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Chemical Characterization and Bioactivity Studies of Methanolic Extract of Edible Alga Spirulina

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ABSTRACT

Spirulina is a microscopic and filamentous edible fresh water alga that derives its name from the spiral or helical nature of its filaments. It is a Cyanobacterium (blue green alga) used as a dietary supplement or concentrated whole food for human consumption and as a feed supplement in aquaculture, aquarium and poultry industry.

Spirulina is considered to be incredibly healthy possessing some pretty unbelievable health benefits. The present study is aimed at exploring the freshwater alga Spirulina for its dietary and pharmacological uses. It involves determination of Fatty Acid Composition or Lipid profile by Gas-Liquid Chromatography, elemental analysis by X-ray Fluorescence and Bioactivity of spirulina. The investigation highlights the importance of Spirulina as a potential source of antimicrobial compounds and warrants further studies to isolate its active principle(s).

Introduction:

Spirulina is a microscopic and filamentous fresh water alga that derives its name from spiral or helical nature of its filaments (P. D. Karkos, et. al., 2010). It is a Cyanobacterium (blue green alga) cultivated worldwide and available in tablet, flakes and powder form. It is used as a dietary supplement, whole food and as a feed supplement in aquaculture, aquarium and poultry industry (Vonshak et. al., 1997). It is known to be rich in nutrients and vitamin content.

Spirulina thrives at pH of about 8.5 and temperature of 30°C. In addition, it has to have an ensemble of nutrients to thrive in a home aquarium or pond. A simple nutrient feed for its growth requires Baking soda, Potassium nitrate, Potassium phosphate, Sea salt, Iron sulfate, which can all be found in the aquarium.

Most cultivated Spirulina is produced in open channel raceway ponds; commercial producers of Spirulina are located in United States, Thailand, India, China, Bangladesh, Burma (Myanmar), Greece etc.

Spirulina is considered to be one of the few most nutritious super foods known to humankind, possessing some pretty unbelievable health benefits. It is known to be incredibly healthy with its nutritional profile signifying a number of health benefits like strengthened immune system, boosted energy levels and supported cellular health. It is also known to aid blood sugar control**,** lower the blood pressure**,** reduce LDL Cholesterol and Triglyceride levels thereby lowering the chances of stroke, speed up weight loss, eliminate Candida, alleviate symptoms of allergic Rhinitis and improve muscle strength and endurance besides possessing powerful antioxidant and anti-inflammatory properties.

Provided in its typical supplement as a dried powder, Spirulina can supply good amount of calories and is a rich source of numerous nutrients, particularly B Vitamins and dietary minerals such as Iron and Manganese, lipids and fatty acids. Dried Spirulina contains about 51-71% Protein (Bhadouria et. al., 2005, Campanella et. al., 2002).

Multiple uses of Spirulina as dietary supplement and medicine have been well recognized. However, according to the regulatory authorities, scientific evidence is insufficient to recommend its supplementation for any human condition and further research is required. Moreover, this vast and economically important resource requires conservation, protection and utilization on sustainable bases. This is possible by following proper scientific standardized sampling procedure, correct species identification and documentation as well as quantitative assessment to evaluate standing stock and standing crop of the economically valuable species.

Advanced analytical methods like GLC and XRF have made the determination of lipid and fatty acid composition much easier. They are also helpful in determining elemental composition of extracts of algal samples. TLC separation method can be used to carry out qualitative and semi quantitative estimation of the algae and illustrate their individual metabolite contents.

Materials and Methods:

Collection and Pre-treatment of Samples:

The algal samples were collected during low tide from Ratnagiri, Maharashtra located at 16.98° N and 73.3° E on the west coast of India. It is predominantly a coastal line and majorly inhabited by patchy reefs present in intertidal areas at sub-tidal depths. The samples were collected in 250 ml sampling vials (Tarson, Mumbai) and transported to temperature controlled refrigeration system in the laboratory based at Mumbai.

The samples were gently rinsed with tap water and then with filtered fresh sea water to remove any traces of salt, sand and epiphytes. Total algal sample was analyzed for its species diversification. Approx 91% of the total sample was identified as Spirulina using standard characteristic key. A sample specimen slide was deposited in herbarium, University of Mumbai. The material was dried at room temperature for 24h and stored at -20°C until further use.

Preparation of Extract:

100g of dry weight of sample was extracted with 500 ml of Methanol for 24h in rotatory evaporator at room temperature (Remi, India). The extraction process was repeated twice. The final extract obtained was evaporated under vacuum to dryness. The lyophilized sample obtained after evaporation was stored at -20°C and used for further analyses.

Characterization of Crude Extract:

Reagents and Chemicals:

All the chemicals used for crude extract characterization were of AR grade and were procured from Qualigens, India.

Thin Layer Chromatography:

For TLC, the methanolic crude extract was reconstituted in solvent and 5µl extract was spotted on silica gel coated alumina backed sheets (Silica gel 60 F254, 0.25 mm thick, Merck). Chromatogram was developed in a closed tank saturated with eluent vapors (15% Ethyl Acetate: Hexane; 10% Methanol: Chloroform; BAW). Developed chromatograms were viewed both in daylight and under UV light (254 nm) in Gel-Doc (Shimadzu, Japan). The compounds separated on TLC chromatogram were characterized by their R_f values calculated as the ratio of distance travelled by solute to the distance travelled by mobile front. (Fried B., Sharma J. 1999).

Elemental Analysis:
The crude extract was dry digested into a muffle furnace and the ash was scanned in X-ray Fluorescence elemental analyzer. The concentrations of different elements were displayed on instrument screen. The elements which were determined by XRF method include Sodium, Magnesium, Silicon, Phosphorus, Sulphur, Cobalt, Nickel, Copper, Zinc, Arsenic, Cadmium, Mercury, Lead etc. Their percentage composition was obtained even if the elements were present in ppm concentrations.

Determination of Fatty Acid Composition:
The methyl esters were formed using boron trifloride and alkali and separated by Gas-Liquid Chromatography using a flame ionization detector. The pattern of methyl esters was compared with authentic oils for identification.

Spirulina lipid content is known to be reasonable; providing Palmitic, Stearic, Myristic, Lauric, Oleic and Linolenic acids (Colla et. al; 2003, Golmakani Mohammad et. al; 2012), Stearidonic, Eicosapentaenoic, Decosahexaenoic and Arachidonic acids (Jubie et. al; 2012, Tokusoglu et. al; 2003).

Antimicrobial Activity:
For evaluating the antimicrobial efficacy of crude methanolic extract of Spirulina against resistant strains, requisite assay was performed using Agar Well method. Pure test cultures of resistant strains of human clinical bacterial pathogens viz. Escherichia coli, Staphylococcus aureus, Salmonella typhi, Bacillus subtilis (MRSA subspp. Rosenbach ATCC® 33591 ™) were procured from Nikhil Analytical and Research Laboratory, Sangli M.S and revived in glycerol for further use. The glycerol stock cultures of human pathogens were added to the standard Nutrient agar plates with composition: Peptone: 0.5%, Yeast Extract: 0.3%, NaCl: 0.5%, Agar: 2%, pH: 6.8, concentration: 100% and incubated at 28 $\pm$ 2^{0}C for 24-48h.

After incubation, plates were observed for zone of inhibition. The inhibition zones shown by the extracts against the test pathogenic cultures were measured in mm along with the disc diameter and tabulated.

Results and Discussion:
Thin Layer Chromatography:
TLC severance showed only one well distinguished band with R_f Value **0.89.** The distinguished spot with bioactive molecules was scrapped in separate vial, reconstituted in required amount of methanol and stored at -20^{0}C for the next phase of study.

Elemental Analysis:
Analysis of methanolic crude extract using XRF elemental analyzer showed the presence of 18 different elements. The concentration of elements present even at ppm level was determined. The results are summarized in Table 2.

Table 2. Elemental Analysis of crude methanolic extract of Spirulina:

Sr. No.	Element	Unit	Value
1.	Sodium	%	02.40
2.	Magnesium	%	00.21
3.	Silicon	%	00.05
4.	Phosphorous	%	00.78
5.	Sulphur	%	00.22
6.	Potassium	%	01.12
7.	Calcium	%	00.39
8.	Chromium	ppm	< 0.1
9.	Manganese	ppm	06.14
10.	Iron	ppm	348.01
11.	Cobalt	ppm	< 0.1
12.	Nickel	ppm	< 0.1
13.	Copper	ppm	258.39
14.	Zinc	ppm	48.70
15	Arsenic	ppm	< 0.1
16.	Cadmium	ppm	< 0.1
17.	Mercury	ppm	< 0.01
18.	Lead	ppm	< 0.1

Lipid Profile or Fatty Acid Composition:
The results of fatty acid composition of the extract are given in Table 3. The sample showed substantial percentages of saturated and unsaturated fatty acids.

Table 3. Fatty Acid Composition of crude methanolic extract of Spirulina

Sr. No.	Fatty Acid	Percentage Composition
1.	Lauric Acid (C_{12})	47.50%
2.	Myristic Acid (C_{14})	02.10%
3.	Palmitic Acid (C_{16})	29.87%
4.	Stearic Acid (C_{18})	05.72%
5.	Oleic Acid ($C_{18:1}$)	14.80%

Antimicrobial activity:
Antimicrobial activity was performed in triplicate and the results are summarized in Table 3. In this assay, crude extract showed significantly strong antimicrobial activity against all the test organisms as evident from the considerable size of Inhibition Zone.

Table 3. Antimicrobial activity of the crude methanolic extract of Spirulina

Sr. No.	Microbial Cultures	Inhibition Zone Diameter (mm)
1.	*Bacillus subtilis*	23
2.	*Staphylococcus aureus*	26
3.	*Escherichia coli*	25
4.	*Salmonella typhi*	30

Discussion:
The algae base dynamic species counts show that there are hundreds of species of freshwater algae present worldwide. Their vast variety along with the specificity of their action and applications has attracted worldwide attention to explore their applications. Considering their widespread applications and invincible role in various fields, they are a subject of great interest for researchers. The survey of literature reveals that exploration of freshwater algae for their delectability and therapeutic possibilities is being extensively studied as they contain chock-full of vitamins, minerals, fatty acids and fiber.

The significance of Spirulina in different fields has been stressed by a number of researchers. Chemical characterization of crude methanolic extract of Spirulina showed the presence of fatty acids and their esters which may be responsible for its antimicrobial activity. Similar results were obtained by Plaza et. al; who identified several compounds including fatty acids, phenols, phytol etc in the ethanol extracts of sea weeds. Long chain unsaturated fatty acids (C16-C20) like Oleic, Palmitoleic, Linoleic, Linolenic acids as well as long-chain saturated fatty acids like Stearic and Palmitic acids are proven potential antimicrobials (John Peter et. al; 2013). Presence of these compounds in methanolic extract of Spirulina may be attributed to its antimicrobial activity against resistant strains. Gonzalez del Val et. al; also used methanol as solvent for extraction of bioactive compounds from algal samples and evaluated their antibacterial activities.

Further detailed analysis is required to evaluate the spectral composition, effectiveness and potential use of Spirulina bioactive compounds for medicinal purposes.

Conclusion:
The crude methanolic extract of Spirulina showed significant antimicrobial activity against resistant strains of human bacterial pathogens. This investigation highlights the importance of the edible algae Spirulina as a source of potent antimicrobial compounds and warrants further studies to isolate the active principle(s) for their therapeutic applications.

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