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Pharmaceutical Applications of Extracted Phycocyanin from Arthrospira Platensis

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Abstract:- Diabetes is caused by irregular insulin secretion and activity levels, leading to cellular hypoglycemia and extracellular intolerance to glucose. It is triggered by non-enzymatic protein glycation and oxidative stress, which damage cells in several ways. Oxidative damage is an imbalance between reactive oxygen species (ROS) production and a biological system's capacity to detoxify reactive intermediates or restore damage, which can lead to metabolic abnormalities in obesity, diabetes, and cardiovascular disease. Plant-based medicines have gained popularity, but their efficacy may be affected by dosage, frequency, and genetic and metabolic variations. Cyanobacteria produce beneficial chemicals such as phycocyanin, which can modulate blood sugar via distinct biological pathways.

Keywords: *Non-Enzymatic Protein Glycation, ROS, Cyanobacteria, Phycocyanin.*

I. INTRODUCTION

Diabetes is hyperglycemia caused by irregular insulin secretion and activity levels. Diabetes is rapidly expanding in low- and middle-income nations such as India. By 2025, 69.9 million Indians will have diabetes, most of whom will remain undiagnosed and untreated [1].

In diabetes mellitus, insufficient insulin or insulin resistance decreases glucose absorption by tissues, leading to cellular hypoglycemia and extracellular intolerance to glucose. During prolonged hyperglycemia in diabetes, glucose glycates plasma proteins, resulting in covalent structural modifications. oxidative stress damages cells induced by hyperglycemia. Increased free radicals accompany hyperglycemia. Weak immune systems are overwhelmed by reactive oxygen species. Consequently, reactive oxygen species outnumber their protective properties, which favors oxidative stress. Numerous organs, such as the eyes, kidneys, liver, nerves, heart, and circulatory system, are at risk for long-term harm, disruption, and disintegration when glucose levels are chronically elevated. Hyperglycemia develops reactive oxygen molecules which damage cells in several ways [2].

Cell damage causes diabetic problems. The presence of diabetes, high blood sugar, nonketotic hyperosmolar coma, and hyperglycemia increases the likelihood of developing acute metabolic disorders such as ketoacidosis[2]. In addition to foot ulcerated wounds, diabetics are also at risk for cardiovascular disease, retinal disease, nephropathy, and neuropathy[3].

Diabetes is triggered by non-enzymatic protein glycation and oxidative stress [4].

➢ *Nonenzymatic Protein Glycation:*

Protein glycation is the reduction of sugars (fructose and glucose) by protein[5]. Glycation is a category of non-enzymatic post-translational protein modifications induced by reducing carbohydrates and their carbonyl derivatives. Glycation generates an unstable secondary Schiff base aldimine. This is further rearranged to produce a stable Amadori product, resulting in advanced glycation end products that are irreversible. (AGE). These rearrangements produce highly reactive carbonyl species (RCS), which induces carbonyl stress. MGO and 3-deoxyglucosone (3DG) are examples. Glycoxidation and lipid peroxidation result in the formation of RCS and AGE. This process is accelerated by glycated or oxidative stress, which may influence short-lived substrates and cause structural changes [6].

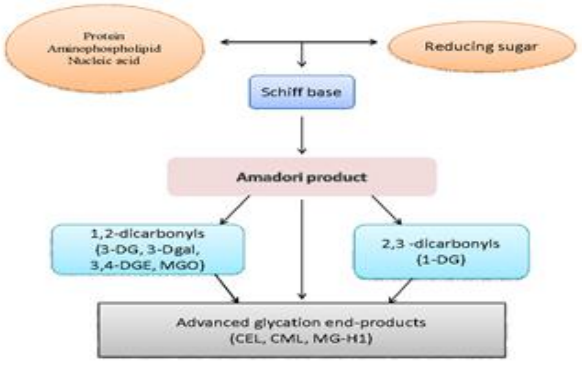

Fig 1 Advanced Glycation End-Products

Oxidative damage: An imbalance between reactive oxygen species (ROS) production and a biological system's capacity to detoxify reactive intermediates or restore damage, which can affect all cell components, including proteins, lipids, and DNA. ROS include superoxide anions, hydrogen peroxide, and hydroxyl radicals[7]. When ROS production exceeds intrinsic ROS production, oxidative stress occurs. Reactive oxygen species are second mediators in numerous intracellular signaling cascades that maintain the equilibrium of the cell's environment. At large concentrations, they are able to destroy biological molecules, causing functional degradation and cell mortality [8].

Numerous studies have established a connection between oxidative stress and metabolic abnormalities in obesity, diabetes, and cardiovascular disease. Alternately, research has demonstrated that focusing on ROS generation pathways as opposed to antioxidants may be advantageous. Several studies have demonstrated that the cell's apparatus will become increasingly active in ROS production and that the expanding molecular mechanism activates inflammatory signal transduction pathways and prolongs inflammation. Mitochondrial dysfunction-induced Ros production, insufficient cellular antioxidant machinery, irregular NADPH oxidase activation, and lipid peroxidation are oxidative causes [9].

Diabetes, atherosclerosis, and hypertension are all caused by oxidative stress.Insulin and tablets are used to treat diabetes. Insulin therapy can reduce postprandial hyperglycemia and prevent between-meal hypoglycemia. Insulin therapy carries hazards. Mismatching insulin injections and meals lead to weight gain and hypoglycemia. Oral hypoglycemic medications include sulphonylureas, glibenclamide and glipizide, and biguanides, such as metformin and phenformin. Sulfonyl increases ß-cell insulin secretion and hypoglycemia in the pancreas. They bind to ß-cell plasma membrane sulfonylurea (SUR) receptors, thereby blocking ATP-sensitive potassium channels and restricting cell activity. Through voltage-gated channels, calcium ions transport insulin granules. Injections of acute sulphonylurea into patients with type 2 diabetes increase pancreatic insulin secretion and decrease hepatic hormone clearance. An intact pancreas is required for sulphonylurea-induced hypoglycemic reactions, according to an early study[10].

Due to an increase in traditional medicine research, plant-based medicines that are environmentally benign, biodegradable, cost-effective, and relatively safe have gained popularity in recent decades. The WHO has recognized 21,100 flora as medicinal. 150 of India's 2500 species are used for commercial purposes. India's medicinal flora and botanical gardens are well-known [11].

Not all secondary plant metabolites inhibit diabetes. Tea and coffee polyphenols may increase insulin resistance and lead to diabetes in certain individuals. Diabetes-controlling efficacy of plant secondary metabolites may be affected by dosage, frequency, and genetic and metabolic variations[11].

Cyanobacteria produce beneficial chemicals such as the anti-inflammatory and antioxidant phycocyanin. These chemicals may modulate blood sugar via distinct biological pathways [11].

Ancient photosynthetic cyanobacteria are found in a variety of aquatic habitats. Its high nutritional value has contributed to Spirulina platensis popularity. Spirulina is "blue-green algae." Blue-green, filamentous spirulina is unicellular. Spirulina platensis is found in tropical lakes with elevated Na-Cl and bicarbonate concentrations and a pH of 11. Other microorganisms cannot thrive in open reactors, but microalgae can. Light is captured by chlorophyll-a, carotenoids, and phycobiliproteins in photosynthetic bacteria. Allophycocyanin, phycocyanin, and phycoerythrin are linear tetra-pyrrole proteins. Spirulina is the best source of protein [12].

C-phycocyanin (CPC), a pigment primarily found in cyanobacteria, may transmit energy from the filament to the core[13]. X-ray crystallographic and EM investigations revealed that two trimers face-to-face form a closed toroidal 6 hexamer with D3 symmetry, the CPC quaternary structure.Phycocyanin, which contains all essential amino acids, is utilized as a dietary supplement. Since it is water-soluble, it is used as a food coloring, cosmetic additive, and fluorescent reagent for clinical diagnostics and immunochemistry. Additionally, phycocyanin has anti-inflammatory, hepatoprotective, and radical-scavenging properties[14]. The anticancer, anti-diabetic, and anti-inflammatory properties of phycocyanin have been examined in vitro [15].

II. MATERIAL AND METHOD

Arthrospira platensis powder is sourced from an authentic marketplace.

➢ *Extraction of Phycocyanin:*

- *Ultrasonic Method:*

Powdered forms of Arthrospira sp have been suspended in 1:25 (w/v) distilled water for 24 hours at 4°C. After 1 hour of 40 kHz irradiation, the solution was spun in a centrifuge at 4°C for 15 minutes at 10,000 rpm in and 10,000 rpm. After eliminating the sediment, the filtrates of the crude extract have been preserved at 4°C [16].

- *Phosphate Buffer Method:*

In a 50 ml conical tube, 2.4 g of Spirulina biomass was hydrated with 37.6 ml of 100mM phosphate buffer at pH 6. Biomass has been distributed and extracted in phosphate buffer for a period of two hours. Using UV-vis spectroscopy, centrifuged unprocessed extract samples had been observed [17].

- *Enzymatic Method:*

In 200 ml sodium phosphate buffer at a concentration of 0.1 M, pH=7, 2 g of dried-out Spirulina was diluted in 10 mmol EDTA and 100 µg/ml lysozyme. At 30°C, the enzymatic breakdown of the algal cell wall needed 24 hours. After 24 hours, the solution had been centrifuged for 1 hour at 8000 rpm at 4°C. [16].

- *Homogenization Method:*

Mortar and pestle destroy cells in pH 6 buffer containing 0.1M phosphate. The extraction was carried out by centrifuge at 6000 rpm for twenty minutes[18].

➢ *Phycocyanin Extract Purification:*

- *Ammonium Sulfate Precipitation Method:*

Solid ammonium sulfate was gradually added to the crude extract while mixing till it reached 50% saturation. After one hour of agitation, it was kept overnight in the dark at 4 °C. At 4 °C, the suspension was centrifuged at 10000 rpm for thirty minutes. The colorless supernatant was discarded, and the phycocyanin-containing blue precipitate was dissolved in a small amount of sodium phosphate buffer (0.025 M for the enzymatic method and 0.005 M for the ultrasonic technique), pH = 7, in a brown container at 4 °C.[16].

➢ *Characterization of Phycocyanin:*

- *Thin-layer Chromatography:*

Phycocyanin has been separated from bioactive substances by TLC. The present study utilized TLC plates made of silica gel. A drop of pigment was extracted and a standard of phycocyanin was deposited on the TLC plate using a microcapillary tube in a TLC vessel containing 100% methanol. When the methanol level reached 2 cm below the top of the tank, the plates were removed and inspected for spots[16].

- *FT-IR Evaluation:*

FT-IR spectroscopy analyzed the composition of substances and their molecular bonds. FTIR spectrum in absorbance mode of cross-linked polysaccharides. Utilization of Perkin Elmer Frequency Band Two with ATR reflectance cell and diamond crystal. At ambient temperature, 64 images were acquired with Happ-Genzel apodization and 2 cm1 resolution. C-phycocyanin and starch cross-linking-C-phycocyanin composite materials were analyzed spectrally. The particles were directed and evaluated by the manufacturer [16].

➢ *Quantitative Analysis:*

- *Total Flavonoid Content:*

The aluminum chloride colorimetric assay was carried out by evaluating the total flavonoid content. the extract had been combined with 1.5 mL methanol, 0.1 mL AlCl3 (10%), 0.1 mL sodium acetate (1M), and 2.8 mL distilled water. After 30 minutes, absorbance at 415 nm was measured. A quercetin curve of calibration (R2= 0.9961) determined the total flavonoid content.[19].

- *Total Phenolic Content:*

The sample and Ciocalteu's Folin-reagent (1 mL, 1.0 mg/mL) were combined with care. This mixture was thoroughly combined with 4 mL of sodium carbonate (7.5%) and 10 mL of distilled water. Phycocyanin extract activity in vitro. After 90 minutes at ambient temperature, the absorbance of the reaction mixture reached 550 nm. Using gallic acid curves for calibration (R2 = 0.9699), the total phenolic concentration was estimated. The results were expressed as mg of the equivalent of gallic acid per gram of dried extract (GAE/g). All samples were analyzed in duplicate and the results have been presented as percentages [19].

➢ *Antioxidant Capability:*

- *Test for DPPH Radical Scavenging:*

Investigating the antioxidant activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical. Before being detected at 517 nm, 0.5mL tests were mixed with 2.5mL of 0.004% DPPH in methanol and kept at ambient temperature for 1 hour. The controls and standards for the analysis had been distilled water and the antioxidant vitamin C. The percentage of inhibitory activity was computed using [(A0 A1)/A0], where A0 was the absorbance of the negative control substance and A1 was the absorbance of the extract. A linear regression analysis (IC50) determined the sample concentration required to reduce 50% of DPPH radicals[20].

- *Assay for Nitric Oxide Scavenging:*

Kumaran and Karunakaran (2006) improved their method for measuring the scavenging activity of nitric oxide. 0.5mL of samples were dissolved in 2.2mL of phosphate-buffered saline pH 7.4 (545 mg Na2HPO4, 160 mg NaH2PO4, 4.5g NaCl in 500mL of distilled water) along with 0.1mL of sodium nitroferricyanide (III) dehydrate. Griess reagent was administered after 150 minutes. The absorbance after twenty minutes was 546 nm. The controls and standards for the analysis were distilled water and vitamin C. The percentage of inhibitory activity was calculated using [(A0 A1)/A0], where A0 was the absorbance of the negative control and A1 was the absorbance of the extract. A linear regression analysis (IC50) was all that was required to determine the sample concentration required to absorb 50% of NO radicals [21].

➢ *Antiglycation Properties Determination:*

- *BSA Fructose Model:*

At 37 degrees Celsius for 1, 2, 3, and 4 weeks, the sample was glycosylated in 10 mg/ml BSA with 1.1 M fructose in 0.1 M phosphate buffer at pH 7.4 with 0.2% sodium azide. At 355 nm excitation and 460 nm emission wavelengths, a glycated BSA composition was found. The positive control was aminoguanidine (AG) [22].

- *Fructosamine Inhibition Evaluation:*

Amadori's fructosamine was quantified in glycated albumin samples and controls using the NBT assay. 0.1 M carbonate buffer with a pH of 10.35 contains 0.75 mM NBT. At 37 degrees Celsius, 40 lL of glycated samples were

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incubated with 0.8 mL of NBT solution for 30 minutes. Thermo Scientific's Genesys 10S UV-Visible measured absorbance at 530 nm. The 1-deoxy-1-moepholinofructose standard curve (Y 14 0.00X 0.017, r 14.981) was utilized to determine the fructosamine concentration in lM/mg protein [23].

- *A Test for Congo-Red Binding:*

Absorbance of 530 nm was utilized to determine the connection between Congo-red and amyloid cross-structure. 500µL of glycated samples were incubated for 20 minutes at room temperature with 100µL of 100M Congo red (Merck, Germany) in PBS with 10% [v/v] ethanol. The background absorbance and Congo-red incubated extracts were evaluated[24].

➢ *Anti-Diabetic Properties:*

- *α-Amylase Assay:*

The alpha-amylase inhibition assay was utilized to evaluate antidiabetic activity. After solution preparation, five test containers for each solvent consisted of 0.01 mg/ml of phycocyanin extract. With a micropipette, 500µl of phycocyanin is extracted into each test tube. 500 µl of alpha-amylase solution per test tube. After 10 minutes at 25oC, 500 µl of a starch solution and 10 minutes were added to the process. After adding 1 ml 3, 5-Dinitrosalicylic acid to the solution, it was heated in a simmering water bath for 5 minutes. The process of cooling the mixture. Water distillation diluted it. Finally, absorbance at 540 nm was measured[25].

Inhibition (%) = (Ablank-Asample/Ablank) × 100.

➢ *Dna Protective Properties Determination:*

With minor modifications, the DNAprotection assay was used to evaluate the ability of phycocyanin to protect human genomic DNA from Fenton's reagent-generated hydroxyl radicals. The reaction mixture contained 20 µL of double-distilled water, 3 µL of human genomic DNA, 10µ L of Fenton's reagent (30 mM H2O2, 50 mM Ascorbic acid, and 80 mM FeCl3), human genomic DNA extract, and 3 L of human genomic DNA. The reaction mixtures were incubated at 37°C for 10 minutes. After 30 minutes of incubation, 0.25% bromophenol blue in 50% glycerol was added. The reaction solutions (20 µL) were electrophoresed at 90 V for 1 hour on a 1.3% agarose gel (prepared by dissolving 0.4 g of agarose in 50 mL of 1 TBE Buffer). EtBr staining then ensued[26].The LAS-4000 MINI Gel Documentation system displayed and estimated closed circular, linear, and relaxed DNA shapes.

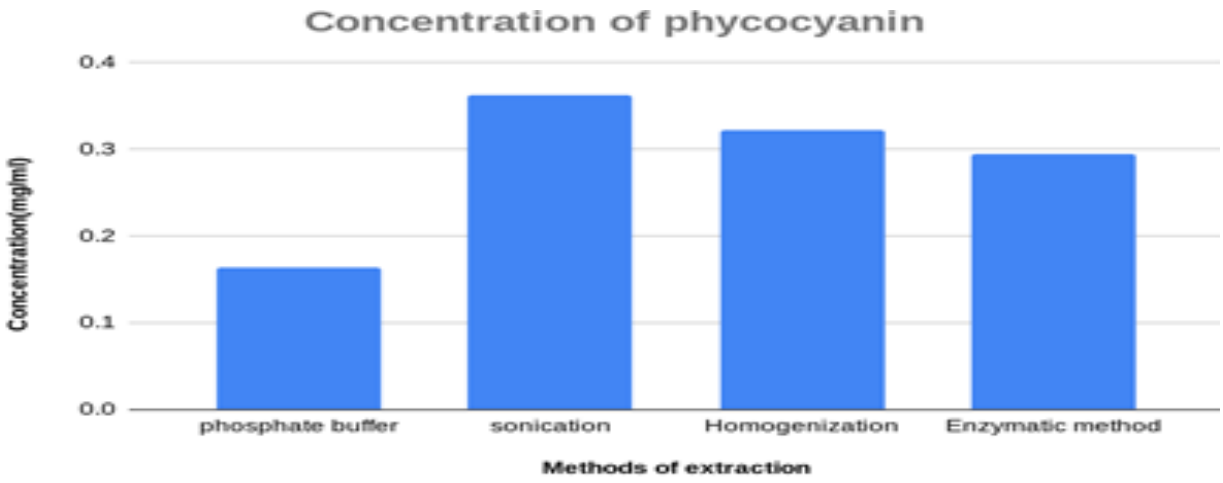
III. RESULTS & DISCUSSION

Fig 2 Arthrospira Platensis Collection

➢ *Extraction of Phycocyanin from the Different Methods:*

Table 1 Concentration, Yield & Purity of Phycocyanin from Different Methods

	Sonication	Phosphate Buffer	Enzymatic	Homo-genization
Concentration (mg/ml)	0.363	0.164	0.295	0.323
Purity	5.52	2.06	5.50	5.48
Yield	9.07	6.56	7.37	3.23



Graph 1 Concentration of Extracted Phycocyanin

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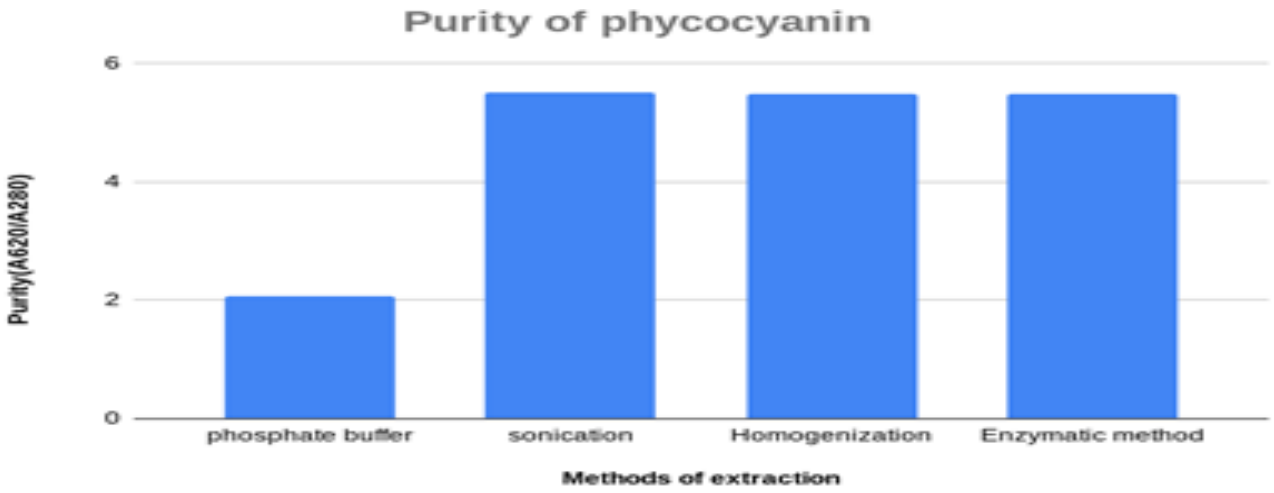
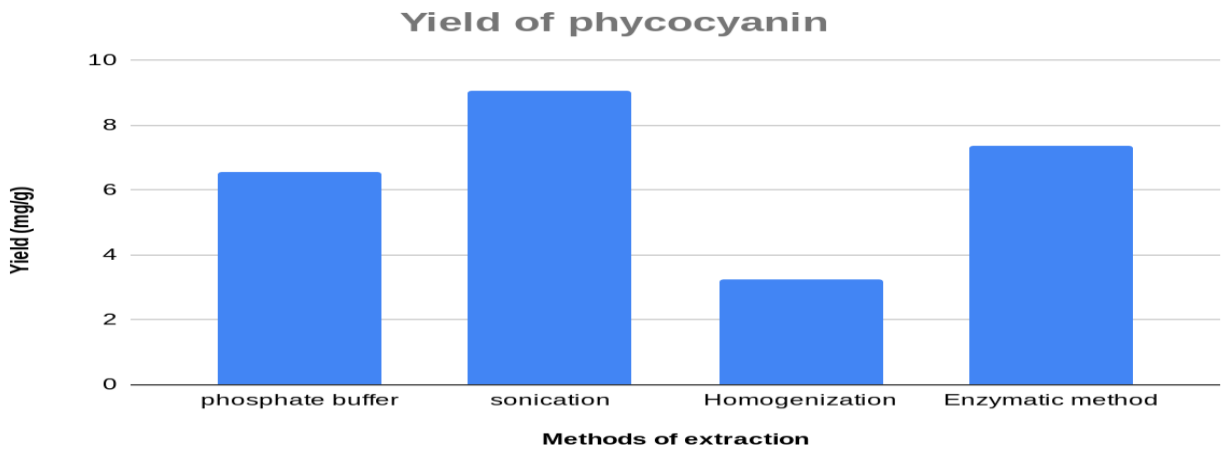


Graph 2 Purity of Extracted Phycocyanin



Graph 3 Yield of Extracted Phycocyanin

According to the graph data, the phycocyanin concentration is maximum in the sonication method, the purification is similar to sonication, homogenization, and the enzymatic method, and the yield is maximum in the sonication method. So, based on the data and interpretation described above, the sonication method is the most practical method for phycocyanin extraction.

- *Phycocyanin Purification:*

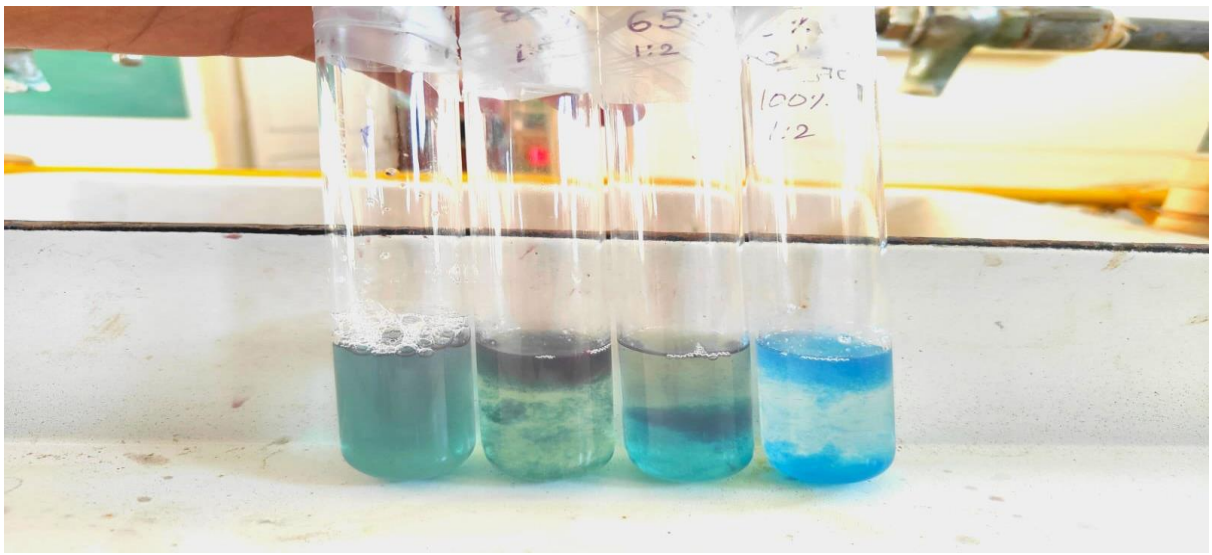


Fig 3 Ammonium Sulfate Precipitation by using different Concentrations of Ammonium Sulfate

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Phycocyanin purification often uses ammonium sulfate precipitation. Ammonium sulfate concentration for phycocyanin precipitation depends on the source and purification method. Ammonium sulfate salting out precipitates and purifies phycocyanin. Because ammonium sulfate makes protein solutions less soluble and precipitates them out. we Start with a low ammonium sulfate concentration (e.g., 40%) and gradually raise it to obtain the necessary purity. Purification used 100% ammonium sulfate. 80% and 100% ammonium sulfate precipitated heavily.

- *Characterization of Phycocyanin:*

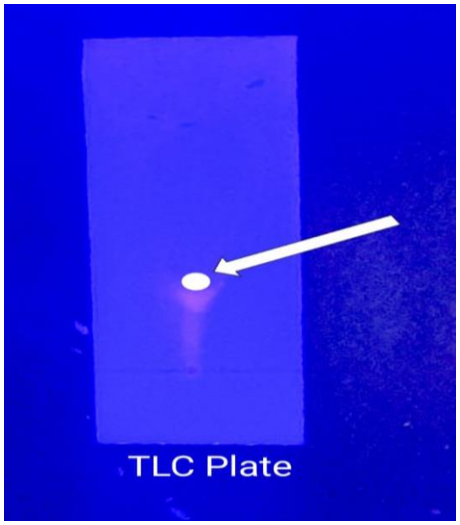
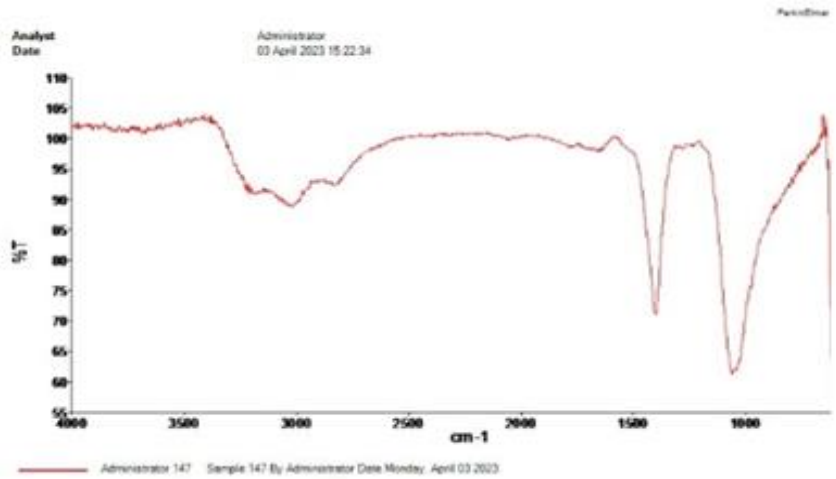


Fig 4 Confirmation test using TLC Method

Spirulina platensis phycocyanin was tested on a TLC plate after extraction. A blue spot indicates the presence of phycocyanin in the sample, which was confirmed by TLC [27]. Analytic thin-layer chromatography of the extract shows phycocyanin and an Rf value of 0.65 [16].



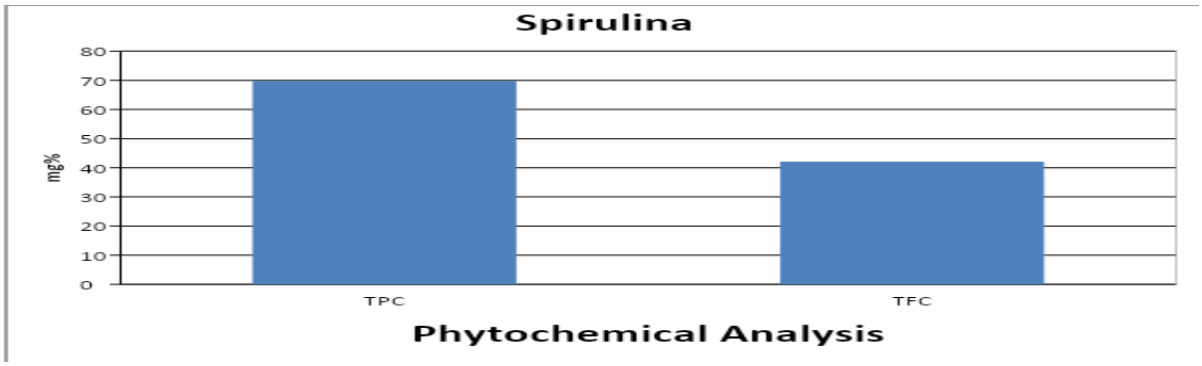
Graph 4 FTIR Spectra for Phycocyanin Extract

Table 2 Transmittance Wave Length Different & their Related Functional Group

Wavelegth	**Functional group**
3049 cm-1	O-H stretching
1404 cm-1	CH3 group
1065 cm-1	C=O group

FTIR spectrum of the extract of *Arthrospira platensis* shows various transmittance bands. A transmittance band 3049 suggested O-H stretching, 1404 suggested CH3 group, and 1065 suggested C=O functional group. Phycocyanin contains an aromatic ring, and the protein part of phycocyanin does contain CH3 groups & C=O which are in line with Al-Malki and Abdulrahman.

➢ *Phytochemical Analysis:*

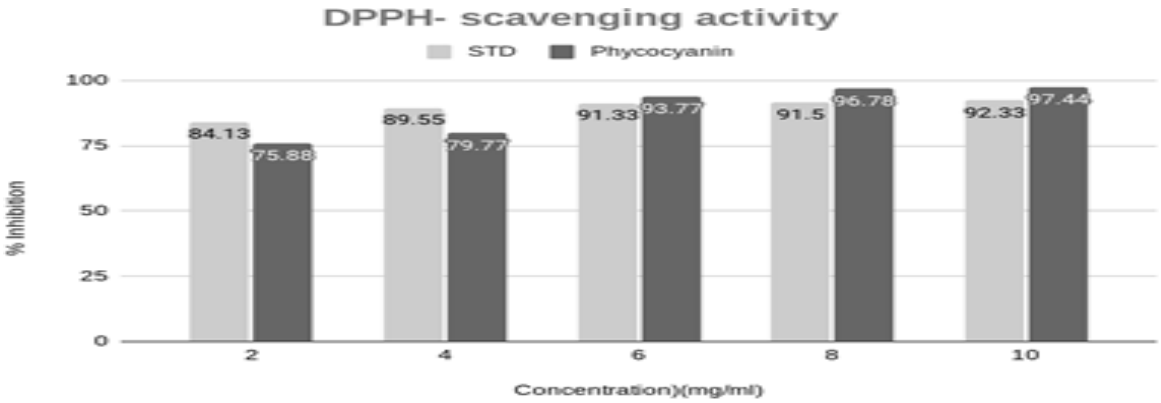


Graph 5 Estimation of total Phenolic and Flavonoid Content of Arthrospira Platensis

The first graph depicts the total phenolic and flavonoid content of Arthrospira platensis microalgae. The presence of 70mg% phenolics and 42.5% flavonoids were detected. Antioxidant and anti-inflammatory properties of phenols may have helped them combat diabetes. Phenols enhance cellular glucose assimilation and insulin sensitivity, which may aid in blood sugar regulation. Similar to phenols, flavonoids are anti-inflammatory and antioxidant. (Hidayati et al., 2020) Flavonoids may diminish diabetes by enhancing insulin sensitivity, cell glucose absorption, and alpha-glucosidase restriction.

➢ *Antioxidant Capability of Phycocyanin:*

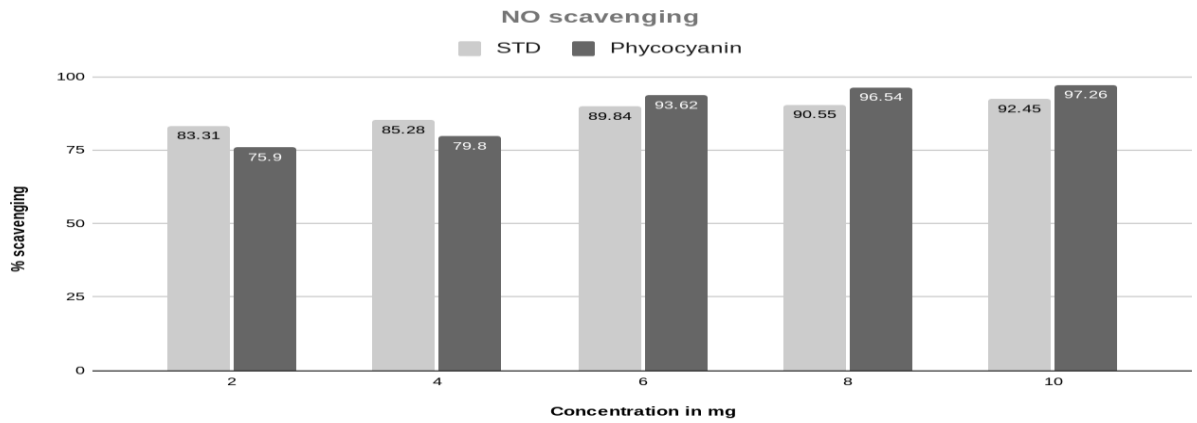
- *DPPH Free Radical Scavenging Assay:*



Graph 6 DPPH Scavenging Capability of Phycocyanin

According to the graph, a low concentration standard gives slightly higher inhibition & the high concentration of the sample (10mg) gives 96.44% DPPH scavenging activity related to the standard.

- *NO Scavenging Assay:*

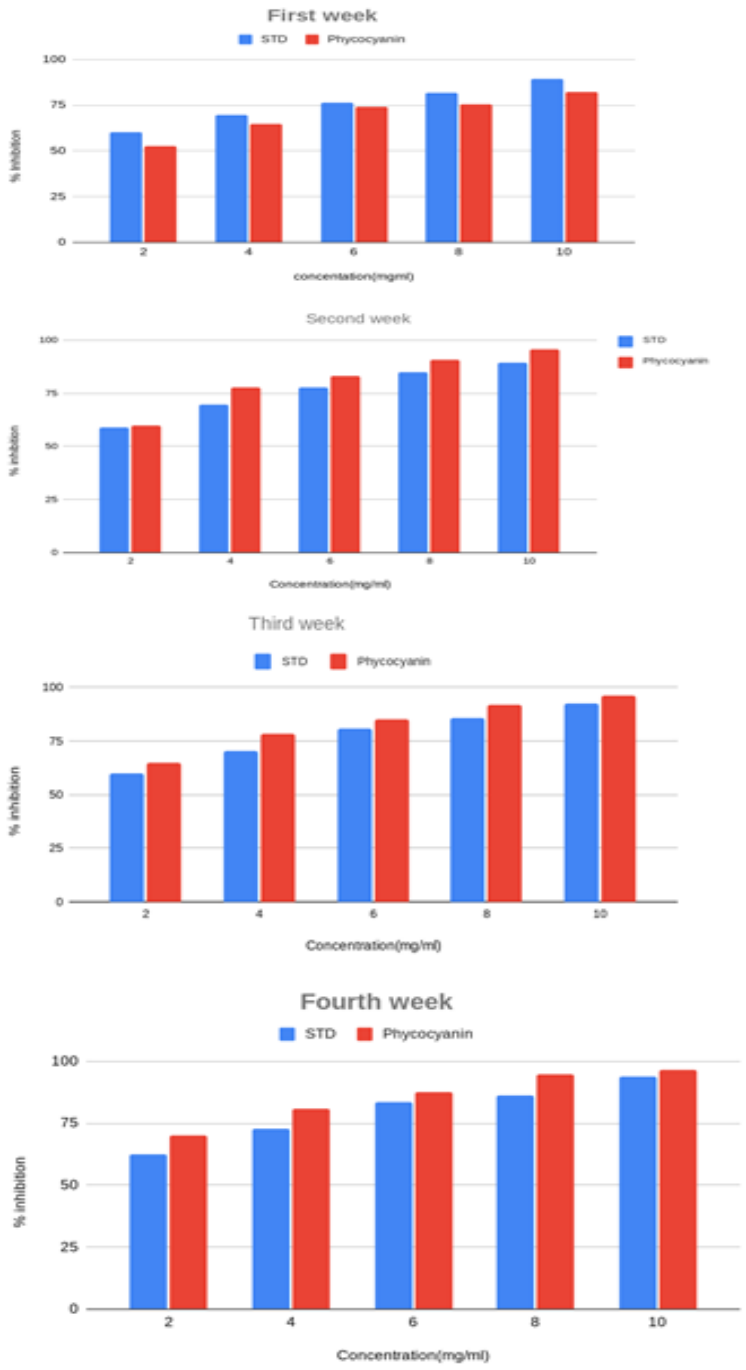


Graph 7 NO Scavenging Capability of Phycocyanin

Low concentration of the standard gives higher inhibition and the high concentration of the sample gives a maximum(97.76%) % NO scavenging activity compared to the standard.

➢ *Antiglycation Capability of Phycocyanin:*

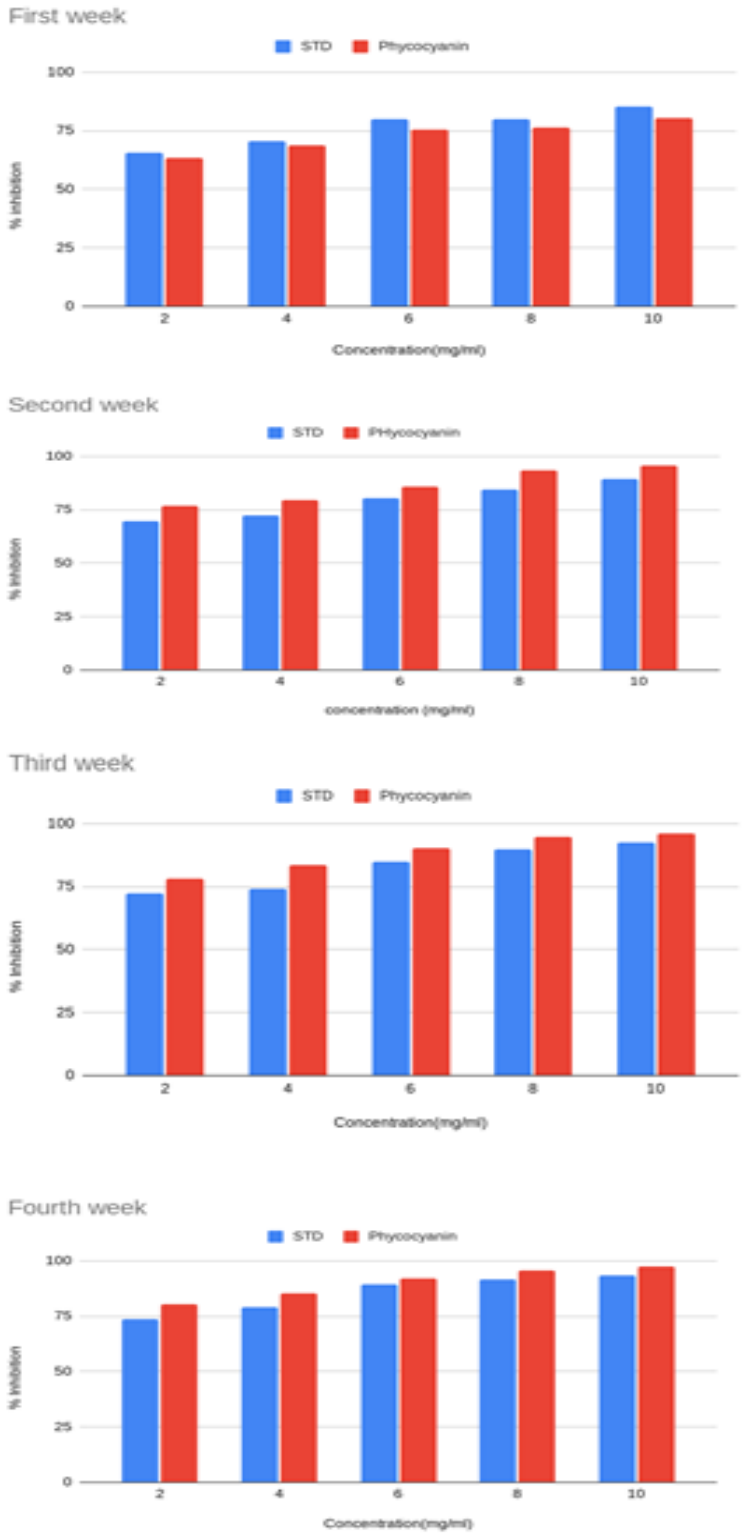
- *Fructosamine Assay:*



Graph 8 Four Weeks' Fructosamine Inhibition Activity of Phycocyanin

Phycocyanin and aminoguanidine inhibit the formation of AGEs in Figure 6. At 3 weeks, the suppression of AGEs by fructose-glycated BSA increased significantly. Phycocyanin decreased AGEs in a concentration-dependent manner. Phycocyanin doses of 2, 4, 6, 8, and 10 mg/mL inhibited AGE development by 65.2%, 78.24%, 85.166%, 92.173%, and 95.99%, respectively, at 21 days and sample. Fructosamine indicates impending glycation. After two and four weeks of incubation, the levels of fructosamine and fructosamine component inhibition decreased. Early on in the glycation process, unstable Schiff's bases are formed and converted into Amadori products such as fructosamine, which can be used to regulate blood sugar levels in diabetic patients. Thus, reducing fructosamine delays the onset of vascular issues.

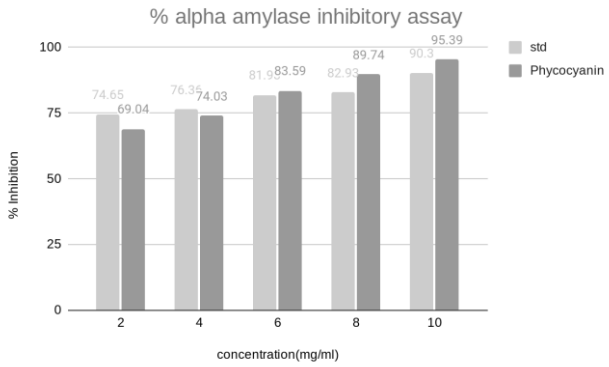
- *Congo-Red Binding Assay:*



Graph 9 Four Weeks'Congo-red Binding Assays of Phycocyanin

The concentration of Congo red was measured, and the percentage of inhibition declined during the second and fourth weeks of incubation. 10mg/ml aminoguanidine inhibited. Glycation increases amyloid cross-structure, resulting in protein conformational alterations and protein aggregate formation. Protein aggregation has been linked to hemodialysis-associated amyloidosis, diabetes, Parkinson's disease, and Alzheimer's disease (Chiti and Dobson, 2006). Glycation increases amyloid cross b-structure, thereby modifying protein conformation and aggregation. Higher concentrations of phycocyanin inhibit 96.17 percent more than normal.

➢ *Anti-Diabetic Activity of Phycocyanin:*



Graph 10 Alpha-amylase Inhibition Activity of Phycocyanin

As a whole, the alpha-amylase inhibitory screening test is a beneficial means of evaluating both synthetic and natural compounds for their capacity to inhibit alpha-amylase activity, which could assist in the discovery of novel causes for anti-diabetic drug development. According to the graph, we estimated that a high concentration of phycocyanin gives a 95.39% of alpha-amylase inhibition activity.

➢ *DNA Protective Ability of Phycocyanin:*

Fig 5 DNA Protective Capability of Phycocyanin

The DNA protection test examines how different chemicals or compounds shield DNA from reactive oxygen species (ROS) and other DNA-damaging agents. Gel electrophoresis measures DNA fragmentation in order to detect reactive oxygen species and other forms of DNA damage. The DNA protection test aids in analyzing the protective effects of medications or substances on DNA damage, which is essential to numerous biological processes such as aging, cancer, and neurological disorders. DNA may be protected by the anti-inflammatory properties of phycocyanin. Inflammatory oxidative stress can disrupt DNA (Suwanwong, 2013). DNA is safeguarded by phycocyanin, as depicted in the illustration.

IV. CONCLUSION

Spirulina-isolated phycocyanin was analyzed for its antioxidant, anti-diabetic, anti-glycation, and DNA-protective properties. According to this study, phycocyanin possesses antioxidant, anti-glycation, and anti-diabetic properties. Recent research produced these findings. Phycocyanin also prevents DNA oxidation caused by free radicals for a certain extent. This makes phycocyanin a promising pharmaceutical and nutritional component. This research establishes the foundation for phycocyanin action mechanisms and therapeutic applications. They also contribute to the potential health benefits of phycocyanin. This study highlights the importance of researching natural bioactive compounds and their medical applications.

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