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Research Article

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Non-inhibitory effects of the potent antioxidant *C-phycocyanin* from *Plectonema* sp. on the *in vitro* glycation reaction

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Abstract

When glucose and Amadori products are auto-oxidized, glycation occurs, resulting in the formation of early (Amadori) and late advanced glycation end products (AGEs), as well as free radicals. Glycation and an increase in free radical activity induce diabetic complications. Antioxidant and antiglycation compounds may aid in the prevention of oxidation and glycation. The goal of this study was to assess the antiglycation and antioxidant capacity of C-phycocyanin (C-PC) derived from Plectonema sp. The DPPH (1, 1-diphenyl-2-picrylhydrazyl), nitric oxide, hydroxyl radical scavenging activities and ferric ions reducing antioxidant power (FRAP) assays were used to assess antioxidant activity, while an in vitro bovine serum albumin-methyl glyoxal glycation (BSA-MG) model was used to assess glycation inhibitory potential. Glycation inhibition was measured using a variety of spectroscopic and biochemical parameters, including UV-visible & fluorescence spectroscopy, ketoamine, carbonyl and hydroxymethyl furfural content, as well as free lysine & free arginine estimations. In vitro, C-PC exhibited dose-dependent potent antioxidant activity, but lacked significant antiglycation potential. As a result, it is recommended that further studies be conducted to evaluate the antiglycation potential of C-PC.

Keywords: *glycation, antioxidant, antiglycation, plectonema sp., C-phycocyanin, Bovine Serum Albumin (BSA), Methylglyoxal (MG)*

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Introduction

Numerous diseases [such as diabetes mellitus (DM), cancer, cardiovascular disease, metabolic disorders, and atherosclerosis] are exacerbated or progressed by oxidative stress. The overproduction of free radicals such as $O_2^{\cdot-}$, ·OH and $ONOO^-$ in a cellular system causes oxidative stress, which is caused by an imbalance between the capacities of the biological system to detoxify these reactive products (1). If cellular antioxidants fail to eliminate these free radicals, they may target proteins, lipids and nucleic acids, causing damage. Glycation, glucose oxidation, and lipid peroxidation are all exacerbated as a result of free radical generation, which damages enzymes and cellular machinery, while also increasing insulin resistance.

Glycation is a non-enzymatic condensation reaction between reducing sugars and amino groups of proteins that results in the development of advanced glycation end products (AGEs) via rearrangement, dehydration and cyclization to stable ketoamines. It is a natural process that is influenced by hyperglycemia severity, duration, the half-life of the protein and the permeability of the tissue to free-reducing sugars and reactive dicarbonyls. Oxidative stress is one of the key processes involved in the glycation reaction, which is generated not only by increased formation of oxygen-free radicals, but also by reduced scavenging of those molecules, resulting in AGEs as the end product of the glycation reaction (2). AGEs trigger oxidative stress production and consequently affect enzymatic activity, reduce ligand binding, modify protein half-life, alter immunogenicity and produce inflammatory and thrombogenic responses that further cause DM and its complications. The synthesis of dicarbonyl compounds such as glyoxal (GO), methylglyoxal (MG) and 3-deoxyglucosone (3-DG), during the glycation process, is a key step in the formation of AGEs, among which methylglyoxal (MG) is one of the most highly reactive carbonyl species (RCS) in the human body. In addition to exploring the impacts of AGEs, current research has also addressed the effects of MG on numerous chronic diseases and aging-related disorders in clinical practice. DM patients had considerably greater MG concentrations in their blood than non-DM individuals. Under pathological situations, the body accumulates an even larger quantity of MG, which is associated with an imbalance in MG detoxification through the glyoxalase pathway. As a consequence, the build-up of RCS in organisms, such as MG and the related metabolic imbalance would result in the development of diseases such as DM and its complications (3).

Antiglycation has recently been deemed a useful method for slowing the progression of DM and its consequences. The antioxidant and antiglycation properties of natural products have been widely researched, and their antiglycation activities have been tested in a variety of *in vitro* experimental systems. Natural compounds, by their antioxidant characteristics, metal-chelating capacity, protein interaction, MG trapping and/or inhibiting the receptor for advanced glycation end products (RAGE) may limit the formation of AGEs (4).

Antioxidants may slow or stop the oxidation process, as well as the harmful effects of reactive oxygen species. Antioxidants play a critical part in the defensive system of the body against free radical assault. They are also utilized to keep food, medicines, and cosmetics stable. Endogenous and exogenous antioxidants are the two forms of antioxidants (5). Exogenous antioxidants and endogenous antioxidants work together to protect the body from free radicals. Antioxidants originating from natural sources such as bacteria, algae, and plants are known as exogenous antioxidants (6).

Blue-green algae or cyanobacteria are a storehouse of bioactive chemicals. Because of their

protein nature, unique color, fluorescence, and well-established antioxidant properties, phycobiliproteins are the most abundant proteins among the wide range of bioactive compounds produced by cyanobacteria and are promising tools in diagnostics, biomedical research, and therapeutics (7). Some eukaryotic algae, such as Rhodophyta and Cryptomonads, include phycobiliproteins, which are light-harvesting pigments. Based on their intrinsic color and absorption properties, cyanobacterial phycobiliproteins are divided into three groups: phycocyanin (C-PC), phycoerythrin (C-PE), and allophycocyanin (C-APC) (8). C-PC is a natural edible pigment, food and cosmetic ingredient, and health product in addition to being a form of pigment-protein complex. C-PC, as the primary pigment protein, is a nutraceutical having biological action in this respect. It is distinguished by its dark blue color and phycocyanobilins, which are protein and non-protein components in its structure (9). C-PC derived from *Lyngbya, Phormidium,* and *Spirulina* scavenges reactive oxygen and nitrogen species (ROS and RNS, respectively) and protects against oxidative damage, which may explain its benefits at least in part (10). C-PC, derived from *Spirulina platensis*, has a wide variety of physiological and pharmacological properties (for example, anticancer, antioxidation, antidiabetic, anti-inflammation, and immune regulation) with hardly any toxicity and side effects (11). C-PC may operate as an antiglycation agent due to its antioxidant capabilities, while it has been documented that substances with antioxidant properties also have antiglycation effects (12).

As glycation is commonly thought to be the fundamental molecular foundation of various diabetic complications, the antiglycation capability of a novel molecule might be studied to identify its role in the treatment of Type II diabetes mellitus (T2DM). In contemporary times, the treatment of DM is dependent on synthetic drugs that very frequently have adverse effects. Alternative bioactive compounds produced from natural sources like plants, blue-green algae and naturopathic remedies have created a path to search for more effective agents with lower adverse effects. As a consequence, the activity of C-PC isolated from *Plectonema* sp. utilizing an *in vitro* MG-BSA glycation system will be investigated in the current investigation to determine its antioxidant and antiglycation potential, which might be used as a ray of hope for therapeutic purposes.

Materials and Methods

Materials

C-PC was isolated and purified from *Plectonema* sp. of cyanobacteria as described in our previous reports (13) and BSA was obtained from Hi-media, sodium carbonate, sodium dihydrogen phosphate, sodium bicarbonate, disodium hydrogen phosphate, methylglyoxal, sodium chloride, oxalic acid, nitroblue tetrazolium (NBT), thiobarbituric acid (TBA), trichloroacetic acid (TCA), sodium hydroxide, phosphotungstic acid, sodium dodecyl sulphate (SDS) and sodium chloride were purchased from Hi-Media. Sodium azide (Na_3N) and ethanol were obtained from Merck and 2,4,6 trinitrobenzene sulphonic acid (TNBS) was purchased from G-Biosciences. Thioflavin-T (ThT), congo red, phenanthrenequinone, 1-anilinonapthalene-8-sulfonic acid, 5,5-dithiobis-2-nitrobenzoic acid (DTNB), 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium ferricyanide [$K_4Fe(CN)_6$], ferric chloride ($FeCl_3$), sodium nitroprusside, sulfanilic acid, naphthalene diamine chloride and triphenylphosphine were purchased from Sigma Aldrich. Ethylacetate was purchased from Emplura, and dinitrophenylhydrazine (DNPH) was purchased from Rankem. Guanidium hydrochloride was purchased from SD Fine Chemical Limited. Hydrochloric acid (HCl) was purchased from Fisher Scientific. All other chemicals used in this study were of analytical grade.

Antioxidant Assays

DPPH Radical Scavenging Activity

The DPPH test was used to determine C-PC free radical scavenging activity. Three ml of C-PC were mixed with one ml of 0.1 mM methanolic DPPH. After 30 minutes, the absorbance was measured at 517 nm, and the percentage scavenging activity was determined using the formula below. As a control, DPPH was utilized without the test sample (14).

% Scavenging activity = [(A0–A1)/A0]*100

Where,
A0 = Absorbance of the control.
A1 = Absorbance of the test sample.

Nitric Oxide Scavenging Assay

The nitric oxide scavenging test was carried out using the method previously described (15). We used 2 ml of 10 mM sodium nitroprusside in 0.5 ml of phosphate buffer saline (PBS) (pH-7.4) as the reaction mixture. Then, 0.5 ml of C-PC in concentrations ranging from 20 to 100 µg/ml was added to the reaction mixture, mixed, and incubated at room temperature for two and a half hours. In a separate test tube, 0.5 ml of the mixture was combined with 1 ml of 0.33 percent sulfanilic acid and left to stand at room temperature for 5 minutes. Subsequently, 1 ml of 0.1 % naphthalene diamine chloride was added and incubated for 30 minutes at room temperature. The absorbance was measured at 540 nm. The % inhibition was calculated as discussed above in section *DPPH Radical Scavenging Activity*.

Ferric Ions Reducing Antioxidant Power (FRAP) Assay

We mixed 0.75 ml of 0.2 M phosphate buffer (pH=6.6) and 0.75 ml of % potassium ferricyanide [$K_4Fe(CN)_6$] with C-PC. The reaction mixture was incubated at 50 °C for 20 minutes. We added 0.75 ml of 10% trichloroacetic acid to the reaction mixture, which was centrifuged for 10 minutes at 3000 rpm. 2.5 ml distilled water and 0.1 ml 0.1 percent $FeCl_3$ was added to the top layer of the solution (16).

Hydroxyl Radical Scavenging Assay

The hydroxyl radical scavenging test was carried out using the method described previously (15). The amounts of 60 µl of 1 mM ferrous chloride, 90 µl of 0.2 M phosphate buffer (pH 7.8), 150 µl of 0.17 M hydrogen peroxide, and C-PC concentrations of 20–100 µg/ml were combined. The mixes were shaken and incubated for 5 minutes at room temperature. At 560 nm, the absorbance was measured, and the percent inhibition was computed as stated in section 2.2.1.1 above.

In vitro Glycation-Inhibition Assays

For glycation assay, Bovine serum albumin (BSA) was glycated with methylglyoxal (MG). C-PC was used as an antiglycating agent. BSA (0.3 mg/ml) was combined with MG (10 mM) and C-PC (100, 200, 500, and 1000 µg/ml) in 10 mM PBS (pH=7.4) containing 0.05 percent sodium azide (for avoiding bacterial contamination). Under rigorous sterile conditions, the reaction mixture was incubated at 37 °C for three days. The reaction mixture was dialyzed after three days to remove any unattached MG molecules from the BSA as well as any other contaminants (17).

UV-vis Spectroscopic Measurement

The absorption profiles of native BSA (N-BSA), MG glycated BSA (BSA-MG), and C-PC treated glycated BSA samples (MG-BSA-CPC) were measured in quartz cuvettes with a 1 cm path length on an Eppendorf UV-visible spectrophotometer. The absorbance of individual aliquots of the reaction mixtures was measured at 280 nm (18).

NBT Reduction Assay

The ketoamine moieties produced during glycation were determined using a nitroblue tetrazolium (NBT) reduction assay. At 525 nm, the absorbance of N-BSA, BSA-MG, and MG-BSA-CPC samples was measured, and the quantity of ketoamine moieties (nmol ml^{-1}) was estimated using an extinction coefficient of 12,640 $M^{-1}cm^{-1}$ for monoformazon (19).

Fluorescence Spectroscopic Measurement

The Agilent Cary Eclipse fluorescence spectrophotometer was used to capture the spectra. Fluorescence AGEs were identified in N-BSA, BSA-MG, and MG-BSA-CPC using a 370/450 nm excitation/emission wavelength (20). The slits were set at 5 nm and the path length was set at 1 cm. The equation was used to calculate the fluorescence emission:

$$\% \text{ Increase in FI} = \frac{\text{FI (Inhibited)} - \text{FI (Glycated)}}{\text{FI (Inhibited)}} \times 100$$

Where,

FI (Glycated) = Fluorescence intensities of MG-BSA.

FI (Inhibited) = Fluorescence intensities of MG-BSA-CPC.

Intrinsic Fluorescence Studies

The Cary Eclipse fluorescence spectrophotometer was used to record the intrinsic fluorescence spectrum profile. The intrinsic fluorescence of N-BSA, BSA-MG, and MG-BSA-CPC samples was measured at 280 nm (specific for tryptophan and tyrosine residues), and the emission spectra were recorded in the 300-600 nm range (21).

Determination of Hydroxymethyl Furfural (HMF) Content

One ml of N-BSA, BSA-MG, and MG-BSA-CPC samples were combined with one ml of 1M oxalic acid and incubated at 100 °C for two hours. The protein was isolated using a 40 percent TCA precipitation method. TBA (0.05 mol/l) was added to the protein-free filtrate, which was then incubated for 40 minutes at 40 °C. The molar extinction coefficient value of 4×10^4 $cm^{-1}mol^{-1}$ at 443 nm was used to calculate the HMF content (22).

Determination of Carbonyl Content

A total of 400 µl of 2,4-DNPH reagent (10 mM 2,4-DNPH in 2.5 M HCl) was combined with 100 µl of N-BSA, BSA-MG, and MG-BSA-CPC samples. After 1 hour of incubation at room temperature, 0.5 ml of ice-cold 20% trichloroacetic acid (TCA) solution was added and left for 5 minutes then centrifuged at 1000 rpm for 10 minutes at 4 °C. The pellet was rinsed three times with a 1:1 (v/v) ethanol/ ethylacetate solution. In the end, the pellet was dissolved in 6 M guanidine hydrochloride. At 370 nm, the absorbance was measured. The results were derived using a 22,000 M^{-1} cm^{-1} extinction coefficient (18).

Determination of Free Lysine

2,4,6-trinitrobenzene sulphonic acid (TNBS) method was used to evaluate the free ε-amino groups of lysine amino acids in N-BSA, BSA-MG, and MG-BSA-CPC samples. In brief, 100 ml of 0.5 percent (w/v), TNBS was mixed with 0.5 ml of N-BSA, BSA-MG, and MG-BSA-CPC samples and incubated for 1 hour at 37 °C. The samples were then solubilized in 0.25 ml of 10% sodium dodecyl sulphate (SDS), followed by 0.1 ml of HCl (1 N). In comparison to a blank, the absorbance was measured at 420 nm (23).

Determination of Free Arginine

The free arginine content of native and glycated proteins was calculated using the phenanthrenequinone method. One ml N-BSA, BSA-MG, and MG-BSA-CPC samples were combined with 200 M phenanthrenequinone and 2N sodium hydroxide (NaOH) for 60 minutes at 30 °C. After that,

a solution of 1.2 M HCl was added in an equal amount. The fluorescence intensity of samples was measured at 395 nm using a 5 nm slit width and an excitation wavelength of 312 nm (24).

Statistical Analysis

The data are represented as mean ± standard deviation (± SD). Statistical significance of the results was determined using one-way and two-way ANOVA followed by Tukey post-test using graph pad Prism (version 5.01).

Results

Antioxidant Assays

DPPH Radical Scavenging Assay

The stable radical DPPH was reduced by C-PC to the yellow-colored diphenyl picryl hydrazine. C-PC's scavenging action was shown to be highest at a concentration of 1000 µg/ml (58.75 %). The minimum scavenging activity of 12.25% was noted at the tested minimum concentration of 50 µg/ml of C-PC. The activity of ascorbic acid, on the other hand, was more prominent than that of C-PC. Figure 1 depicts C-PC dose-dependent DPPH radical scavenging activity.

Nitric Oxide Scavenging Activity

At a concentration of 50 µg/ml, the minimum nitric oxide scavenging activity was 11.75 %, while the maximum activity was 58.4 % at a dosage of 1000 µg/ml. With increasing C-PC concentrations, the percentage inhibition increased. However, the activity of ascorbic acid was more pronounced than that of C-PC. Figure 2 indicates the dose-dependent nitric oxide scavenging activity of *C-PC.*

Ferric Reducing Antioxidant Power Assay

The antioxidant capability of C-PC in terms of ferric reducing capacity is visible from the color change in the reaction mixture, which was determined to be 0.13 (absorbance) at 1000 µg/ml concentration. At 50 µg/ml, the tested minimum concentration, the absorbance was 0.04. The absorbance of ascorbic acid, which was employed as a standard, was 0.24 at 1000 µg/ml (Figure 3). The findings suggest that the C-PC may act as an electron donor, reducing oxidized intermediates in lipid peroxidation reactions.

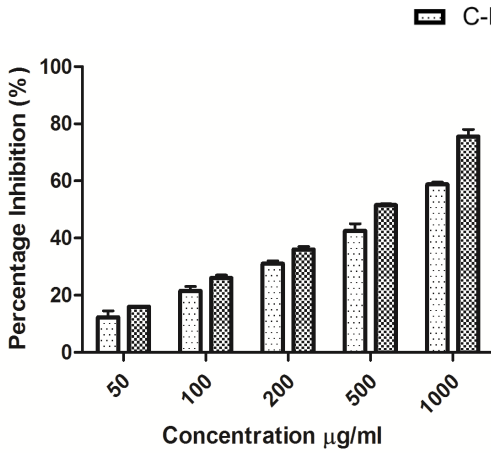


Fig. 1. DPPH radical scavenging activity of C-PC and Ascorbic acid. The data are mean ± SD of three independent experiments.

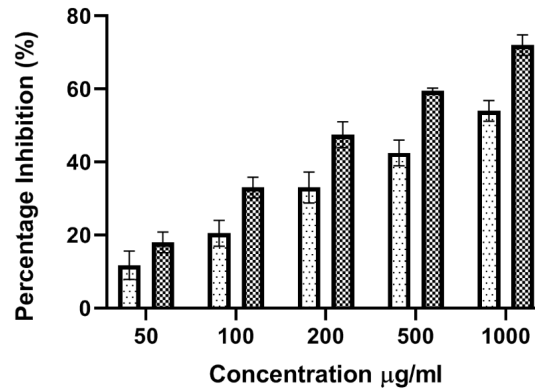


Fig. 2. Nitric oxide scavenging activity of C-PC and Ascorbic acid. The data are mean ± SD of three independent experiments.

Hydrogen Peroxide Radical Scavenging Activity
C-PC had a maximum hydrogen peroxide radical scavenging activity of 61.5 % at a concentration of 1000 µg/ ml and a minimum of 26.75 % at 50 µg/ml. For the same concentrations of ascorbic acid, maximum and minimum activities were 72 % and 33 %, respectively (Figure 4).

In vitro Glycation-Inhibition Assays

UV-visible Spectroscopic Measurement
The absorbance of all samples was recorded on a UV-vis spectrophotometer at 280 nm. The absorbance (hyperchromicity) of MG-BSA was significantly increased at 280 nm. Treatment of MG-BSA with C-PC enhances the peak of protein highlighting non-inhibitory effects on glycation. The hyperchromicity of MG-BSA, MG-BSA-CPC (100 µg/ml), MG-BSA-CPC (200 µg/ml), MG-BSA-CPC (500 µg/ml) and MG-BSA-CPC (1000 µg/ml) were recorded to be 46.77 %, 57.14 %, 63.93 %, 68.557 %, 71.3 % respectively when compared to N-BSA (Figure 5).

NBT Reduction Assay
The ketoamine moieties generated by glycation of BSA were assessed colorimetrically using the NBT assay to detect early glycation products. On the second day of incubation, N-BSA alone had insignificant ketoamine concentration, but MG-BSA had the highest ketoamine level. The ketoamine content of MG-BSA-CPC (100 µg/ml), MG-BSA-CPC (200 µg/ml), MG-BSA-CPC (500 µg/ml), and MG-BSA-CPC (1000 µg/ml) was significantly higher than that of glycated samples, which was 14.07 %, 33.33 %, 46.15 %, and 58.82 % respectively (Figure 6).

Fluorescence Spectroscopy
The characteristic excitation and emission wavelengths, 370 nm (excitation) and 450 nm (emission) verified the development of fluorescent AGEs. Figure 7 shows the fluorescence intensity of the experimental groups incubated with BSA-MG and MG-BSA-CPC at different concentrations; the intensity of fluorescence was shown to be dependent on the C-PC concentration. In contrast to N-BSA, the spectrum revealed a rise in F.I in MG-BSA, MG-BSA-CPC (100 µg/ml), MG-BSA-CPC (200 µg/ml), MG-BSA-CPC (500 µg/ml), and MG-BSA-CPC (1000 µg/ml) which were 89.9%, 92.3 %, 92.5 %, 92.9 %, and 95.4 %.

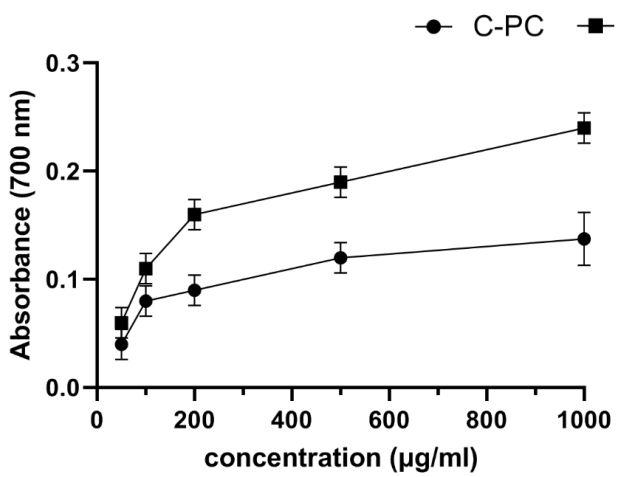


Fig. 3. Ferric reducing antioxidant power assay of C-PC and ascorbic acid. The data are mean ± SD of three independent experiments.

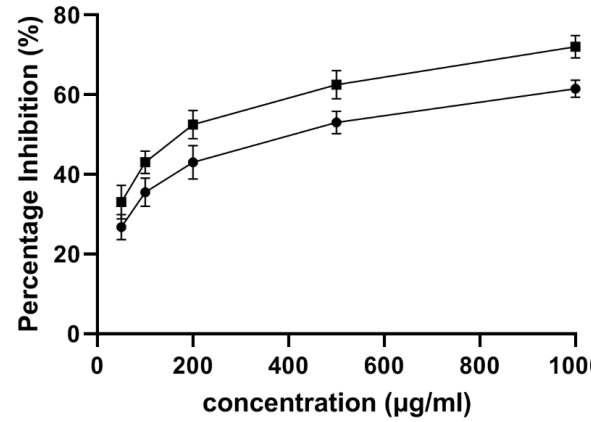
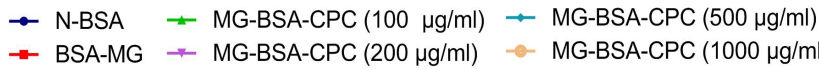


Fig. 4. Hydrogen peroxide radical scavenging activity of C-PC and ascorbic acid. The data are mean ± SD of three independent experiments.

Intrinsic Fluorescence Detection

Phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp) are the amino acids in proteins having inherent fluorescence characteristics, although only Tyr and Trp quantum yields (exited photons/emitted photons) are high enough to provide a stronger fluorescence signal. In contrast to N-BSA, the F.I of MG-BSA, MG-BSA-CPC (100 µg/ml), MG-BSA-CPC (200 µg/ml), MG-BSA-CPC (500 µg/ml), and MG-BSA-CPC (1000 µg/ml) decreased by 25%, 42.8 %, 55.5 %, 68.8 %, and 79.63 % (Figure 8).

Fig. 5-8 legend



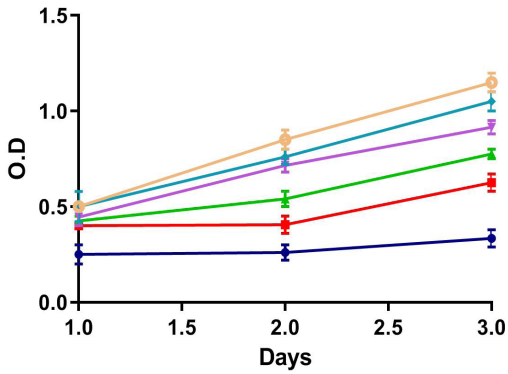


Fig. 5. Ultraviolet absorption spectra of N-BSA, MG-BSA, and MG-BSA-CPC at varying time intervals and concentrations. The data are mean ± SD of three independent experiments.

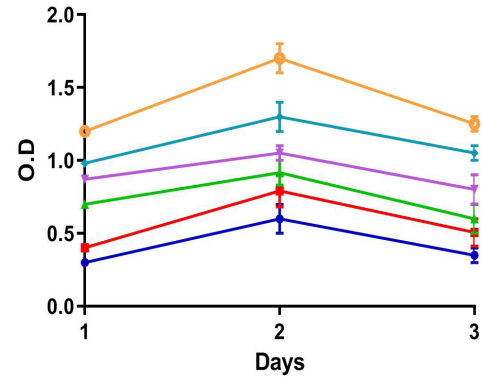


Fig. 6. NBT reduction assay (λ=525 nm) to estimate ketoamine content in N-BSA, MG-BSA, and MG-BSA-CPC at varying concentrations of 1–3 days. Maximum ketoamine content was found on the second day of reaction. The data are mean ± SD of three independent experiments.

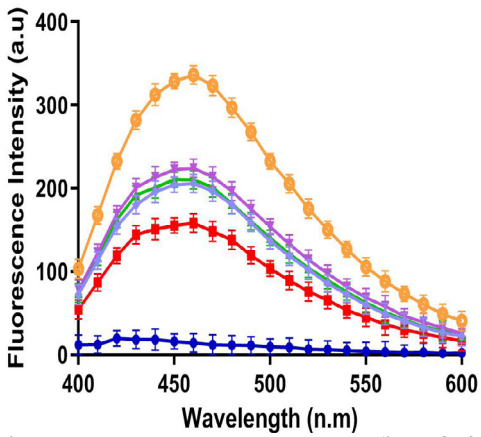


Fig. 7. Fluorescence spectroscopy (λex=370 nm; λem=450 nm) for the detection of AGEs in N-BSA, MG-BSA and MG-BSA-CPC at varying concentrations. The data are mean ± SD of three independent experiments.

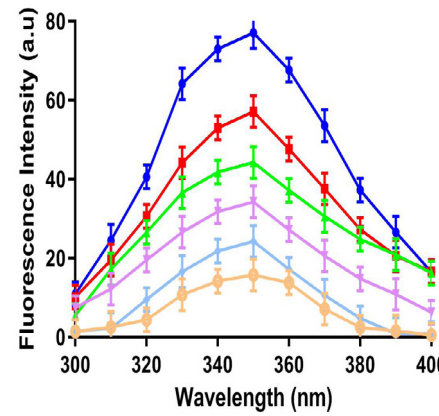


Fig. 8. Intrinsic fluorescence spectra (λex=280 nm; λem=350 nm) in N-BSA, MG-BSA, and MG-BSA-CPC at varying concentrations. The data are mean ± SD of three independent experiments.

Detection of HMF Content

Hydroxymethyl furfural (HMF) is a well-known heterocyclic early glycation reaction product. It is studied due to its potential toxic and harmful effects. The HMF content were 4.20 ± 0.33 nmol ml^{-1}, 6.04 ± 1.8 nmol ml^{-1}, 7.24 ± 1.1 nmol ml^{-1}, 8.16 ± 0.9 nmol ml^{-1}, 9.18 ± 1.01 nmol $ml^{-1}$and 10.7 ± 1.2 nmol ml^{-1} in N-BSA, MG-BSA MG-BSA-CPC (100 µg/ml), MG-BSA-CPC (200 µg/ml), MG-BSA-CPC (500 µg/ml), and MG-BSA-CPC (1000 µg/ml) respectively (Figure 9).

Detection of Carbonyl Content (CC)

Carbonyl content is generated as a result of glycation or their oxidation products. Carbonyl content is the most common biomarker of oxidative stress (25). The maximum carbonyl contents were found in MG-BSA-CPC (1000 µg/ml). While CC in N-BSA, MG-BSA, MG-BSA-CPC (100 µg/ml), MG-BSA-CPC (200 µg/ml), MG-BSA-CPC (500 µg/ml), and MG-BSA-CPC (1000 µg/ml) were 14.45 ± 1.4 nmol ml^{-1}, 22.57± 0.2 nmol ml^{-1}, 22.95 ± 1.2 nmol ml^{-1}, 23.75 ± 1.2 nmol ml^{-1}, 24.55 ± 1.2 nmol ml^{-1} and 25.75 ± 1.2 nmol ml^{-1}respectively (Figure 10).

Detection of Free Lysine and Arginine Residues

The detection of free lysine and arginine residues was performed as the lysine and arginine residues are more prone to glycation and form crosslinks (26). The depreciation in availability of free lysine residues was found to be 31.5 %, 37.6 %, 40.76 %, 51.5 %, 60 % and arginine residues were 9.8%, 16.3 %, 24.5 %, 32.4 %, 43.9 % respectively in MG-BSA, MG-BSA-CPC (100 µg/ml), MG-BSA-CPC (200 µg/ml), MG-BSA-CPC (500 µg/ml) and MG-BSA-CPC (1000 µg/ml) when compared to N-BSA [Figure 11 (A) and (B)].

Discussion

The production of advanced glycation end products (AGEs) is accelerated by oxidative stress,

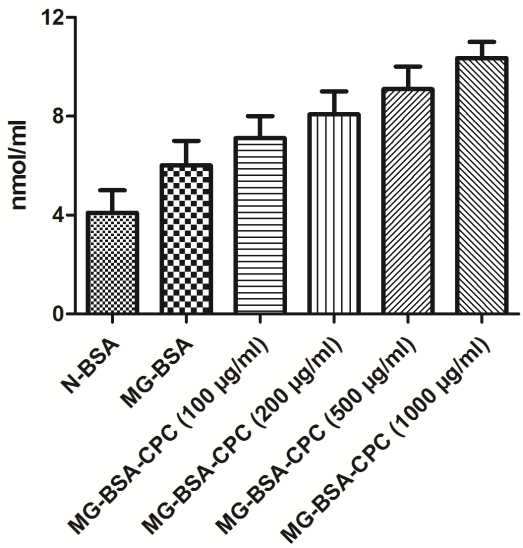


Fig. 9. Quantitative estimation of hydroxymethyl furfural (λ=443 nm) in N-BSA, MG-BSA and MG-BSA-CPC at varying concentrations. The data are mean ± SD of three independent experiments.

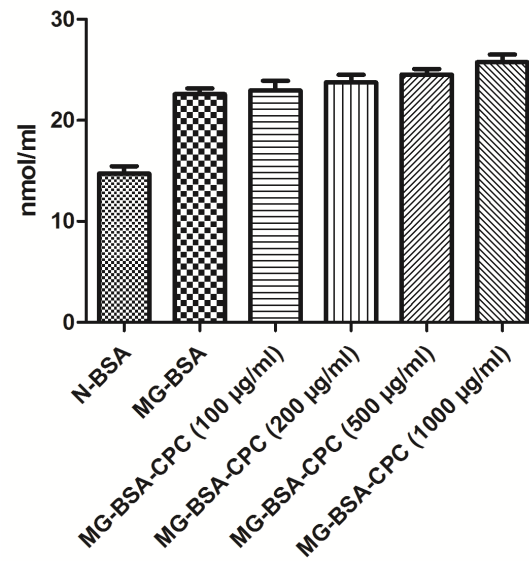


Fig. 10. Carbonyl content (C.C) (λ=370 nm) in N-BSA, MG-BSA, and MG-BSA-CPC at varying concentrations. The data are mean ± SD of three independent experiments.

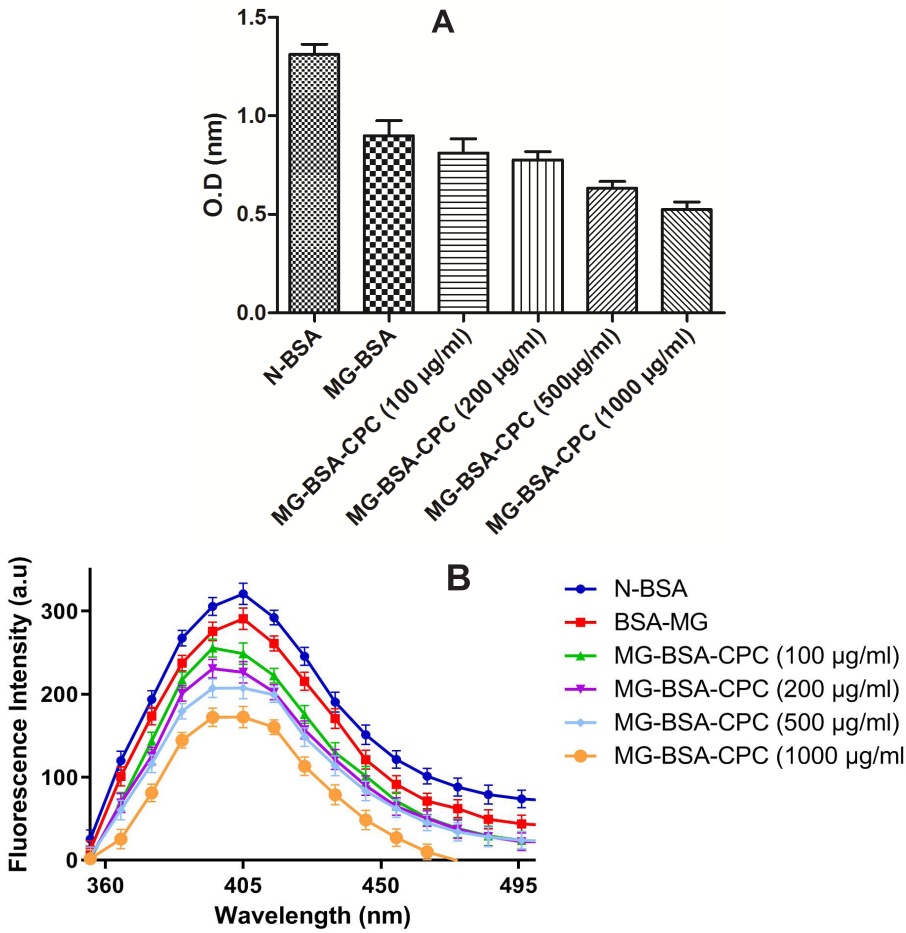


Fig. 11. (A) Free lysine group estimation (λ =420 nm) in N-BSA, MG-BSA, and MG-BSA-CPC at varying concentrations. (B) Free arginine group estimation (λ=315 nm) in N-BSA, MG-BSA, and MG-BSA-CPC at varying concentrations. The data are mean ± SD of three independent experiments.

according to several studies. Under hyperglycemic conditions in diabetic patients, AGEs have the ability to create reactive oxygen species and promote protein glycation. Oxidative stress is considered to have a key role in the development of DM and its complications. The generation of free radicals in DM is caused by the non-enzymatic glycation of proteins, which causes cellular machinery dysfunction (14, 27). During oxidative metabolism, a portion of the used oxygen is converted to water, and the remaining oxygen is turned to oxygen-free radical (O•), an essential reactive oxygen species (ROS) that is converted to $ONOO^-$, OH^-, and H_2O_2. The basic processes of DM development include hyperglycemia, energy imbalance, chronic inflammation, and insulin resistance. Furthermore, AGEs play a role in diabetic complications (28). As a result, we focused on the antioxidant and antiglycation effects of C-PC isolated from *Plectonema* sp. in this study.

In this investigation, C-PC demonstrated high antioxidant activity *in vitro*. Because of C- PC's high antioxidant properties, it may have sup-

pressed the production of free radicals, which are crucial in glucose autoxidation and non-enzymatic protein glycation. According to a previous study, increased free radical generation due to enhanced glycolysis, activated sorbitol, glucose autoxidation, and non-enzymatic protein glycation leads to a poor antioxidant defense system and insulin resistance (29).

DPPH is often used as a reagent to assess the free radical scavenging capability of antioxidants. DPPH is a stable free radical that receives an electron or hydrogen radical to form a diamagnetic molecule. Because of the interaction with an antioxidant, the DPPH radical is reduced to 2,2-diphenyl-1-picrylhydrazine (DPPH-H) by attaching a hydrogen atom or an electron to the radical centre. The reduction may be seen as a color shift from purple to yellow, and the DPPH capacity of the radical to reduce is measured by a reduction in absorbance at 517 nm. C-PC was able to convert the stable radical DPPH to the yellow diphenylpicryl hydrazine. The scavenging action of C-PC was highest at a concentration of 1000 µg/ml (58.75%). Free radical involvement, particularly increased generation, seems to be a characteristic of most, if not all, human diseases, including diabetes, cardiovascular disease, and cancer. Because of its high radical scavenging action, C-PC is a promising option for treating the aforementioned diseases (30).

Under aerobic circumstances, nitric oxide is a very unstable species. Through the intermediates NO_2, N_2O_4, and N_3O_4, it interacts with O_2 to form stable products nitrates and nitrites. The quantity of nitrous acid decreases in the presence of a test substance, which is a scavenger. The magnitude of the decrease shows the degree of scavenging (31). When compared to ascorbic acid, the percentage inhibition of C-PC was determined to be highest (58.4%) at 1000 µg/ml.

The ferric reducing antioxidant power assay assesses the ability of the pigment to convert ferric ion to ferrous ion. Antioxidant chemicals often form a Prussian blue-color combination with potassium ferricyanide. Antioxidants act as electron donors, donating electrons to reduce the oxidized complex. In this assay, the electron donor (reducer) converts ferric to ferrous. Fe^{3+} is employed as an indication of electron-donating activity and is linked to phenolic antioxidant activity as well as other antioxidant features (32). A rise in the absorbance of the reaction mixture at 700 nm shows that the reducing power of the compound has increased. The color change in the reaction mixture demonstrates the antioxidant capability of C-PC in terms of ferric reducing capacity, which was determined to be highest at 0.13 (absorbance) at 1000 µg/ml concentration. They may operate as an electron donor and decrease the oxidized intermediates of lipid peroxidation processes, according to the findings.

Hydrogen peroxide is a powerful oxidizing agent. The superoxide dismutase enzyme produces them in the body. It has the capacity to oxidize biomolecules by crossing the cell membrane. The capacity of the C-PC to scavenge hydrogen peroxide was measured and compared to that of ascorbic acid in this study. At a concentration of 1000 µg/ml, C-PC had the highest scavenging activity of 61.5 %. Although H_2O_2 is not reactive, the production of hydroxyl radicals in the cell may make it hazardous to cell components. C-PC may be used to detoxify hydrogen peroxide, superoxide and hydroxyl radicals, which is necessary for the protection of biomolecules, pharmaceuticals, and nutraceuticals (33). We may deduce from these findings that C-PC might be a promising antioxidant compound.

Glycation occurs when the amino group of a protein reacts with the carbonyl group of a reducing sugar or another reactive di-carbonyl species like methylglyoxal, glyoxal, or 3-deoxyglucosone. Glycation is a significant element in the development of chronic diseases. Glycation has been linked to the development of insulin resistance and low-grade inflammation,

both of which are known to precede the onset of T2DM. Micro- and macro-vascular consequences, such as nephropathy, neuropathy, retinopathy and cardiovascular diseases (CVDs), are caused by the accumulation of RCS-induced AGEs in tissues (34). Hence, we aim to analyze the inhibitory impact of C-PC on *in vitro* glycated protein model. We identified a non-inhibitory impact of C-PC towards the MG glycated BSA system. We detected enhanced hyperchromicity in C-PC treated glycated BSA. Maximum hyperchromicity (71.3 %) was observed in MG-BSA-CPC (1000 µg/ml) as compared to N-BSA. The UV-vis spectroscopy analysis indicates time and concentration-dependent rise in hyperchromicities in MG-BSA-CPC. The rise in hyperchromicities was due to the unfolding of the protein component of C-PC that resulted in the exposure of chromophoric aromatic amino acids Phe, Tyr, and Trp. Ketoamine moieties were identified during the transformation of yellow color NBT dye into purple color monoformazon crystals upon interaction with Schiff's base (35). Ketoamine contents were found to be maximum on the second day of reaction in MG-BSA-CPC (1000 µg/ml) after that it steadily reduced until the third day. The result of the NBT test illustrates the progressing of glycation reactions from the early glycation state to the end-stage generation of AGEs.

Protein carbonyls and HMF were also discovered to be in greater concentration in comparison to that of native analogs. Protein carbonyls were synthesized owing to oxidative stress-induced during glycation, whereas HMF was formed by the dehydration of sugars (23).

Furthermore, fluorescence spectroscopy was conducted for the identification of fluorescent AGEs. The rise in fluorescence intensity was observed in glycated and anti-glycated samples which show the production of AGEs. The maximum AGEs production was found in MG-BSA-CPC (1000 µg/ml) sample considering native analog. The intrinsic fluorescence was maximum for native BSA, which progressively declines with an increase in the quantity of C-PC in glycated samples. The tryptophan and tyrosine residues are buried inside the hydrophobic environment and have high quantum yield, hence, they exhibit high fluorescence intensity with regard to unfolded protein. During glycation, tryptophan and tyrosine residues are exposed on the surface and consequently display low fluorescence intensity (36).

Glycation happens more often at lysine and arginine amino acid residues that contain the ε-amino group, hence calculating free lysine and arginine residues is a crucial metric to consider when predicting glycation process (37). Evidence shows that non-enzymatic glycation does not include all lysine and arginine residues; rather, certain particular lysine and arginine residues are engaged in the glycation process. The extent of glycation was demonstrated by the lower proportion of free lysine and arginine residue in the samples. The proportion of free lysine and arginine residues in MG-BSA-CPC samples was lower than in MG-BSA samples, indicating that C-PC has a non-inhibitory impact.

During the early and propagation stages of the glycation process, a high number of free radicals, carbonyl species, and reactive dicarbonyl species are produced, the most reactive of which is methylglyoxal (MG), which may generate dicarbonyl stress and interfere with normal physiological functioning. Antioxidant activities have a critical role in avoiding oxidative stress and the production of AGEs. Free radicals, such as reactive oxygen species (ROS) and their derivatives, may be neutralized and prevented from harming cells by antioxidant activities (38). Many studies have shown distinct ways by which most antioxidant-potent substances suppress the production of AGEs. Mechanisms proposed include radical scavenging, reducing power, lipid peroxidation suppression, carbonyl trapping, and metal ion

chelation (39). C-PC extracted from *Plectonema* sp. did not exhibit substantial anti-AGEs action since it did not inhibit the MG-BSA glycation system *in vitro*. This impact might be attributed to C-PC containing both protein and non-protein components (40). The protein part of C-PC itself may be glycated due to the presence of MG under *in vitro* conditions. C-PC, blockage of free radicals reduces oxidative stress but did not decrease the further production of reactive carbonyl and dicarbonyl species such as MG. The decrease in ketoamine content, carbonyl content and HMF content was not observed profoundly through C-PC. Fluorescence intensities were observed to be increased *in vitro*, demonstrating that C-PC had no inhibitory impact on the MG-BSA glycation system. The formation of AGEs increases with the formation of aldimines (Schiff's bases) and ketoamines (Amadori products); as a result, C-PC attaches to MG and thus leads to the formation of aldimines and ketoamines via rearrangement, dehydration, and cyclization, resulting in the formation of fluorescent cross-links (e.g., pentosidine) and non-fluorescent compounds (e.g., Nε-carboxymethyl lysine, CML), referred to as AGEs. In addition, C-PC increases the concentration of free lysine and free arginine residues, resulting in the generation of glycation end products, indicating that C-PC has a negligible antiglycation impact.

Conclusion

This study reveals the antioxidant potential of C-PC isolated from *Plectonema* sp. by suppressing the production of free radicals. Furthermore, the study tries to document a probable antiglycation effect of C-PC. The combination of glycation reaction and C-PC resulted in an antagonistic relationship as it resulted in an enhanced glycation reaction. This may be due to *in vitro* conditions that do not suppress the production of dicarbonyl (MG). Therefore, ketoamines, intermediate of glycation are formed and the vicious cycle of glycation reaction continues and C-PC might be glycated due to the fact that the C-PC is a pigment-protein complex. Hence, *in vivo* conditions may be a plausible way to circumvent the non-inhibitory effect of C-PC on glycation due to the availability of the enzyme system. It can be concluded that C-PC possesses profound antioxidant potential but not antiglycation potential under *in vitro* conditions. However, as this is only a preliminary and first study of its kind, further study was taken up to identify the antioxidant, antiglycation and antidiabetic potential of C-PC in *in vivo* model.

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Authors' contribution

AH, AK and AF designed, conceptualized, investigated and prepared the first draft of this manuscript; Saheem Ahmad did the validation and funding Acquisition. SA, RA, MK, and SK collected and analyzed experimental data and did the funding acquisition. All authors have approved the submission and publication of this manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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