treatment such as antiviral activity, blood nourishment, enhancing immunity against bacteria or foreign substances for body and recovery during convalescent period (Quoc, 1996) [11]. The microbial ecosystem of this Lonar lake has not been studied in detail specially with reference to *Spirulina*. Hence the attempt of this work was to isolate *Spirulina* from the Lonar lake and identified their antibacterial activity and presence of phytochemicals.

Materials and Methods
Collection of *Spirulina* species: *Spirulina* is isolated from Lonar Lake and the coomercial Spirulina sample were purchased from the local market of Kopargaon

Identification of lonar lake sample: Identification of the *spirulina* was done by wet mounting and observing morphological characters

Preparation of *Spirulina* extracts: The commercial *Spirulina platensis* powder and isolated *Spirulina* sp. from Lonar lake were successfully extracted with aqueous and organic solvent (ethanol, methanol and acetone).

a. **Preparation of aqueous extract:** A 20g of freshly dried Spirulina sp. from Lonar lake and Spirulina platensis, were soaked separately in 200 mL of sterile distilled water and kept at room temperature for 48h. Each extract was stirred every 10 to 12 h using a sterile glass rod and filtered through muslin cloth. The obtained aqueous extract was concentrated in oven. The extracts were stored at 40C for further use.

b. Preparation of solvent extract: The organic solvent extracts were prepared by adding 20 g of freshly dried Spirulina sp. from Lonar lake and Spirulina platensis (powder) separetely in 200 mL of organic solvent (acetone, ethanol and methanol) in screw-capped reagent bottles and then kept at room temperature for 48h. Then the supernatant of each bottle was filtered with ordinary filter paper and each extract was concentrated in oven. Dried extracts were stored in labeled sterile screw capped bottles at 4^0 C and later used for the *in vitro* study.

Phytochemical screening: Preliminary qualitative phytochemical screening was carried out with the following methods (Harborne, 1998; Khandelwal, 2001) [7, 9].

Test for Tannins: To 0.5 ml of extract solution, 1 ml of distilled water and 1 to 2 drops of ferric chloride solution was added, observed for blue or green black coloration.

Test for Saponins: Two ml of distilled water was added to 2 ml of the test solution shaken well and observed for frothing.

Test for Flavonoids: A volume of 1.5 ml of 50 % methanol was added to 4 ml of the extracts. The solution and magnesium metal was added and warmed. Then, 5 to 6 drops of concentrated hydrochloric acid was added to the solution and observed for red coloration.

Test for Steroids (Salkwoski's test): Five drops of concentrated sulphuric acid (H2SO4) was added to 2 ml of each extract and observed for red coloration.

Test for Glycosides: To 4 ml of extract solution and add few drops of glacial acetic acid, few drops of ferric chloride and concentrated sulphuric acid and observed for a reddish brown coloration at the junction of 2 layers and bluish green colour in upper layer.

Test for Alkaloids: To 4 ml of extract filtrate, a drop of Mayer"s reagent was added along the sides of test tube. Creamy yellow or white precipitate indicates that the test is positive.

Test for Anthraquinones: One gram of powdered plant material was taken and extracted with 10 ml of hot water for five minutes and filtered. Filtrate was extracted with 10 ml of CCl4 then CCl4 layer was taken off. Five ml water and 5 ml dilute ammonia solution was added. No free anthraquinones were revealed as absence of appearance of pink to cherry red colour. One gram of second sample of the same plant material was extracted with 10 ml of ferric chloride solution and 5 ml of hydrochloric acid then it was heated on water bath for 10 minutes and filtered. Filtrate was cooled and treated as mentioned above.

Test for phenolic compounds: Two ml of extract was diluted to 5 ml with distilled water. To this a few drops of neutral 5 % ferric chloride solution was added. A dark green colour indicates the presence of phenolic compounds

Bacterial cultures: The standard pathogenic bacterial cultures were procured from IMTECH, Chandigarh, India and used in the present study (Table 1). The bacterial cultures were rejuvenated in Mueller- Hinton broth (Hi-media laboratories, Mumbai, India) at 37^0C for 18h and then stocked at 4^0C in Mueller-Hinton Agar. The inoculum size of the bacterial culture was standardized according to the National committee for Clinical Laboratory Standards (NCCLS, 2002) [10] guideline. The pathogenic bacterial culture was inoculated into sterile Nutrient broth and incubated at 37^0C for 3h until the culture attained a turbidity of 0.5 McFarland units. The final inoculum size was standardized to 10^5 CFU/mL with the help of SPC and Nephlo-turbidometer.

Table 1: Bacterial cultures used in study (IMTECH, Chandigarh, India)

Bacterial Pathogens	MTCC Number
Proteus vulgaris	426
Staphylococcus aureus	96
Escherichia coli	739
Pseudomonas aeruginosa	424
Klebsiella pneumoniae	109
Salmonella typhi	733

Preparation of disc for antibacterial activities: The Methanol, n-Hexane and Petroleum ether extracts were prepared in their respective solvents and the sterile blotting paper disc (10 mm) were soaked in the diluted extract in such concentration that the amount of solution absorbed by each disc was 1mg, 2mg, 3mg, 4mg, 5mg of each extracts of resins of *Spirulina platensis*. The prepared disc were dried in controlled temperature to remove excess of solvent and used in study.

Antibacterial activity using disc diffusion method: The modified paper disc diffusion method was employed to determine the antibacterial activity of Methanol, n-Hexane and the Petroleum ether extracts of resins of *Spirulina platensis*. Turbidity of inoculums was matched with McFarland turbidity standard (NCCLS, 2002) [10]. Inoculums were spread over the Nutrient agar plate using a sterile cotton swab in order to get a uniform microbial growth. Then the prepared antibacterial disc were placed over the lawn and pressed slightly along with positive and negative controls. Ampicillin 10 mcg/disc (Hi-Media, Mumbai) were used as positive control while disc soaked in various organic solvents and dried were placed on lawns as negative control. The plates were incubated for 18h at 37^0C. The antibacterial activity was evaluated and diameters of inhibition zones were measured. Experiment was carried out in triplicate and the averages diameter of zone of inhibition was recorded. The antibacterial activity was classified as strong (>20mm), moderate (16-19mm) and mild (12-15mm) and less than 12mm was taken as inactive.

Results and Discussion

The potential use of algal biomass for the benefit of mankind has been intensively reviewed in the last few years. Algae are considered to be the most efficient biological system for harvesting solar energy and for the production of organic

compounds via the photosynthetic process. Many species of algae can be induced to produce particularly high concentrations of chosen compounds (proteins, carbohydrates, lipids and pigments) that of commercial value (Vonshak, 1997) [13]. *Spirulina platensis* is a plankton, filamentous, spiral-shaped, multicellular photosynthetic, blue-green microalga. *Spirulina* is considered as an excellent food, lacking toxicity and having corrective properties against viral attacks, anemia, tumor growth and malnutrition.It has been reported as animal and fish food supplements (Becker, 2004). Other benefits of *Spirulina* as antifungal (Moore *et al.*, 1984; Clardy *et al.*,1990), herbicidal compounds (Entzeroth *et al.*, 1985), larvicidal agent for mosquitos (Ahmad *et al.*, 2004) and biofertilizer (Aly *et al.*, 2004).

It is dominating the microflora of alkaline saline waters with pH of up to 11.0 and can exist in various types of habitats, namely soils; marches, fresh, brackish and seawaters, thermal springs and waters of industrial and domestic uses.

In the present study *Spirulina* sp. was isolated from Lonar lake and their antibacterial activity compared with commercially available *Spirulina platensis*. Lonar is the third largest natural salt-water lake in the world, with a diameter of 1800 meter. Very few study carried out on *Spirulina* of Lonar lake.

Antibacterial activity of *Spirulina platensis* against tested bacterial pathogens by disc diffusion method represented in Table 1. Aqueous extract of *Spirulina platensis* showed strong antibacterial activity against *S. aureus* while moderate antibacterial against *S. typhi* and *P. aeruginosa*, and mild antibacterial against E. coli, *P.vulgaris* and *K. pneumoniae.* Ethanol and methanol extracts of *Spirulina platensis* were mild antibacterial against *E.coli, S. aureus, S. typhi* and *K. pneumoniae* whereas resistant to *P. vulgaris* and *P. aeruginosa*. Acetone extract of *Spirulina platensis* was mild antibacterial against *S. aureus* and *S. typhi* whereas resistant to *E. coli, P. vulgaris* and *K. pneumonia* and *P. aeruginosa*.

Table 2: Antibacterial activity of *Spirulina species* isolated from Lonar Lake, extracted with different solvents by Disc diffusion method. (Zone of inhibition in mm / disc size 5 mm)

SN	Microorganism	Methanol	Ethanol	Acetone	Aqueous
1	*Escherichia coli*	14	9	9	11
2	*Staphylococcus aureus*	12	8	12	13
3	*Salmonella typhi*	12	10	15	17
4	*Proteus vulgaris*	-	-	-	12
5	*Klebsiella pneumonae*	12	11	10	16
6	*Pseudomonas aeruginosa*	-	-	-	13

Where: Antibacterial activity (Zone of inhibition in mm) Mild=9-12, Moderate=13-16, Strong=17-20.

Table 3: Antibacterial activity of *Spirulina platensis* from Market extracted with different solvents by disc diffusion method. Zone of inhibition in mm / disc size 5 mm

S. No	Microorganism	Aqueous	Ethanol	Acetone	Methanol
1	*Escherichia coli*	11	9	-	10
2	*Staphylococcus aureus*	20	12	11	10
3	*Salmonella typhi*	15	10	10	9
4	*Proteus vulgaris*	11	-	-	-
5	*Klebsiella pneumonae*	12	10	-	9
6	*Pseudomonas aeruginosa*	14	-	-	-

Where: Antibacterial activity (Zone of inhibition in mm) Mild=9-12, Moderate=13-16, Strong=17-20

Antibacterial activity of *Spirulna* sp. from Lonar lake against tested bacterial pathogens by disc diffusion method (Table 1and 3). Aqueous extract of *Spirulina* sp. from Lonar Lake was strong antibacterial against *S. typhi* whereas moderate antibacterial against *K. pneumoniae, S. aureus* and *P. aeruginosa* and mild antibacterial against *P. vulgaris* and *E.coli*. Acetone extract of *Spirulina* sp. was moderate antibacterial against *S. typhi* whereas mild antibacterial against *E. coli, S. aureus* and *K. pneumoniae.* Methanol extract of *Spirulina* sp. was moderate antibacterial against *E. coli* whereas mild antibacterial against *S. aureus, S. typhi* and *K. pneumoniae*. Ethanol extract was mild antibacterial against all tested bacterial pathogens except *P. vulgaris* and *P. aeruginosa.* Acetone and methanol extracts were resistant to *P. vulgaris* and *P. aeruginosa.*

The comparative study of antibacterial activity of *Spirulina* from lonar lake was showed that the maximum antibacterial activity than the commercial *Spirulina*. Similarly Abd El-Baky *et al., (*2009) reported that the cell extract of *S. maxima* has shown antimicrobial activity against *Bacillus subtillis, Streptococcus aureus, Saccharomyces cerevisiae* and *Candida albicans.*

As the isolated Spirulina sp. as well as commercial Spirulina platensis showed antibacterial activity, both strains can be prevent or treat enteric infection caused by *E. coli, S. aureus, K. pneumoniae, Pr. vulgaris, S. typhi* because these pathogens are the main cause of diarrhoea, dysentery, typhoid and gastroenteritis.

Phytochemical Analysis

Spirulina platensis chemical composition includes proteins (55%-70%), carbohydrates (15%-25%), essential fatty acids (18%) vitamins, minerals and pigments like carotenes, chlorophyll *a* and phycocyanin (Ciferri, 1983). *Spirulina* excretes variable quantities of products from its metabolism such as organic acids, vitamins, and phytohormones (Sánchez *et al.*, 2003) [12].

Similarly in the present study aqueous extract showed the presence of alkaloids, carbohydrates, glycosides, sterols, terpenoids, proteins, tannins, saponins, and flavonoids whereas proteins and tannins were not present in methanol extract. Ethanol extract showed the presence of terpenoids and flavonoids while including these glycosides are absent in acetone extract. Saponins present in all the extracts (Table 5).

Table 4: Phytochemical analysis of commercially available *Spirulina platensis*

Phytochemical Constitutes	Methanol Extract	Ethanol Extract	Acetone Extract	Aqueous Extract
Alkaloids:	+	+	+	+
Carbohydrates:	-	-	+	+
Glycosides:	-	-	-	+
Sterols:				
Terpenoids	-	+	-	+
Proteins and amino acids:	-	+	+	+
Tannins	-	+	+	+
Saponins:	+	+	+	+
flavones and flavonoids:	-	+	-	+

In the present study results indicate that *Spirulina* sp. from

Lonar lake as well as commercial *Spirulina platensis* having antibacterial activity against tested pathogens. Both of these isolates showed the presence of all tested phytochemicals except tepenoids and flavonoids were absent in *Spirulina* sp. isolated from Lonar lake. These isolates also having various amino acids. Hence these isolates might be used as food supplement as well as for the treatment of various diseases.

Table 5: Phytochemical analysis of Spirulina sp. from Lonar Lake

Phytochemical Constitutes	Methanol Extract	Ethanol Extract	Acetone Extract	Aqueous Extract
Alkaloids	+	+	+	+
Carbohydrates	-	-	-	-
Glycosides	-	-	-	-
Sterols	+	+	+	-
Triterpenoids	-	-	-	-
Tannins phenolic compounds	-	-	-	-
Proteins and amino acids	+	+	+	-
Saponins	+	+	+	+
Flavones and flavonoids	-	+	+	+

Phytochemical analysis of *Spirulina* sp. from Lonar Lake is given in Table 6. Aqueous and methanol extracts showed the presence of alkaloids, carbohydrates, glycosides, sterols, proteins, tannins, and saponins whereas flavonoids were present in methanol but absent in aqueous extract. Ethanol and acetone extract showed the presence of alkaloids and flavonoids while glycosides, carbohydrates, were absent in both the extract. Sterols tannins and proteins were present in ethanol extracts whereas absent in acetone extracts. Saponin was present in all the extracts.

The results obtained from the present study concerning the antibacterial activity of *Spirulina platensis* extracted with different solvents against different species of bacteria are recorded in table 1-4. Aqueous extract of *Spirulina* isolated from Lonar Lake showed maximum antibacterial activity of 16.0 mm against *Klebsiella pneumonae* both agar well and disc diffusion methods. Proteus vulgaris and *Pseudomonas aeruginosa* are resistant to both methanol, ethanol and aqueous extracts of *Spirulina platensis* commercially available and Spirulina sp. from Lonar Lake. *Staphylococcus aureus, Salmonella typhi* and *Escherichia coli* showed the zone of inhibition in the range of 10.0 mm to 15.0 mm in methanol, ethanol, acetone and aqueous extracts. Aqueous extract of *Spirulina platensis* showed strong antibacterial activity of 20.0mm and 18.0 mm against Staphylococcus aureus by the both disc diffusion and agar-well diffusion methods respectively. Methanol, ethanol, acetone and aqueous extracts showed average antibacterial activity of 9.0mm to 13.0mm against *Staphylococcus aureus, Salmonella typhi* and *Escherichia coli* by both diffusion methods. The result showed the potent antibacterial activity against the bacteria. Extracts of *Spirulina platensis*, obtained by different solvents exhibited different degrees of antimicrobial activity on both Gram-positive and Gram-negative organisms (Ozdemir, *et al.* 2001). Most antimicrobial active components that have been identified are not water soluble and organic solvent extracts have been found to be more potent. This is contradictory to our results because our water extracts are more potent than organic solvent extracts. Extract of water have been found to show more consistent antimicrobial activity as compared to organic solvent extracts. The intensity of the inhibitory effects was variable between the extracts. The phytochemical analysis of alkaloids shows no variation, in carbohydrates aqueous extract gives positive results in Benedict`s test while negative in Fehling's test, in Glycosides aqueous shows positive result in Baljet`s test,Legals test and Killer-killiani test, it is contradictory to previous work. steroids also shoes positive result in aqueous extract that is Liebermann's and Burchard test and Salkowski`s test. Therefore the aqueous extract is the good source of dietary supplement than organic solvent extracts.

Conclusion

Phytochemical analysis of *Spirulina* it was confirmed that it contains carbohydrates, fats, proteins, and both essential as well as non-essential amino acids. These are important to us because lacking of these amino acids consequently will result in the lack of raw materials that produce hormones and support brain. Insufficient amino acids will cause several diseases. As *Spirulina* is composed of complete amino acids, it can replenish the nervous system, protect the brain cell and improve the brain system. The results show that the food products based on *Spirulina* can be considered a valid alimentary source for the chemical and nutritional composition. *Spirulina* is suitable as a nutritious food not only for healthy, unhealthy person but also for those who have the following symptoms: easily get tired, easy to catch cold, insufficient consumption of green or yellow vegetables, dizziness, lack of some nutrients, skipping of meals like breakfast or person under diet.

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