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Phytochemical analysis, antioxidant and antifungal activity of different solvent extracts of *Spirulina platensis* collected from Rankala Lake, Kolhapur, Maharashtra

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Abstract:

The objective of the present work was to evaluate the phytochemical analysis, antioxidant and antifungal activity of different extracts obtained from *Spirulina platensis*. The *Spirulina platensis* was collected from Rankala Lake, Kolhapur (MH), India and grown in CHU-10 medium for 10 days. After 10 days the culture was recovered, dried, powdered and extracted using different solvents such as methanol, ethanol, petroleum ether, acetone and water by solvent- solvent extraction method. The qualitative phytochemical analysis of all extracts revealed the presence of alkaloids, terpenoids, steroids, tannins, saponins, flavonoids, phenols, coumarins, quinones and glycosides. Quantitative phytochemical analysis revealed that the aqueous extract possessed high phenolic content (27.0090±0.04129 mg) and flavonoids content (63.47± 0.88059 mg) when compared to other extracts. Ferric ion reducing power assay was performed with chloroform, ethanol, methanol and aqueous extracts by using ascorbic acid as standard. In this assay, aqueous extract (1.2407±0.00702) showed highest antioxidant activity among the extracts used for the assay which was comparable to standard. Among all the extracts, aqueous extract showed highest antioxidant capacity in Phosphomolybdenum assay and H_2O_2 scavenging assay at the rate 0.8983±0.00351 and 76.8233±0.09074 respectively. The aqueous extract showed effective antifungal activity against *Candida albicans*, and *Aspergillus fumigatus* with 22mm and 18mm zone of inhibition respectively. So it can be concluded that, the aqueous extract of *Spirulina platensis* possesses potent bioactive compounds with antioxidant and antifungal activity as compared with other solvent extracts.

Key words: *Spirulina platensis*, phytochemical analysis, Antioxidant activity, Antifungal activity.

1. Introduction:

Phycology or algology is the study of the algae (3). The word Phycology is derived from the word phykos, which means "seaweed". The term algology, describe in Webster's dictionary as the study of algae has fallen out of favor because it resembles the term Algogenic which means "producing pain" The algae are thallophytic in nature (4). The history of Phycology is the scientific study of algae. Human interest in plants as food goes back into other origins of algae can be traced back more than two thousand years. However, only in the last three hundred years the knowledge has evolved into a rapidly developing science, the first attempts at plant cultivation were believed to have made shortly before 10,000 BC in western Asia (7). According to records as far back as 30,000 BC indicates that algae were used by the emperor of China as food. The first algae were recognized as living organism and were probably coralline, by Pliny the Elder in first century AD (6). The development of the study of Phycology runs in a pattern comparable with and parallel to other biological field but at a different rate.

In 3.5 billion years ago prokaryotic life began on the planet in the absence of oxygen (7-9). The Cyanobacteria arose and began releasing oxygen into the atmosphere as a waste product of chlorophyll-a mediated photosynthesis. The prokaryotic blue-green algae, those photosynthesized and that changed the atmosphere of the planet (13-15). Like other prokaryotes, the blue-green algae are abundant and present in almost every conceivable habitat from oceans to lakes, ice, snow, thermal hot springs, and deserts. Since more than 99% of all species ever evolved on Earth have gone extinct, it is probable that human will be a relatively short-term component of life on Earth, but the blue-green

algae that were main players since 3.5 billion years ago during the creation of life (16-17). The primary groups of algae are rich in species variety with their distribution. Rhodophyta is also called as red algae and there was 4000–6000 species in Marine and freshwater systems. The big algae are also called as seaweed. They found near to ocean coasts or around lakes and ponds. These macroscopic algae are typically divided into three groups such as jade algae, red algae, and brown algae. Green algae are called as Chlorophyta. Total there are an estimated 6,000 to 8,000 species of green algae and 90% of them are freshwater rather than marine. The green algae and the land plants share a common ancestor, and all descendants of that widespread ancestor are either green algae or land plants (10-12).

In 17 the century, there was a great revolution in scientific interest all over Europe and after the invention of the printing press; various books on botany were published. There was no exact progress done in the scientific study of algae until the invention of the microscope in about 1600 by Antonio van Leeuwenhoek (7). Carl Linnaeus was the father of modern nomenclature who not only made an easy way for the naming system of plants and animals but also made a systematic study of their naming system. He developed a coherent system for naming organisms and divided the plant kingdom into 25 classes one of which is Chyptogamia (6). He divided Thechyptogamia into four orders: Flices, Muscle (mosses), Algae (lichens and liverwort), and fungi. Johann Hedwig (1730-1799), a German botanist provided further evidence of sexual process in algae and figured conjugation in Spirogyra. During 18th century, there was a huge controversy on the topic that whether coralline algae are plants or animals. Up to mid of 18th century, coralline algae were treated as plants (18) but up to 1768 some of the researchers accepted as an animal. After five years it was concluded that Coralline algae are definitely plants (4-7).

Spirulina is being produced commercially from the last 20 years and the current manufacture universal is estimated to be about 3,000 metric tons. The chief commercial large-scale culture of Spirulina was started in the early 1970s at Lake Texcoco, Mexico [4]. Commercial production of Spirulina in man-made ponds was pioneered by Dainippon Ink and Chemicals Inc. (DIC) in 1978 in Bangkok, Thailand [2,3]. Earthrise farms were found in 1981, by the Proteus Corporation of the USA and later incorporated with DIC of Japan in 1982 [4,5]. Commercial production of Spirulina at Earthrise farms in California started in 1983. It is being sold as a health drink as well as in tablet form for more than ten years without undesirable effect on humans [6,7]. Spirulina is marketed and consumed in several countries, including, U.S.A, Thailand, Taiwan, Vietnam, China, India, and Cuba [6,7]. Spirulina is a multicellular, filamentous *cyanobacterium*, belonging to Phormidiaceae family which under the microscope appears as blue-green filaments composed of cylindrical cells arranged in unbranched helicoidal trichomes [1-5]. The trichomes are arranged in open left-handed helix pattern along the entire length. The cell wall is made of four numbered layers, LI, III, LIU, and LIV from the innermost to outward [4-8]. All the layers are very weak except LII, which is made up of peptidoglycan, and responsible for the rigidity. Spirulina is a non-heterocystous and a non-nitrogen fixer [10]. The helical shape of the trichome is characteristic of the genus which is due to hydration/dehydration of oligopeptides in the peptidoglycan layer [8]. Spirulina is natural food belongs to the Plantae kingdom which consists of different phytochemicals [1-6].

These phytochemicals are biologically significant and play vital role in medicinal applications. Mainly laboratory experiments revealed that phytochemicals from *Spirulina* and their use in cancer, tuberculosis, inflammation, and many other blood-related diseases [4]. But each and every Spirulina is varying from each other in the production of these compounds. Somehow these production depends on environmental conditions such as temperature, pH, nutrients, metal ions and other chemicals [4,8]. The phytochemical research loom is measured effective in discovering novel bioactive compounds from Spirulina. There are two main methods for the analysis of phytochemical screening such as qualitative and quantitative analysis. The qualitative tests are used to identify the constituents [4,6,9].

The objectives of the present research study were (I) Selection of study area and collection of *Spirulina platensis* (II) Extraction and phytochemical analysis of bioactive compounds (III) Antioxidant and antifungal activity of *Spirulina platensis* derived bioactive compounds.

Materials and Methods:

Study area

Rankala Lake is located in Kolhapur District, Maharashtra, India and was selected as the study area. It is 16042" North 74015" East on the North West plateau of Maharashtra. The district is bordered by the steep ridges of Sahyadri to the west, the Deccan plateau on the east, and boundaries of Goa on the south and Karnataka on east. The area of the district is 7746 sq. km. and it is 2-5% of the state area.

Sample Collection

Spirulina platensis was collected from the Rankala Lake Kolhapur (MH), India and was used as the experimental algae to that biodiversity. Samples were thoroughly washed under running tap water, air dried and then homogenized to fine powder and stored in airtight bottles in refrigerator.

Spirulina platensis Cultivation

Spirulina platensis was axenically grown in CHU-10 medium and incubated in a culture room at temperature of 25℃ ± 2℃ and illuminated with day- light fluorescent tubes. During the process of growth the flask was shaken at every half hour per day. The experiments were run in triplicates. All culturing were carried out under aseptic conditions in a laminar flow.

Preparation of Extract

Sample extracts were prepared by Soxhlet extraction method. 20 g of powdered material was uniformly packed into a thimble and extracted with 250 ml of different selected solvents such as methanol, ethanol, petroleum ether, and acetone separately. The process of extraction has to be continued for 24 hours or till the solvent in siphon tube of extractor become colorless. After that the extract was taken in a beaker and kept on hot plate and heated at 30-40ºC till all the solvent got evaporated. Dried extract was kept in refrigerator at 4ºC till future use.

Phytochemical analysis

Qualitative phytochemical analysis

Qualitative phytochemical analysis of all extracts of *Spirulina platensis* was employed for the detection of alkaloids, terpenoids, steroids, tannins, saponins, flavonoids, and phenols, coumarins, quinones and glycosides by preferring standard protocols of Mane and Vedamurthy et al. 2018.

Quantitative phytochemical analysis

Estimation of total Flavonoids and Phenol content

The Flavonoids content and phenol content were estimated by using Folin-Ciocalteu colorimetric method as described by Mane and Vedamurthy et al. 2018. Quantification was done on the basis of the standard curve of gallic acid and results were expressed as gallic acid equivalent.

In Vitro Antioxidant Activity

Ferric ion reducing power assay (FRAP)

Ferric ions reducing power was measured according to the method proposed by (16) with minimal modifications.

Phosphomolybdenum Assay (PM assay)

Total antioxidant activity was estimated by PM assay using standard procedure of (19).

Hydrogen Peroxide (H_2O_2) Scavenging Assay

In-vitro H_2O_2 scavenging activity was performed in accordance with the method developed by (5). The percentage of inhibition was calculated by the formula

% Inhibition = $[(A_c-A_t)/A_c] \times 100$

Where

Ac is the absorbance of the control containing phosphate buffer and H_2O_2,

A_t is the absorbance of the test samples.

Antifungal activity

Test Fungi

The all extracts of *Spirulina platensis* were used for *in-vitro* antagonistic activity against *Candida albicans*, and *Aspergillus fumigates* by well diffusion method.

Well diffusion method

The fungal inoculum was prepared to the concentration of 1.0 × 10^4 CFU/ml adjusted with saline. The culture suspension was prepared and used as a stock culture for the experiment purpose. The culture suspension was spread on nutrient agar medium for verification of other microbial contamination. Telithromycin (10mg/mL) was used as positive control and solvent DMSO was used as negative control. The verified microbial culture suspensions were spread on Muller-Hinton agar medium plates and purified extract samples were added in the wells with standard antibiotics. Plates were incubated at 25℃ for 72 hrs and zone of inhibition was recorded with the help of zone reader. All experiments were performed in triplicates.

Statistical Analysis:
All the results (triplicates) were represented as Mean ± Standard Deviation (SD). One way ANNOVA was carried out to check the variation between the samples using SPSS software version 20.0 IBM. The levels of significance was considered as $p< 0.01$ and $p <0.05$ for the comparison.

Results

Phytochemical Analysis:

Phytochemical screening of ten different chemical compounds (alkaloids, terpenoids, steroids, tannins, saponins, flavonoids, phenols, coumarins, quinones and glycosides) were tested in five different extracts such as methanol, ethanol, petroleum ether, acetone and aqueous extracts of *Spirulina platensis.* The qualitative phytochemical analysis of distilled water, methanol, ethanol, petroleum ether, and acetone extracts of *Spirulina platensis* revealed different bioactive compounds. The methanol extract showed presence of all bioactive compounds excepting steroids. The quinones, tannins, and glycosides were absent in ethanol extract while all other bioactive compounds were present. The aqueous extract showed negative results for coumarins and glycosides while positive results for other bioactive compounds. Petroleum ether showed positive results for all bioactive compounds while negative results for Tannins Coumarins, and glycosides. Acetone extract showed presence of all bioactive compounds and negative results for coumarins. The results are shown in table 1.

Table I. Phytochemical analysis of *Spirulina platensis* extracts (Distilled water, Methanol, ethanol, Petroleum Ether and acetone were used as solvents for extraction of bioactive compounds at room temperature 25℃ ±2^{O}C, [+] = Presence, [-] = Absence).

Sr. No	Tests	Sample extracts				
		Distilled water	Methanol	Ethanol	Petroleum Ether	Acetone
1	Alkaloids	+	+	+	+	+
2	Terpenoids	+	+	+	+	+
3	Steroids	+	-	+	+	+
4	Tannins	+	+	-	-	+
5	Saponins	+	+	+	+	+
6	Flavonoids	+	+	+	+	+
7	Phenols	+	+	+	+	+
8	Coumarins	-	+	+	-	-
9	Quinones	+	+	-	+	+
10	Glycosides	-	+	-	-	+

Estimation of total Phenolic and Flavonoids Content:

The total phenolic content of the selected extracts was estimated with Gallic acid as a reference standard. The aqueous extract showed high phenolic content (27.0090±0.04129 mg) when compared to ethanol extract (10.0991±0.09487 mg) and acetone (8.40±0.05000 mg). The results were expressed as mg/g GAE per gram of plant extract. Total flavonoid content was performed using $AlCl_3$ method using quercetin as a standard. The aqueous extract showed high flavonoid content (63.47± 0.88059 mg) whereas methanol extract showed moderate flavonoid content (51.9221±0.07300 mg) and aqueous extract (20.3568±0.05064 mg). The results were expressed as mg/g of quercetin equivalent.

In-vitro Antioxidant Activity

Ferric ion reducing power assay:

Ferric ion reducing power assay was performed with chloroform, ethanol, methanol and aqueous extracts by using ascorbic acid as standard. In this assay, aqueous extract (1.2407±0.00702) showed highest antioxidant activity among the extracts used for the assay which was comparable to standard.

Phosphomolybdenum Assay:

PM assay was performed for four different extracts with standard ascorbic acid. The results revealed that the aqueous extract showed 0.8983±0.00351 higher antioxidant capacity than the other extracts and was compared to the standard ascorbic acid.

H_2O_2 scavenging Assay:

H_2O_2 radical scavenging assay was performed with four extracts and ascorbic acid standard. The aqueous extract showed potent scavenging activity (76.8233±0.09074) and also the acetone extract showed better scavenging activity (67.7133±0.07506) which was comparable to the standard.

Antifungal activity

The highest antifungal activity showed by aqueous extract while other extracts showed less activity. The results were shown in figure: 1. The aqueous extract showed effective antifungal activity against *Candida albicans*, and *Aspergillus fumigatus* with 22mm and 18mm zone of inhibition respectively.

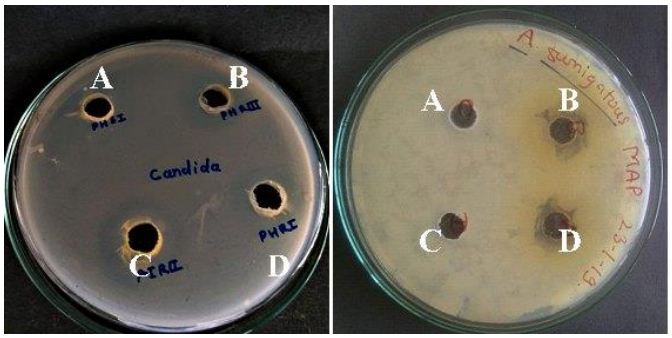

Plate 1 Antifungal activity of different extracts. The plate of *Candida albicans,* and *Aspergillus fumigatus*. The well A contains aqueous extract, B contains acetone extract, C contains DMSO and D contains Telithromycin. The all obtained pure cultures were spread on Muller Hinton agar and wells were prepared. Addition of all prepared suspensions, positive control, and negative control was done and incubated at 25℃ for 72 hrs and after 72 hrs zone of inhibition was recorded with the help of zone reader.

Discussion

The potent therapeutic agents are also vast collection of chemical agents which are potent drugs in treating many diseases or conditions (4). These phytochemicals are more promising with lesser side effects. In recent days, the

modern medicine systems are using these phytochemicals as clues for designing newer drugs. The present study has noted the isolation of various secondary metabolites which are known to possess different protective mechanisms to dreadful diseases and conditions (5). In the recent years, there is an increased demand for traditional medicines which are based on these phytochemical principles. The major secondary metabolites which can potentially inhibit the oxidative stress are mainly alkaloids, flavonoids, phenols, tannins, sterols, glycosides and terpenoids (6).

The bioactive compounds obtained from *Spirulina platensis* are used in the treatment of various diseases by traditional practitioners and it is due to these phytochemical principles play role in controlling the disease condition. Flavonoids are involved in the free radical scavenging, anticancer and antimicrobial properties (6-9). These flavonoids are also involved in improving the blood circulation to the brain in Alzheimer's disease. Alkaloids are mostly used in the treatment of alzheimer's disease, parkinson's disease, as antimicrobial agents, as antimalarial agents. Phenols are a kind of natural products and antioxidant substances which are competent scavengers of free superoxide radicals, anti-aging and reducing the risk of cancer. Tannins may be employed medicinally in anti-diarrheal, haemostatic, and anti hemorrhoidal compounds (10-13). The anti-inflammatory effects of tannins help control all indications of gastritis, esophagitis, enteritis, and irritating bowel disorders. Sterols are the biological molecules which are mainly used in the treatment of cardiovascular disease, in lowering the LDL-cholesterol levels, in the treatment of breast cancer and prostate cancer (14-17). Terpenoids are having various medicinal properties such as anticarcinogenic, antimalarial, anti-ulcer, hepaticidal, antimicrobial or diuretic activity and anticancer activity. The antioxidant defense mechanism is an in-built mechanism found in humans. But nowadays due to life style changes and varied food habits the antioxidant balance to the oxidants produced has been reduced. So there is an immediate need of potent antioxidant molecules which can stabilize the Reactive Oxygen Species (ROS) and free radicals generated during the metabolic processes (18-19). In order to search for potent antioxidant molecules, the present study aimed to evaluate the leaf extracts of *Spirulina platensis,* for different *In vitro* antioxidant assays. Three different assays were conducted to check the antioxidant power of *Spirulina platensis* extracts such as ferric ion reducing power assay, Phosphomolybdenum assay and hydrogen peroxide scavenging assay. In ferric ion reducing power assay Fe^{+3} ions donate an electron and converted into Fe^{+2} in presence of plant extracts as reducing agents. This assay has become matter of interest due to its ease in performance and its accuracy. In the present study aqueous extract (1.2407±0.00702) showed highest antioxidant activity when compared to other extracts. Phosphomolybdenum assay is a very useful method to predict the antioxidant activity of crude extracts on the total basis (7). This assay is based on the principle of reduction of Phosphate-Molybdenum (VI) to Phosphate-Molybdenum (V). In the present study chloroform, acetone, methanol and aqueous extracts were subjected to PM assay (9). Out of all extracts, the aqueous extract showed (0.8983±0.00351) potent antioxidant activity. H_2O_2 scavenging assay hydrogen peroxide is a weak oxidizing agent which inactivates certain enzymes directly by oxidation of vital thiol (-SH) groups (12,17). It can pass cell membranes quickly; once inside the cell, it is most likely to react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radicals leading to many toxic effects. In the present investigation, the aqueous extract showed higher scavenging activity (76.8233±0.09074) than the standard and also the aqueous extract showed scavenging activity (67.7133±0.07506) which was comparable to the standard. In all tests aqueous extract showed effective results as compared with other extracts. As far as antifungal activity was considered here also aqueous extract was effective candidate. The aqueous extract showed effective antifungal activity against *Candida albicans*, and *Aspergillus flavus* with 22mm and 18mm zone of inhibition respectively.

Conclusion:

In the study undertaken, phytochemical analysis, antioxidant and antifungal activities of *Spirulina platensis* was evaluated. The *Spirulina platensis* is used as alternative drugs in treating dreadful diseases such as Alzheimer's diseases, Parkinson's diseases, Diabetes, Hepatic damage. The phytochemicals observed in *Spirulina platensis* can be equally potent as the standard drugs used presently as antioxidants, and antifungal agents. The evaluation of total phenolic and flavonoids contents of *Spirulina platensis* revealed that, this alga possess high amount of phenols and flavonoids in different extracts. *In-vitro* antioxidant and antifungal activities of *Spirulina platensis* showed the potential of these phytochemicals as novel drugs in treatment of above mentioned diseases. So these phytochemical principles may replace the current synthetic drugs as strong antioxidant, antifungal agents. Further detailed study is required in order to evaluate the efficacy of these compounds *In-vivo* using different animal models to elucidate the exact mechanism of action of these bioactive compounds.

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