

Research Article

Extraction, Purification, and Biological Evaluation of Flavonoids from *Spirulina* CPCC-695

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Abstract

Flavonoids are low molecular weight plant secondary metabolites having polyphenolic structure and are associated with plant cell stress tolerance, antioxidant properties and detoxification potential. Recently, flavonoids have gained commercial applications in the field of nutraceutical, pharmaceutical, medicinal, and cosmetic industries. This has compelled the scientist to look for good, high yielding, low cost bioresources. Therefore, in present study *Spirulina* CPCC-695 flavonoid was investigated for its bioactivities. HPLC-MS analysis of the flavonoid revealed 2 prominent peaks, identified as quercetin and rutin. The purified flavonoid depicted satisfactory α -amylase and α -glucosidase inhibition activity with $IC_{50} = 112.43$ and $130.68 \mu\text{g/ml}$, respectively along with considerable anti-inflammatory activity ($IC_{50} = 40.14 \pm 0.14 \mu\text{g/ml}$). Among DPPH, ABTS, NOR and PM antioxidant activity the sample showed highest activity in ABTS assay ($IC_{50} = 40.12 \pm 0.10 \mu\text{g/ml}$). Inhibition of hemolysis and thrombosis (1.80% and 81.46%) with $250 \mu\text{g/ml}$ suggested its safe use for human consumption. The findings of the present study suggested that *Spirulina* CPCC-695 may be used as a promising aquatic renewable source of flavonoids for pharmaceutical and nutraceutical applications.

INTRODUCTION

Polyphenols are categorized as phenol carbonic acids (Hydroxybenzoic acid, Hydroxycinnamic acids); flavonoids (flavones, flavonols, flavanones, flavanonols, flavanols, anthocyanins); isoflavones (isoflavones, coumestans); stilbene; lignans and phenolic polymers (proanthocyanidins-condensed and hydrolysable tannins). Metabolic processes like glycosylation, malonylation, methylation, hydroxylation, acylation, prenylation or polymerization of flavonoids are responsible for the synthesis of a wide spectrum of end products [1]. Over 5000 flavonoids, have been characterized from different tissues including leaves, flowers, stems, pollen, and seeds [2].

Due to antioxidative nature, the spectrum of biological activities of flavonoids (polyphenols) is very extensive. Flavonoids help in plant heat acclimatization, freezing tolerance [3] and UV filtration [4]. They protect the organism from different biotic and abiotic stresses through detoxification of reactive oxygen species such as superoxide ($O_2^{\cdot-}$), H_2O_2 , hydroxyl radical (OH \cdot), singlet oxygen (1O_2) and hydroperoxyl (HO_2^{\cdot}) with the help of flavonoid peroxidase [5]. They not only inhibit the autoxidation of lipids, but also retard lipid oxidation by inhibiting lipoxygenase activity. Presence of large number of phenolic

groups, enable the flavonoids effectively chelate heavy metal ions and reduce their toxicity [6]. In animal system, flavonoids exhibit antioxidative, anti-inflammatory, anti-mutagenic and anti-carcinogenic properties coupled with the modulation of cellular enzymatic functions [7].

Currently many flavonoids such as apigenin, hesperitin, naringenin, quercetin, myricetin, rutin, kaempferol, naringin and phloretin have been widely investigated as a new source of bioactive ingredient that can be incorporated into foods for human welfare [8]. Flavonoids have various medical significance such as an antimicrobial, anti-cancer, cardioprotective, immunity booster, anti-inflammatory, anti-diabetic, and antioxidant substance. Genistein, a flavonoid is known for improving endothelial functions in postmenopausal women [9].

Klejduš et al. [10], revealed the presence of isoflavones (e.g., daidzein, genistein and its derivatives) in several algae (*Undaria*, *Sargassum*, *Chondrus*, *Scenedesmus*, *Spongiochloris spongiosa*) (cyanobacterium-*Nostoc*). Shibata et al. [11], detected bromophenols - 2,4-dibromophenol, 2,4,6-tribromophenol and dibromo-iodophenol with U.V. absorbing potential from brown algae *Eisenia bicyclis* and *Ecklonia kurome*. Rico et al. [12], noted heavy metal (Cu and Fe) induced increase in total phenolic

contents with alteration in phenolic profile in *Phaeodactylum tricornutum*. Singh et al. [13], found flavonoids rutin, quercetin and kaempferol from cyanobacterial extracts. Considering the significance of flavonoids, the objective of this study was to isolate, purify and characterize flavonoids present in *Spirulina* CPCC-695 and to detect their biological activities.

MATERIALS AND METHODS

Screening of Cyanobacteria for flavonoids

All the cyanobacterial strains were obtained from germplasm collection center of IARI, New Delhi, University of Allahabad, Canadian Phycological Culture Centre (CPCC), University of Madras and Central Food Technological Research Institute (CFTRI), Mysore. Cyanobacterial strains were grown in BG-11/Zarrouk's/ ASN medium in 500 ml Erlenmeyer flasks placed in a BOD cabinet at 27 ± 2 °C illuminated with 20W fluorescent lamps providing a light intensity of 2000 ± 200 lux under 12:12 light:dark photoperiod [14-16]. Cultures were shaken at alternate days for aeration and nutrient mixing. *Lyngbya* sp. NCCU-102, *Oscillatoria* sp. NCCU-369, *Synechocystis* NCCU-370, *Gleocapsa gelatinosa* NCCU-430, *Chroococcus* sp. NCCU-207, *Plectonema* sp. NCCU-204, *Phormidium* sp. NCCU-104 and *Spirulina* CPCC-695 were grown in BG-11 (N_2 +ve) medium. *Nostoc sphaericum* NCCU, *Scytonema* sp. NCCU-126, *Cylindrospermum stagnale* NCCU, *Tolypothrix tenuis* NCCU 122, *Microcheate* sp. NCCU- 342, *Nostoc muscorum* NCCU-42, *Aphanocapsa* NCCU-542, *Calothrix brevisseima* NCCU-65, *Westiellopsis prolifica* NCCU-331, *Hapalosiphon*, *Anabaena variabilis* NCCU-441 and *Nostoc punctiforme* NCCU were grown in BG-11 (N_2 -ve). *Arthospora platensis* (NEERI), *Arthospora indica* (SOSA-4), *Arthospora indica* (SAE-84), *Aulosira fertilissima* NCCU-443, *Spirulina* NCCU-479, *Arthospora maxima* SAE-49-88, *Spirulina platensis* (2303), *Spirulina platensis* S5 and *Spirulina platensis* (CFTRI, Mysore) were grown in Zarrouk's medium whereas, *Spirulina subsalsa* was grown in Artificial Seawater Nutrient III (ASN III) medium. The analytical grade chemicals used in this study were purchased from Hi-Media and Merck. Double distilled water was used in all the experiments. Biomass was harvested by filtration, thoroughly washed (4 times) with double distilled water and dried at room temperature for 5 to 6 hours. Dried biomass was stored at 20°C for further analysis.

Preparation of extract

Flavonoid was isolated by adopting modified protocol of Altemimi et al. [17]. 3 g biomass and 10 ml methanol were mixed and ultrasonicated (Vibra-Cell™ Ultrasonic Processors, Sonics & Materials, Inc., USA) and stored at -20°C for 48 hours. The extract was then centrifuged at 5000 rpm (revolutions/min) for 20 mins. The supernatant was filtered through Whatman No. 1 filter paper. The extraction process was repeated until the green color of the pellet faded. Extract was separated by two-phase partitioning system in a separating funnel using n-hexane and ethyl acetate as separating solvents. The extract was then concentrated in Buchi type rotatory evaporator.

Total flavonoid content of methanolic fraction was estimated by aluminium chloride method (AlCl₃ test) using quercetin as standard [18]. For this, 1 ml of methanolic extract was mixed with 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of Milli-Q water, incubated for 40

minutes at room temperature and absorbance was measured spectrophotometrically (UV Plus Spectrophotometer, Motras Scientific, India) at 415 nm against blank (10 % Aluminium chloride). To quantify the flavonoid, the standard curve of quercetin was prepared. The calibration curve was described by the equation given below with regression co-efficient (r^2) being 0.9491, which was used to calculate the amount of flavonoid.

$$y = 0.0016X + 0.0457$$

where, Y is absorbance at 415 nm and X is the amount of flavonoid in µg/g.

Optimization of flavonoid extraction conditions

The best strain *Spirulina* CPCC-695 was selected for time course, yield optimization and characterization of cyanobacterial flavonoid. Time course study was performed to investigate the optimal time for flavonoid yield. For this, the cultures were incubated for 21 days, and their flavonoid yield and growth was measured at the interval of 3 days. The effect of temperature (10, 20, 30, 40, 50, and 60°C), pH (4.0 - 12.0) and solvent on flavonoid extraction were also observed. Methanol, ethanol, acetone, acetonitrile, n-Hexane, butanol, ethyl acetate, petroleum ether and di-ethyl ether were used as different solvents. Pretreatment techniques such as agitation and no agitation were also determined.

Purification and characterization of flavonoids

The concentrated extract was then subjected to HPLC analysis. HPLC was performed with Agilent 1260 Infinity II LC System (California, USA) equipped with quaternary pump, an autosampler, and a diode array detector. The analytical column used was C18 column (Eclipse XDB-C18 5µm 4.6x15 mm column) with a mobile phase consisting of acetonitrile and water containing 0.2% phosphoric acid. The flow rate and injection volume were 1.0 ml/min and 20 µl respectively. The column temperature was maintained at 25°C and the detection wavelength was set at 255 nm for rutin and quercetin.

Preparation of standard solutions for HPLC

The stock solution of concentration 1mg/ml was prepared by dissolving 1 mg quercetin and rutin separately in 1 ml HPLC-grade methanol and were protected from light by covering with aluminium foil. A series of standard solution of flavonoids ranging from (5-160 µg/ml) was prepared and filtered by using sterilized 0.2 µm syringe filters.

Determination of Bioactivities of Flavonoid

Anti-diabetic assay: Anti-diabetic efficacy of partially purified flavonoid was evaluated by α-amylase and α-glucosidase assay. α-amylase assay was performed according to the method of Telagari and Hullatti [19]. 20 µl sample of various concentrations (15, 25, 50, 100, 150, 200 and 250 µg/ml) was added to 50 µl phosphate buffer (10mM; pH 6.8) along-with 10 µl α-amylase (2 U/ml). The reaction mixture was incubated at 37 °C for 30 mins. After, the incubation 100 µl of 3,5-Dinitrosalicylic acid (DNS) colour reagent was added and the reaction mixture was boiled for 10 mins. Further, the absorbance was read at 540 nm and acarbose was used as reference standard.

α -glucosidase assay was performed by the method of Sanger et al. [20]. In 10 μ l sample (15, 25, 50, 100, 150, 200 and 250 μ g/ml) added 20 μ l α -glucosidase (0.5 U/ml) along-with 120 μ l phosphate buffer (0.1 M; pH 6.9) and the reaction mixture was incubated at 37°C for 30 mins. Reaction was incited by adding 20 μ l phosphate buffer (5 mM; pH 6.9) and incubated at 37°C for 15 mins. Reaction was ceased by adding 80 μ l sodium carbonate (0.2 M). Absorbance was read at 405 nm and acarbose was used as reference standard. Percentage inhibitions for both the assays were calculated using equation 1:

$$\% \text{ Inhibition} = \frac{(\text{control absorbance} - \text{sample absorbance})}{\text{control absorbance}} \times 100 \quad (1)$$

Concentration of samples resulting in 50% α -amylase and α -glucosidase inhibition (IC50 value) was calculated.

Anti-inflammatory activity: Method of Sakat et al. [21], was followed for anti-inflammatory activity by protein denaturation assay. For this, 0.2 ml of 1% bovine albumin fraction was added to 2 ml of test extract of different concentrations and pH was maintained using 1 N HCl. The reaction mixture was incubated at 37°C for 20 minutes along with further heating at 57 °C for 20 minutes. After cooling, the turbidity was measured at 660 nm using UV-visible spectrophotometer. Acetylsalicylic acid was used as a reference standard. The experiment was performed in triplicates. Percentage inhibition of protein denaturation was calculated using equation 2. Concentration of samples resulting in 50% protein inhibition (IC50 value) was calculated.

Antioxidant Assays: Antioxidant potential of *Spirulina* CPCC-695 flavonoid was assessed by DPPH, ABTS, NOR, and PM assay as detailed below-

During, DPPH (2, 2-Diphenyl-2-picrylhydrazyl) radical scavenging assay of Blois [22], 300 μ l of sample was mixed with 900 μ l of DPPH (1 mM in 80% ethanol) at room temperature in dark for 30 mins. The absorbance was recorded at 517 nm against blank. Ascorbic acid was used as a reference standard. The scavenging ability (%) was calculated using equation 1. Concentration of samples resulting in 50% inhibition on DPPH (IC50 value) was calculated.

For ABTS assay of Re et al. [23], aqueous solution of 7 mM of ABTS [2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] was reacted with potassium persulfate (2.45 mM) and the mixture was allowed to stand in the dark at room temperature for 12-16 h before proceeding for experiment. This reaction produces ABTS radical cation (ABTS^{•+}). Prior to assay, the ABTS^{•+} was diluted with ethanol to give an absorbance of 0.70 \pm 0.02 at 734 nm. 1 ml of ABTS solution was added to 10 μ l of extracts (different concentrations) and the absorbance was measured at 734 nm against blank exactly 30 minutes after the initial mixing. Ascorbic acid was used as positive control. The percentage scavenging activity was calculated using equation 1. Concentration of samples resulting in 50% inhibition on ABTS radical cation (ABTS^{•+}) (IC50 value) was calculated.

Nitric oxide radical scavenging activity was determined according to Lee et al. [24], in which sodium nitroprusside (500 μ l of 5 mM) prepared in phosphate buffer (0.1 M, pH 7.4) was added to 1 ml of sample (5-100 μ g/ml). After 30 mins of incubation at 25 °C, 500 μ l of Griess reagent (1% sulphanilamide and 0.1%

N-(1-Naphthyl) ethylenediamine) was added. After thorough mixing the absorbance of the chromophore formed during diazotization of the nitrite with sulfanilamide and coupling with N-(1-Naphthyl) ethylenediamine dihydrochloride was noted at 550 nm. Ascorbic acid was used as a positive control. Percentage of scavenging ability was calculated using equation 1. Concentration of samples resulting in 50% inhibition on NO radical (IC50 value) was calculated.

In Phosphomolybdenum assay, the reduction of phosphate molybdenum (VI) to phosphate molybdenum (V) was performed by the method of Elkhamlichi et al. [25]. 1 ml of sample was allowed to stand with 1 ml of phosphomolybdate reagent (28 mM sodium phosphate, 4 mM ammonium molybdate and 0.6 M sulphuric acid) at 95°C for 90 minutes in a water bath. The absorbance of the green molybdenum complex was measured at 695 nm against blank (0.3 ml distilled water along-with molybdate reagent) using UV-Visible spectrophotometer. Ascorbic acid was used as a positive control. Percentage of reduction was calculated using equation 1. Concentration of samples resulting in 50% inhibition on phosphate-molybdenum (VI) (IC50 value) was calculated.

Biocompatibility assays: Anti-hemolytic and anti-thrombotic activity was performed to determine the biocompatibility of partially purified flavonoids towards human RBCs.

Anti-hemolytic assay was performed according to the method of de Freitas et al. [26]. Blood from healthy individual were collected in vacuum tubes containing EDTA as anti-coagulant. The erythrocytes were harvested by centrifugation at 1500 rpm for 10 mins at 4 °C. Erythrocytes were separated from plasma and buffy coat and was washed thrice by centrifugation at 1500 rpm for 5 min in phosphate buffer saline (PBS) (10mM; pH 7.2). With each wash supernatant and buffy coat was removed. Pellet was resuspended in 0.5 % saline solution. 500 μ l of flavonoids (10, 20, 40, 80, 160, 320 μ g/ml) was added in 0.5 ml cell suspension and incubated at 37 °C for 30 mins. Reaction mixture was centrifuged at 1500 rpm for 10 mins. Triton X-100 (1%) and phosphate buffer saline was used as positive and negative control, respectively. Anti-hemolytic activity was assessed by measuring absorbance at 540 nm. Percentage of inhibition of hemolysis was calculated using equation 2.

$$\text{Anti-hemolysis \%} = \frac{(\text{Test sample} - \text{blank})}{(\text{Triton} - \text{blank})} \times 100 \quad (2)$$

Anti-thrombotic activity was done according to Prasad et al. [27]. 5 ml of blood was drawn from healthy individual and was distributed as 0.5 ml/tube in pre-weighed sterile micro-centrifuge tubes. Blood was incubated at 37°C for 45 mins. After clot formation, serum was removed without disturbing the clot. Each tube having clot was again weighed to determine the clot weight. 100 μ l of sample of various strength (10, 20, 40, 80, 160, 320 μ g/ml) was added in each tube and was incubated at 37 °C for 90 mins and clot weight was noted. 100 μ l streptokinase and distilled water was used as positive and negative control. Fluid released was removed and tubes were again weighed to observe the difference in weight after clot lysis. Was expressed as percentage of clot lysis. Percentage of clot lysis was calculated using equation 3.

$$\% \text{ Clot lysis} = \frac{\text{weight of the lysis clot}}{\text{weight of the clot before lysis}} \times 100 \quad (3).$$

Statistical analysis

All the experiments were carried out in triplicates (n=3) and all the values are expressed as means \pm SD. Statistical analysis was done using OriginPro 8.5 (2011). One-way ANOVA was performed to determine whether there are any statistically significant differences between the means of two or more independent groups. P-values <0.05 were regarded as significant.

RESULTS AND DISCUSSION

Quantification and optimization of total flavonoids

The screening of 18th day old biomass of selected 30 cyanobacterial strains showed that all of them contain flavonoid that ranged from 0.633 to 1.9266 mg / g of biomass. The highest yield of flavonoids (1.9266 mg / g of biomass) was found in *Spirulina* CPCC-695 (Figure 1). Most of the other studies yielded less than our test organism e.g. 100 μ g / g from *Oscillatoria* sp [28], 91 μ g/g from *Calothrix geitonos*, 88.37 μ g/g from *Microcheate tenera*, 79.43 μ g/g from *Hapalosiphon fontinalis*, 71.1 μ g/g from *Scytonema simplex*, 95.13 μ g/g from *Calothrix brevissima*, 95.20 μ g/g from *Phormidium tenue* [13] and 71.115 μ g/g from *Spirulina platensis* [29]. However, El-Baky et al. [30] and Gabal et al. [31] reported higher flavonoid from *Spirulina maxima* and *Spirulina platensis* respectively.

Flavonoids are the natural polyphenolic compounds produced during the stationary phase and possess a broad spectrum of chemical and biological properties including antioxidant and free radical-scavenging activities. Total phenolic contents exhibit significant variations depending on the geographical, physiological, and environmental factors and culture conditions

[32,33]. In the present study, flavonoid and growth was monitored on every third day and it was found that flavonoid peak was delayed than growth peak (Figure 2). It suggests that after highest growth on 15th day, cells started producing secondary metabolites like flavonoids that reached to its peak on 18th day. *Nannochloropsis* species extracts also showed highest flavonoid during the stationary phase [34].

Therefore, during optimization of culture conditions (light and dark condition (L:D), temperature, and pH parameters) biomass was harvested on 18th day. The highest flavonoid yield was obtained in 16:08 L:D cycle, 50 $^{\circ}$ C temperature and pH 8 condition (Figure 3). Extraction condition was optimized for solvents, contact time, pre-treatment techniques. Methanol gave highest content after 48 hrs without agitation and with cold maceration technique (Figure 4).

Total flavonoid content in *Spirulina* CPCC- 695 increased with increasing extraction temperature and was highest at 50 $^{\circ}$ C (Fig 3C). Other scientists have also reported maximum flavonoid extraction at 50 $^{\circ}$ C e.g. from *Saccharina japonica*-brown algae [35]. However, Messyasz et al. [36], have reported lower temperature (35-40 $^{\circ}$ C) as adequate temperature for extraction of flavonoids from *Desmococcus olivaceus*, *Ulva clathrata*, *Cladophora glomerata* and *Polysiphonia fucooides*. Wang et al. [37], reported higher temperature (65 $^{\circ}$ C) as optimum temperature for extraction of flavonoids from kelp (macroalgae). Higher temperature increases the efficiency of the extraction, since heat escalates the cell walls permeability, increases solubility and diffusion coefficients of the compounds to be extracted.

Kilic et al. [38], and Safafar et al. [33], have also reported higher flavonoid yield in *Spirulina platensis*, *Dunaliella* sp., *Nannochloropsis salina*, *Nannochloropsis limnetica*, *Desmodesmus* sp., *Dunaliella salina*, *Phaeodactylum tricornutum* and *Chlorella*

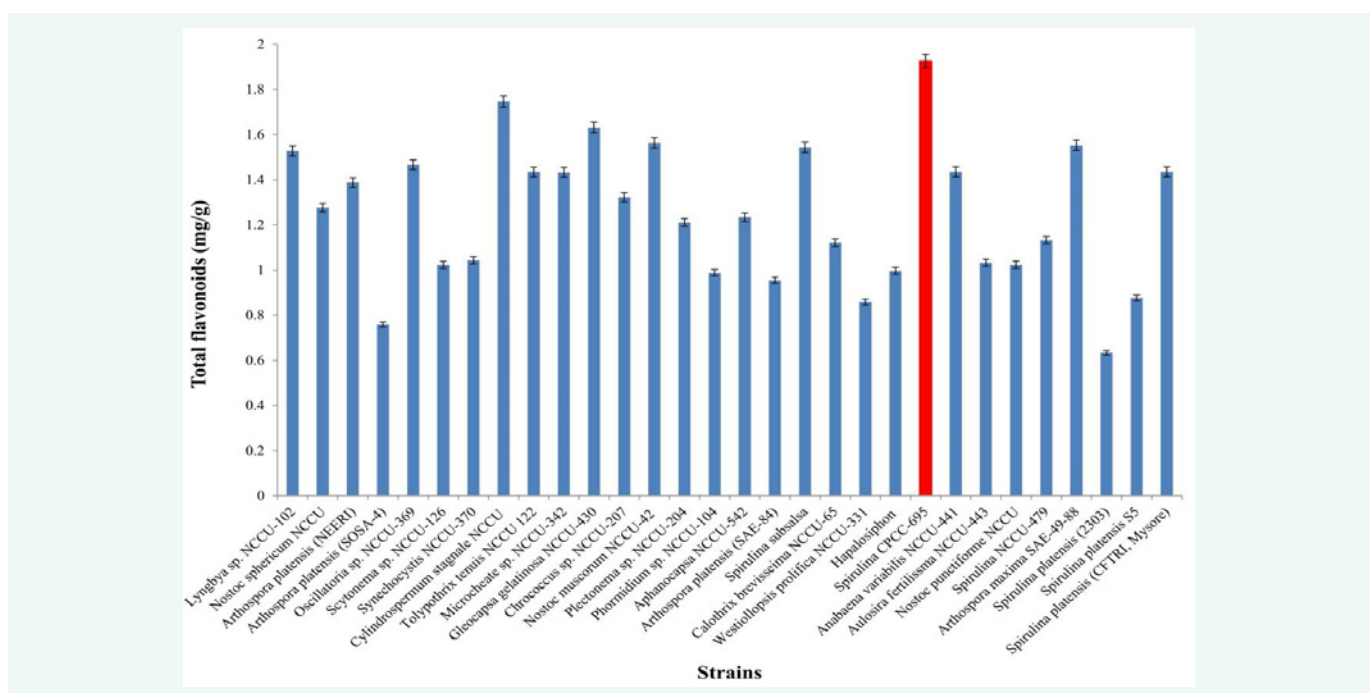


Figure 1 Screening of 30 cyanobacterial strains to determine maximum yield of flavonoid.

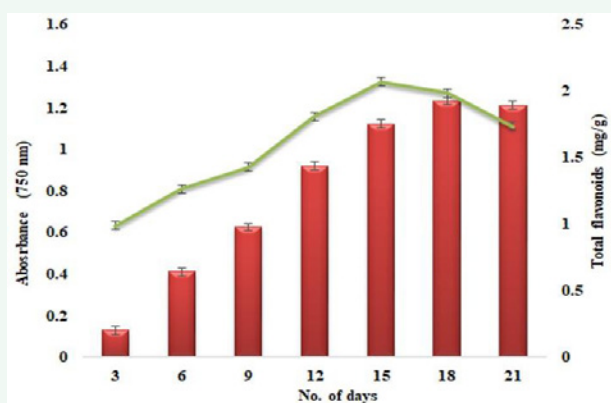


Figure 2 Time course study for growth and flavonoid in *Spirulina* CPCC-695.

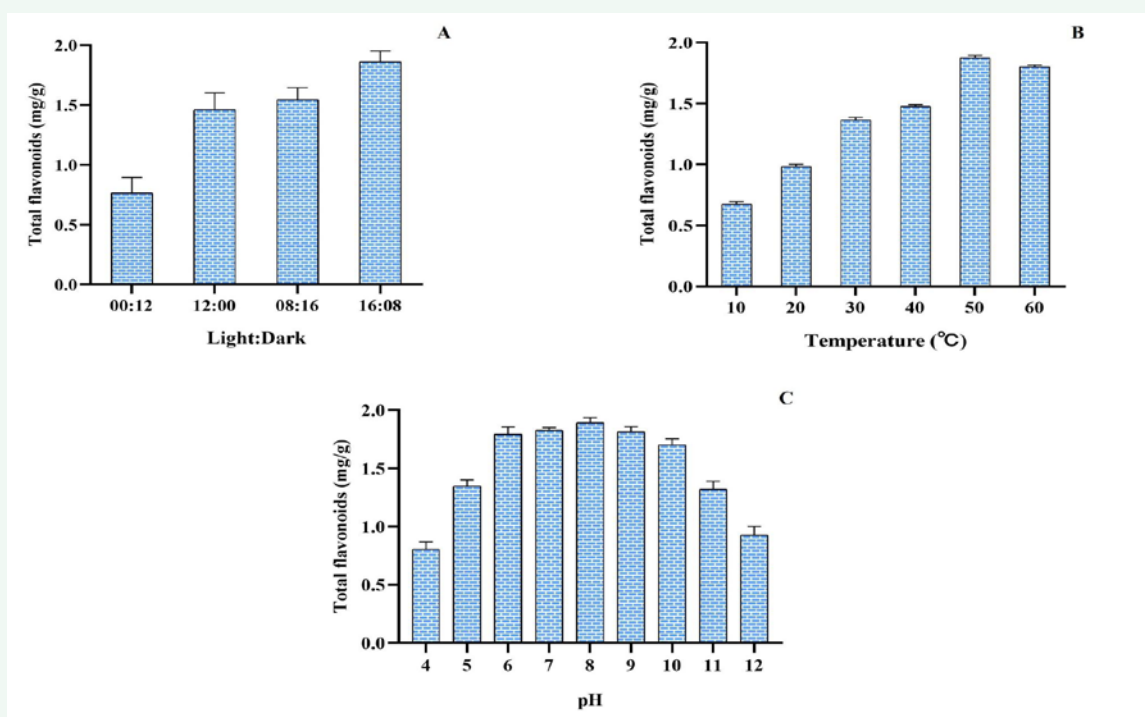


Figure 3 Effect of extraction (a) light: dark (b) temperature (c) pH on flavonoid extraction from *Spirulina* CPCC-695.

sorokiniana at pH 8.0. Sivagnanam et al. [35], found maximum flavonoid extraction at 50 °C e.g., from *Saccharina japonica*-brown algae.

Flavonoids are the natural polyphenolic compounds possessing a broad spectrum of chemical and biological properties. This work aims to study *Spirulina* CPCC-695 as a potential source of bioactive flavonoids for the first time.

HPLC analysis

The purification and characterization of crude extract was done by column chromatography and HPLC respectively. The method developed for HPLC provided a quick analysis of the extract. Quercetin (Rt= 1.680) and rutin (Rt= 1.307) can be identified in the chromatogram (Figure 5a, Table 1). Quercetin

and rutin were identified by their respective standards obtained under the same conditions. The concentration of both the analytes was determined by the calibration curve made with their standards (5-160 µg/ml). Calibration curve was constructed with purity against the peak of quercetin and rutin (Figure 5b and c). The R² for both the analytes was 0.997. The content of the analytes was determined by using the regression equation. Good linearity was obtained in the range of 5-160 µg/ml with coefficient of determination and linear regression equation $y=1.47 X + 1.71$ for quercetin and $y=1.26 X + 5.73$ for rutin respectively.

Antidiabetic Activity

Spirulina CPCC-695 flavonoid exhibited 82.10% inhibition (IC₅₀ = 112.43 µg/ml) and 78.22% inhibition (IC₅₀ = 130.68 µg/ml) in α -amylase and α -glucosidase activity showing anti-diabetic

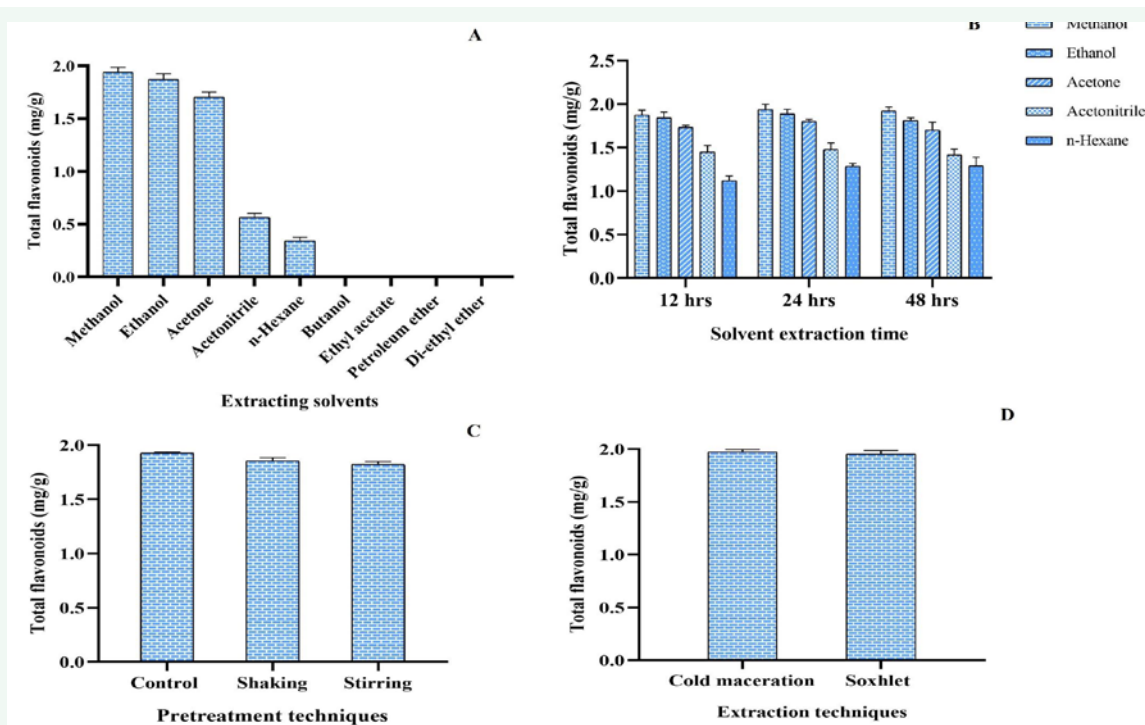


Figure 4 Effect of extraction (a) solvent (b) solvent extraction time (c) pretreatment techniques (d) extraction techniques on flavonoid extraction from *Spirulina* CPCC-695.

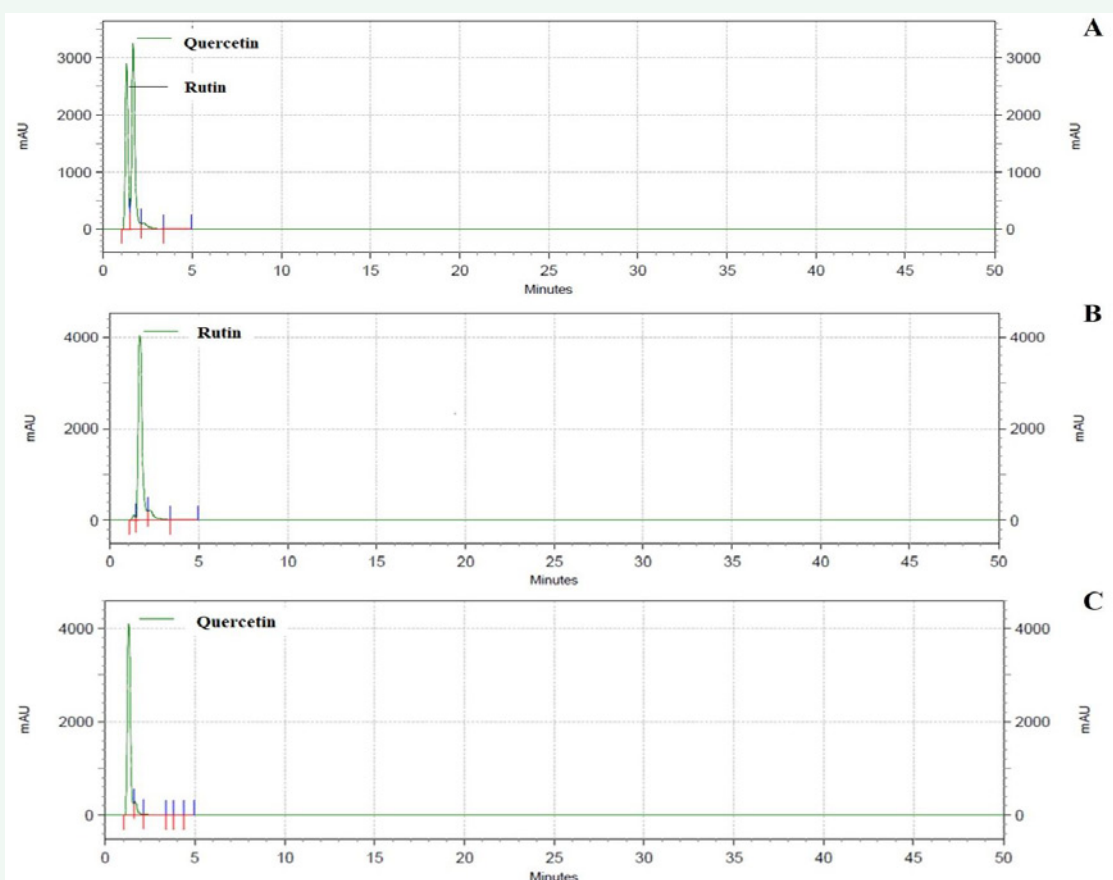


Figure 5 HPLC spectrum of (a) sample (b) rutin standard (c) quercetin standard.

Table 1: Parameters of calibration of rutin and quercetin for HPLC.

Sample	Detected at wavelength λ	Retention time	Regression coefficient (R^2)	LOD ($\mu\text{g/ml}$)	LOQ ($\mu\text{g/ml}$)	Content ($\mu\text{g/ml}$)
Rutin	255	1.307 (Rutin)	0.997	0.6433	1.9494	1959.521
Quercetin	255	1.680 (Quercetin)	0.997	0.6433	1.9494	2829.345

potential, respectively (P-values <0.05) (Table 2, Figure 6). Pirian et al. [39], reported α -amylase inhibition by polyphenols present in *Sirophysis trinodis* (brown algae) at 8000 $\mu\text{g/ml}$ having 97 % inhibition ($\text{IC}_{50} = 420 \mu\text{g/ml}$). Unnikrishnan et al. [40], reported that among four seaweeds *Chaetomorpha aerea* at the concentration of 500 $\mu\text{g/ml}$ showed 86% inhibition of α -amylase and $\text{IC}_{50} = 408.9 \mu\text{g/ml}$. Flavonoids from *Durvillaea antarctica* and *Gelidium* sp. inhibited α -amylase activity to 56.6% and 77.9% respectively at 2000 $\mu\text{g/ml}$ [41]. Sanger et al. [20] reported that flavonoids from *Halimena durvillae* (red algae) showed only 18.71 % inhibition against α -glucosidase activity.

The level of glucose produced by carbohydrate hydrolysis to produce monosaccharide is mediated by α -amylase and α -glucosidase enzymes in small intestine. The inhibition of these enzymes slows the breakdown of dietary polysaccharides into simpler saccharides thus, reducing hyperglycemia [42,43]. Thus, the flavonoid in the present study inhibited α -amylase and α -glucosidase enzymes in a dose dependent manner hence reducing the level of glucose.

Anti-inflammatory Activity (Inhibition of Protein Denaturation)

Inflammation is associated with increased protein denaturation, vascular permeability, membrane stabilization, and pain. Denaturation of proteins involves the disruption of both the secondary and tertiary structures as well [44] as well. Flavonoid exhibited inhibition of albumin denaturation in a dose dependent manner having maximum inhibition of 79.96% with $\text{IC}_{50} = 40.14 \pm 0.14 \mu\text{g/ml}$ in comparison to the standard acetylsalicylic acid (Aspirin) that showed 96.07% with $\text{IC}_{50} = 15.23 \pm 0.04 \mu\text{g/ml}$ (P-values<0.05) (Table 3, Figure 7). SM et al. [45], reported 58.69% inhibition by *Sargassum wightii* flavonoid at 3000 $\mu\text{g/ml}$ with $\text{IC}_{50} = 2500 \mu\text{g/ml}$. Patil et al. [46], and Punitha et al. [47] observed 21.65% and 0.67% at 500 and 125 $\mu\text{g/ml}$ from *Scenedesmus bajacalifornicus* BBKLP-07 and *Spirulina platensis* cell extract rich in flavonoid. The flavonoids obtained from *Spirulina* CICC-695 have shown significant anti-inflammatory property which might be due to inhibition of biosynthesis of prostaglandins, protein kinases and phosphodiesterases [48].

Table 2: IC_{50} of Anti-diabetic assays.

Sample ($\mu\text{g/ml}$)	α -amylase	α -glucosidase
Flavonoid ($\mu\text{g/ml}$)	112.43	130.68
Acarbose ($\mu\text{g/ml}$)	34.75	36.24

Table 3: IC_{50} of Anti-inflammatory activity.

Sample ($\mu\text{g/ml}$)	IC_{50}
Flavonoid ($\mu\text{g/ml}$)	40.14 ± 0.14
Acetylsalicylic acid	15.23 ± 0.04

Antioxidant Activities

Antioxidant nature of polyphenols is responsible for wide range of their biological activities like protection against biotic and abiotic stresses. They get oxidized by radicals, resulting in a more stable, less-reactive form [49]. During present investigation *Spirulina* CICC-695 flavonoid also showed good antioxidant activities in tested bioassays as detailed below---

DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) antioxidant assay is based on electron-transfer that produces violet color in ethanol. This free radical is stable at room temperature and gets reduced in the presence of an antioxidant molecule that gives rise to colorless ethanol solution [50]. Purified flavonoid extract and ascorbic acid showed DPPH radical scavenging activity in a dose dependent manner (P-values <0.05) and % inhibition of free radical was 69.68% with $\text{IC}_{50} = 62.21 \pm 0.84 \mu\text{g/ml}$ and 95.15% with $\text{IC}_{50} = 8.41 \pm 0.08 \mu\text{g/ml}$ respectively (Table 4, Figure 8) (P-values <0.05). Badr et al. [51], reported percentage inhibition as 18.25% for *Nostoc calcicola* (KY250420.1), 19.16% with *Nostoc* sp. (KY296359.1), 22.94% with *Leptolyngbya* sp. (KY321359.1) and 29.33% with *Nostoc* sp. (KU373076.1) using 50 $\mu\text{g/ml}$ cell extract. Ebrahimzadeh et al. [52], noticed 39.03% and 21.68% inhibition by flavonoids in ethyl acetate and methanolic extract of *Nannochloropsis oculata* with 400 $\mu\text{g/ml}$ crude cell extract. Further, they also determined 20.48% and 17.4% inhibition by *Gracilaria gracilis* flavonoid with same concentration. Widowati et al. [53], found that flavonoids from *Dunaliella salina*, *Tetraselmis chuii* and *Isochrysis galbana* clone Tahiti showed 62.19% inhibition at 500 ppm, 71.36% inhibition at 1000 ppm and 67.93% inhibition at 250 ppm respectively. Safar et al. [33], estimated DPPH assay of flavonoids obtained from *Nannochloropsis limnetica*, *Phaeodactylum tricorutum*, *Nannochloropsis salina* and *Chlorella sorokiniana* and they found highest percentage inhibition (35.17%) from *Nannochloropsis limnetica* 1000 $\mu\text{g/ml}$ flavonoid.

ABTS [(2, 2'-Azinobis-(3-ethylbenzothiazole-6-sulphonate)] is a substance that produces stable and green colored ABTS radical cation ($\text{ABTS}^{\bullet+}$). It was calculated from the decolorization of $\text{ABTS}^{\bullet+}$ at 734 nm [23]. For ABTS, purified flavonoid extract and ascorbic acid showed percentage inhibition as 82.66% with $\text{IC}_{50} = 40.12 \pm 0.10 \mu\text{g/ml}$ and 98.31% with $\text{IC}_{50} = 7.76 \pm 0.01 \mu\text{g/ml}$ respectively (P-values <0.05) (Table 4, Figure 8). Wu et al. [54], and Shalaby et al. [55], reported 80.65% and 80% inhibition with 100 $\mu\text{g/ml}$ of *Spirulina platensis* flavonoid. Shanab et al. [56], and Singh et al. [13], reported IC_{50} as 69.80, 72.60, 230 and 970 $\mu\text{g/ml}$ for *Oscillatoria* sp., *Nostoc muscorum*, *Anabaena constricta* and *Oscillatoria acuta*, respectively.

Nitric oxide radical (NO^{\bullet}) scavenging assay is based on the ability of oxygen to scavenge the nitrite radical which is generated from sodium nitroprusside [24]. Purified flavonoid extract and ascorbic acid exhibited maximum percentage inhibition as

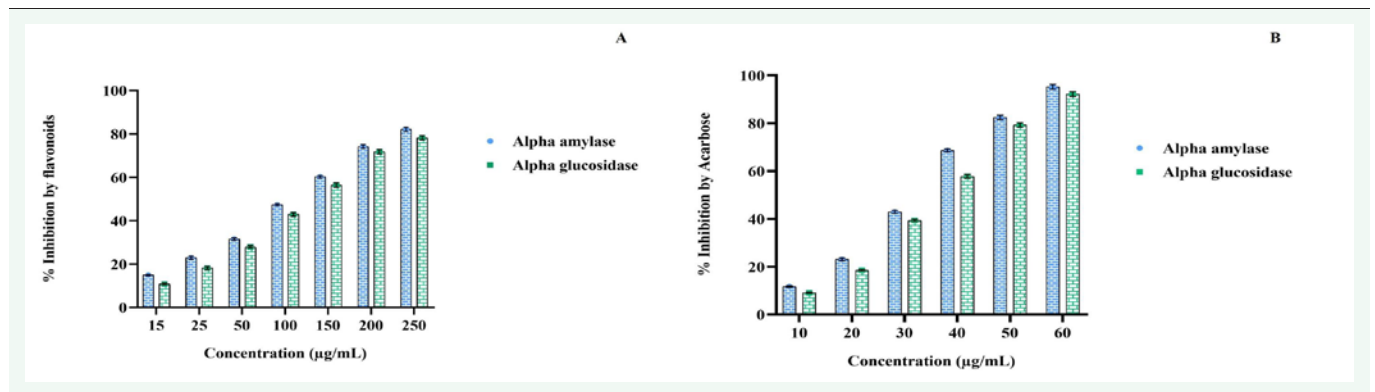


Figure 6 Anti-diabetic activity (a) acarbose (b) flavonoid from *Spirulina* CPCC-695.

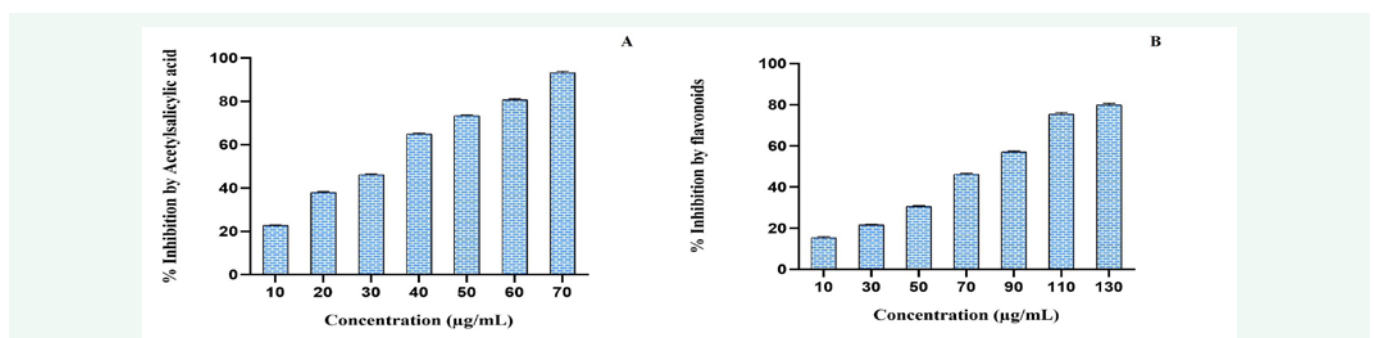


Figure 7 Anti-inflammatory activity (% inhibition) by (a) acetylsalicylic acid (Aspirin) (b) flavonoid from *Spirulina* CPCC-695.

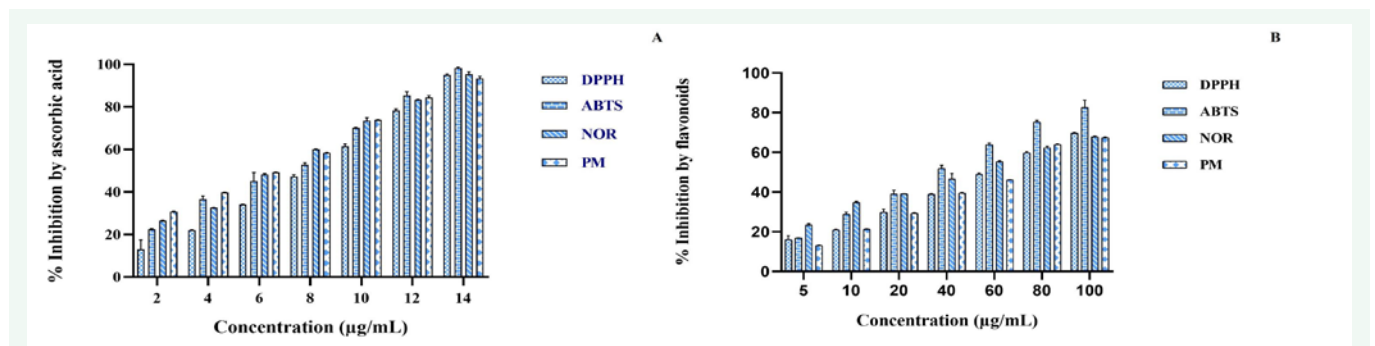


Figure 8 Antioxidant assays (% inhibition) by (a) ascorbic acid (b) flavonoids extracted from *Spirulina* CPCC-695.

67.88% with $IC_{50} = 48.76 \pm 1.01$ µg/ml and 95.33% with $IC_{50} = 6.77 \pm 0.01$ µg/ml respectively (P-values <0.05) (Table 4, Figure 8). Ebrahimzadeh et al. [51] reported 73.73% and 65.6% inhibition with flavonoids from ethyl acetate and methyl acetate extract of *Nannochloropsis oculata* and 65% and 34.8% with *Gracilaria gracilis* flavonoid. Alghazeer et al. [57], observed 56, 53.62, 44.64, 35.73 and 27.49% inhibition by flavonoids in 1000 µg/ml cell extracts of *Cystoseira spicata*, *Gelidium sesquipedale*, *Codium tomentosum*, *Enteromorpha linza* and *Padina pavonica* respectively.

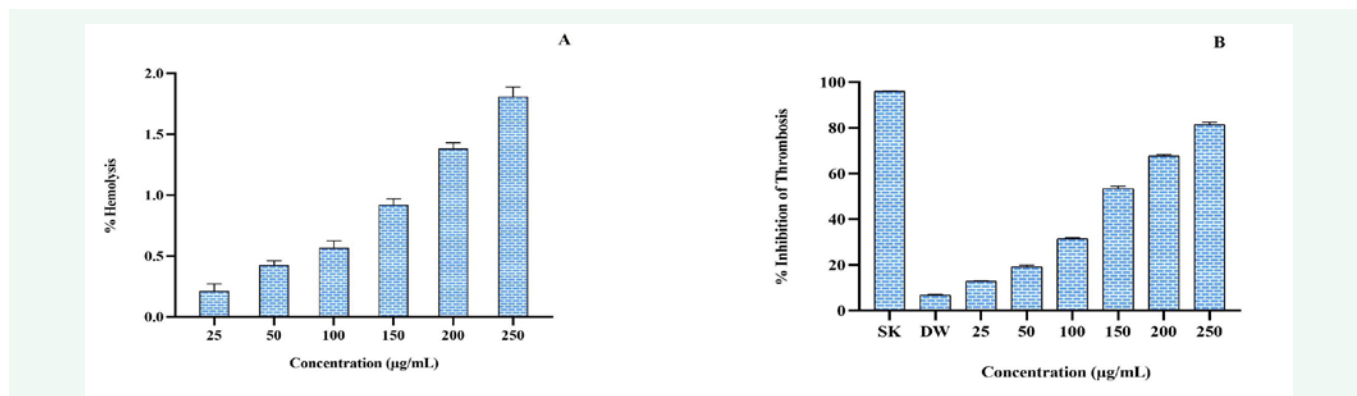
Phosphomolybdate is another important in vitro antioxidant assay to assess the total antioxidant capacity of the flavonoids which forms green phosphate Mo (V) complex due to the conversion of Mo (VI) to Mo (V) in the reaction [58]. In the

present study flavonoid rich extract and ascorbic acid showed 67.48% with $IC_{50} = 62.65 \pm 0.15$ µg/ml and 93.26% with $IC_{50} = 5.87 \pm 0.01$ µg/ml respectively (Table 4, Figure 8) which suggested the presence of effective antioxidant activity (P-values <0.05). Jayshree et al. [56] and Jose et al. [60], found determined 92.9% inhibition with $IC_{50} = 73.06$ µg/ml using 1 mg/ml flavonoid of *Chlamydomonas reinhardtii*, 98% inhibition with $IC_{50} = 106.7$ µg/ml and 95% inhibition with $IC_{50} = 99.5$ µg/ml at 2 mg/ml of flavonoids from the extract of, *Sargassum swartzii* and *Chaetomorpha antennina* respectively.

Antioxidant activity of *Spirulina* CPCC-695 flavonoid observed as above may be due to dominance of quercetin and rutin flavonoid.

Table 4: Comparison of the antioxidant assays.

Sample ($\mu\text{g/ml}$)	DPPH (IC50)	ABTS (IC50)	NOR (IC50)	PM (IC50)
Flavonoid	62.21 \pm 0.84	40.12 \pm 0.10	48.76 \pm 1.01	62.65 \pm 0.15
Ascorbic acid	8.41 \pm 0.08	7.76 \pm 0.01	6.77 \pm 0.01	5.87 \pm 0.01

**Figure 9** (a) Anti-hemolysis (b) Anti-thrombotic activity of flavonoids extracted from *Spirulina* CPCC-695.

Biocompatibility Assays

Biocompatibility assays are necessary for evaluation of toxicity or irritancy potential of compounds/drugs for human use. To determine biocompatibility of *Spirulina* CPCC-695 flavonoid anti-hemolytic and anti-thrombotic activities were performed towards human RBCs.

The flavonoid showed 1.80 % hemolysis at the concentration of 250 $\mu\text{g/ml}$ in a dose dependent manner (P-values <0.05) (Figure 9a). Triton X-100 (positive control) and phosphate buffer (negative control) showed 100 and 0% hemolysis rate. Less than 5% hemolysis rate suggests biocompatible nature of any substance as according to Hou et al., [60]. Flavonoid isolated from *Spirulina* CPCC-695 is in the permissible limit of hemolysis.

During in-vitro anti-thrombotic activity, the partially purified flavonoids depicted 81.46 % clot lysis at 250 $\mu\text{g/ml}$. However, distilled water (negative control) treated clots showed 6.66 % clot lysis while streptokinase depicted 96.03 % clot lysis (P-values <0.05) (Figure 9b). To the best of our knowledge there has not been any previous report on biocompatibility activity of flavonoids from cyanobacteria.

Biocompatibility assays revealed that the purified flavonoid from *Spirulina* CPCC-695 depicted satisfactory inhibition of hemolysis and thrombosis at 250 $\mu\text{g/ml}$ which makes it a biocompatible compound that can further help in fighting several human related diseases.

CONCLUSIONS

Present investigation revealed that flavonoid purified by solvent partitioning from *Spirulina* CPCC-695 showed dose-dependent anti-inflammatory, antidiabetic, and antioxidant (DPPH, ABTS, NOR and PM) activities. The added quality of biocompatibility of *Spirulina* CPCC-695 flavonoid suggested possibility of its use for human consumption as novel drug/

food supplement and its use in pharmaceutical and nutraceutical industry. Further investigations are needed to confirm the pharmacological activities of both the flavonoids.

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AUTHOR CONTRIBUTIONS

RA has done the experiments, designed, and written the manuscript, NS and DY assisted in writing, AZ, STA, and SF helped in experimental work and TF checked, edited, and finalized the manuscript.

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