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ARTICLE

Effect of *Arthrospira platensis* extracts on glycation of BSA

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ABSTRACT Glycation, a non-enzymatic interaction between the carbonyl groups of sugars and the amino groups of macromolecules, leads to the formation of deleterious advanced glycation end-products (AGEs). Diseases such as neurodegenerative disorders, cardiovascular complications, and diabetes are characterized by the involvement of glycated products. This study investigated the antioxidant and antiglycation potential of a well-known cyanobacterium, *Arthrospira platensis*, through its extract. In an in vitro glycation system involving BSA and glucose, *A. platensis* PCC 7345 extracts (prepared in water- APA and buffer- APB) were incubated at 37 °C for 28 days. Antioxidant assays like ABTS, DPPH and NO Radical Scavenging Assay was performed. Quantification of glycation products was performed using spectroscopic methods, including browning, NBT assay, individual AGEs and fluorescent AGEs assessment. Both extracts demonstrated inhibitory effects on fructosamine content and AGEs, within the glycation system. Both APA and APB extracts from *A. platensis* effectively reduced browning reactions, with APA showing a slightly higher efficiency (51.67%) than APB (48.07%). Fructosamine assays revealed substantial reductions in ketoamine formation for both extracts, while the fluorometric determination of total AGEs indicated that APB exhibited superior antiglycating potency compared to APA, consistent with outcomes from fructosamine assays and supporting its effectiveness in attenuating glycation processes.
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Introduction

Diabetes presents a formidable global health challenge with far-reaching consequences. The onset of this condition is characterized by the gradual accumulation of glucose, eventually reaching deleterious levels. The carbonyl group of sugars reacts covalently with amino groups within biomolecules, predominantly proteins, through the glycation or Maillard reaction, a phenomenon attributed to Louise Camille Maillard's discovery (Kikuchi et al. 2003). This sequence culminates in the formation of Schiff's bases, which undergo subsequent conversion into Amadori products. The rearrangement of these metabolites gives rise to advanced glycation end products (AGEs) (Ahmad and Ahmed 2006), compounds detrimentally associated with the glycation process. Notably, these glycation intermediates also play a role in the generation of free radicals, compromising the body's antioxidant defense mechanisms and leading to cellular organelle and enzyme damage (Ahmad et al. 2014).

The rate of AGE formation escalates under conditions of oxidative and carbonyl stress, attributed to the accumulation of reactive oxygen species (ROS) and dicarbonyl compounds (Sadowska-Bartosz and Bartosz 2015). The biological processes leading to AGE formation and the concurrent generation of free radicals are intricately interconnected and are commonly denoted as the glycoxidation process. This phenomenon has been experimentally linked to various pathophysiological conditions, encompassing diabetes, neurodegenerative disorders, and cancer (Ahmad et al. 2014).

Researchers are actively engaged in the development of pharmaceuticals aimed at either inhibiting the formation of AGEs in the body or treating conditions resulting from their accumulation. While certain synthetic drugs, including aminoguanidine, have received approval for treatment, their adverse effects on the body have been documented (Thornalley et al. 2003). Recent advancements in phytochemistry and analytical methods have facilitated the identification of natural products derived from plants or microorganisms, subsequently synthesized and subject to laboratory testing. Several of these natural compounds, exemplified by curcumin, eugenol, rutin, and garcinol, have exhibited noteworthy antioxidant and antiglycation properties (Khan and Gothalwal 2018).

A. platensis, is a filamentous, gram-negative cyanobacterium, has been found to be efficient in reducing glycation and related disorders. Research indicates that specific components inherent in *A. platensis* may contrib-

ute to its remedial properties (Paramanya et al. 2019). Notably, phycocyanin, the pigment responsible for the algae's characteristic blue hue, demonstrates antioxidant attributes, effectively neutralizing free radicals and mitigating oxidative stress (Kumar et al. 2014). By alleviating oxidative stress, phycocyanin holds promise in attenuating the formation of advanced glycation end products (AGEs) and the ensuing damage. Furthermore, the presence of diverse antioxidants, including vitamins C and E, in *A. platensis* serves to counteract oxidative stress, potentially reducing AGE formation. This study aimed to ascertain the suitability of *A. platensis* extracts as an antiglycating agents.

Materials and Methods

Materials

Live axenic culture of *Arthrospira platensis* (spirulina) PCC 7345 was sourced from Birla Institute of Technology & Science, Pilani, Rajasthan, India as a kind gesture from Dr. Vani. BG11 medium was purchased from HiMedia (India). All the other chemicals used in the research were high quality grade and purchased from SD Fine Chemicals (India).

Growth of culture

A. platensis PCC 7345 was cultured in BG-11 liquid medium at room temperature (ranging from 25-28 °C), under constant warm white illumination, within an environment maintained at neutral pH. The number of cells (cells/ml) was calculated using a hemocytometer after every 3 days and growth curve was plotted.

Preparation of the extracts

The growth culture was aseptically transferred to centrifuge tubes, and centrifugation was performed at 7000 rpm for 10 min at a temperature of 10 °C. The resulting pellet was then washed three times with distilled water (D/W). For the extraction process, two different solvents were utilized. A portion of the pellet was suspended in D/W, while another portion was suspended in phosphate buffer (0.01 M, pH 7.4), with both samples having a concentration of 100 mg/ml. Subsequently, the samples were sonicated at 37 °C for 10 minutes with 15 pulses per minute. Following sonication, the samples were centrifuged at 7000 rpm for 10 min at 4 °C, and the supernatant was collected (Paramanya et al. 2023). The extract prepared in D/W was designated as APA, while the extract prepared using the buffer was labeled as APB.

Comparison of antioxidant activity of both extracts

Both the APA and APB extracts were subjected to an assessment of their antioxidant activity employing the ABTS, DPPH and NO radical scavenging assays. The antioxidant potential of each extract was quantified using the percentage reduction formula. This allowed for a thorough comparison of the antioxidant capabilities between the two extracts (Balyan et al. 2022).

In vitro d-glucose induced glycation of BSA

To induce glycation, bovine serum albumin (BSA) at a concentration of 10 mg/mL underwent incubation with 100 mg/mL D-glucose. This process occurred in a phosphate buffer (0.1 M, pH 7.4), supplemented with 0.02% sodium azide at 37 °C over a duration of four weeks, as outlined by Kumar and Ali (2014). Concurrently, samples containing the extracts APA and APB were subjected to similar incubation conditions to evaluate their effectiveness in preventing glycation (Table 1). A positive control, aminoguanidine (AG), was employed at a concentration of 1 mM.

Early Glycation Products estimation: Browning

The assessment of browning intensity was conducted at a wavelength of 420 nm utilizing a Shimadzu UV 1800 spectrophotometer, following the methodology outlined by Rondeau et al. (2007). BSA+G was considered as the control and all other incubated samples (1 mL) were checked for its efficiency by calculating percent reduction. The relative percentage of absorbance reduction was determined using the percentage reduction formula.

$$\text{Inhibition (\%)} = (A_C - A_T)/ A_C \times 100$$

Where A_C = absorbance of the control and A_T = absorbance of the test samples.

Fructosamine assay

Throughout the incubation period, the assessment of ketoamine or early glycation end products, particularly Amadori products, was carried out employing the nitroblue tetrazolium (NBT) assay, following the methodology established by Johnson et al. (1983). The quantification of ketoamine content was ascertained using 1-deoxy-1-morpholino-fructose (1-DMF) as the reference standard. The quantity of ketoamines in each sample was determined using standard graph.

Estimation of late glycation products

Total fluorescent AGEs formation using fluorescence spectroscopy

Formation of fluorescent AGEs resulting from albumin glycation was assessed by measuring their fluorescence at excitation/emission wavelengths of 370 nm and 440

nm, respectively, using an Agilent Cary Eclipse spectrofluorometer (Agilent Technologies, Victoria, Australia). BSA+G (glycated BSA) was considered as positive control and inhibition in all other incubated samples (1 mL) were calculated. To determine the percentage inhibition of glycation (Ding et al. 2020), the calculation is performed as follows:

Inhibition of glycation (%) = (Fluorescence of glycated BSA - Fluorescence of native or SP-treated BSA) / Fluorescence of glycated BSA × 100.

Individual AGEs formation

Certain AGEs exhibit inherent fluorescence and can be detected at specific excitation and emission wavelengths (Thornalley and Rabbani 2014). In this study, the formation of specific AGEs, namely argpyrimidine, pentosidine, vesperlysine, and crossline, was examined. The incubated samples were diluted in the ratio of 1:5 in a sodium phosphate buffer (50 mM, pH 7.4). The emission spectra were recorded in the wavelength range of 300-600 nm using Agilent Cary Eclipse spectrofluorometer (Agilent Technologies, Victoria, Australia), allowing for the estimation of the levels of argpyrimidine, pentosidine, vesperlysine, and crossline in the samples. BSA+G was considered as positive control and inhibition in all other incubated samples (1 mL) were calculated using the above-mentioned formula.

Statistical analysis

The statistical analysis was performed using GraphPad Prism version 9.5.1 for Windows (GraphPad App, San Diego, CA, USA). The data are presented as means ± standard deviation (SD) and are based on three independent experiments. A one-way (ANOVA) was conducted to determine the significance of the data. Tukey's multiple comparison tests were then used to compare the treatments and identify any significant differences. The results are expressed as mean ± SD (n = 3). Statistical significance is indicated as follows: ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results

Growth of A. platensis

A growth versus number of days graph was created using the Exponential (Malthusian) growth command in GraphPad Prism. The rate constant (k) was determined to be 0.0683, indicating the growth rate of the culture. The doubling time of the culture, calculated based on the rate constant, was found to be 10.15 days (Fig. 1).

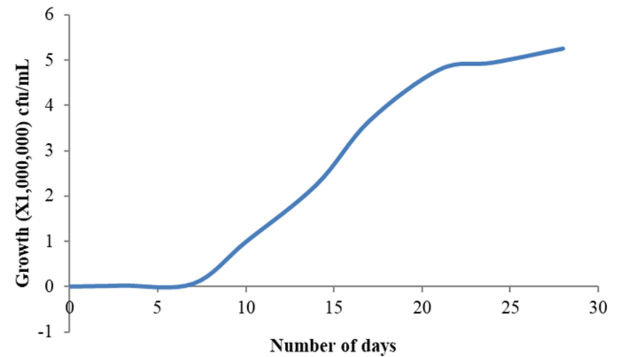

Figure 1. Logarithmic growth curve of *A. platensis* PCC 7345 (n = 3)

This result aligns closely with the findings of Lai et al. (2019), who reported a doubling time of 11.75 days for a similar culture.

Antioxidant potentials of A. platensis extracts

Increasing the concentration of both APA and APB extracts shows a clear uptrend in antioxidant activity across all three assays (Fig. 2). This suggests a positive correlation between concentration and the ability of the substance

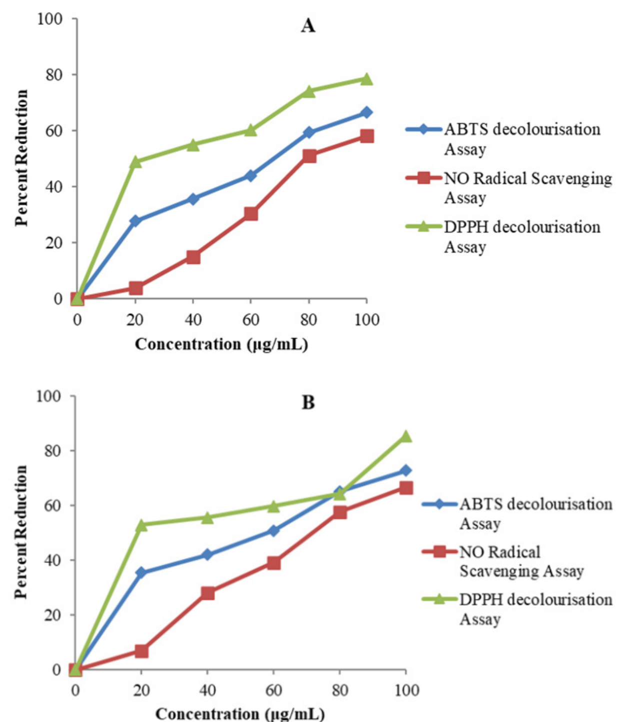

Figure 2. Antioxidant potential of both the extracts. (A) represents aqueous extract (i.e. APA); (B) represents extract prepared in buffer (i.e. APB). ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH: 2,2-Diphenyl-1-picrylhydrazyl; NO: nitric oxide

Table 1. Composition of samples used for incubation to induce glycation and its subsequent prevention

	BSA µL	Glucose µL	Sodium azide µL	Phosphate buffer µL	Sample µL	Distilled water µL
Blank	-	-	200	200	-	1600
BSA	200	-	200	200	-	1400
BSA+ G	200	200	200	200	-	1200
BSA+ APA	200	-	200	200	AP 200	1200
BSA+ G + APA	200	200	200	200	AP 200	1000
BSA+ APB	200	-	200	200	APB 200	1200
BSA+ G +APB	200	200	200	200	APB 200	1000
BSA+ AG	200	-	200	200	AG 200	1200
BSA+ G + AG	200	200	200	200	AG 200	1000

BSA: bovine serum albumin; G: glucose; APA: *A. platensis* aqueous extract; APB: *A. platensis* buffered extract; AG: aminoguanidine. Total volume in each tube was 2000 µL.

to scavenge free radicals, as indicated by higher values in the ABTS decolorization, NO radical scavenging, and DPPH decolorization assays. It can be observed that at each concentration level, APB consistently demonstrates higher values in the assays compared to APA. This suggests that, overall, APB may possess a stronger ability to scavenge free radicals than APA.

The antioxidant assays for APA and APB extracts, measured through ABTS, NO, and DPPH assays, reveal distinct linear relationships represented by the equations and R^2 values (Table 2). In biochemical assays like ABTS, NO, and DPPH, APA and APB show different trends. APA generally has higher R^2 values and steeper slopes in the NO and DPPH assays compared to APB. However, APB demonstrates a slightly lower slope in the ABTS assay. APA also consistently shows higher IC_{50} values across all assays, indicating potential differences in antioxidant effectiveness between APA and APB.

Early Glycation Products estimation: Browning

The browning process, known as the Maillard reaction, arises from the complex chemical interaction between sugars and proteins and is notably influenced by the duration of the reaction and the temperature conditions. To assess the potential reduction of browning, we established used BSA+G as the reference control and checked for reduction in browning in presence of APA and APB.

The percent reduction in browning (Fig. 3.) for APA was measured at 51.67 ± 2.15%, while APB achieved a reduction percentage of 48.07 ± 1.83%. In comparison, the positive control AG demonstrated a reduction percentage of 54.78 ± 1.91%, indicating its comparable effectiveness in mitigating the browning process. These results suggest that both APA and APB possess properties that are effective in reducing the browning reaction, with APA showing

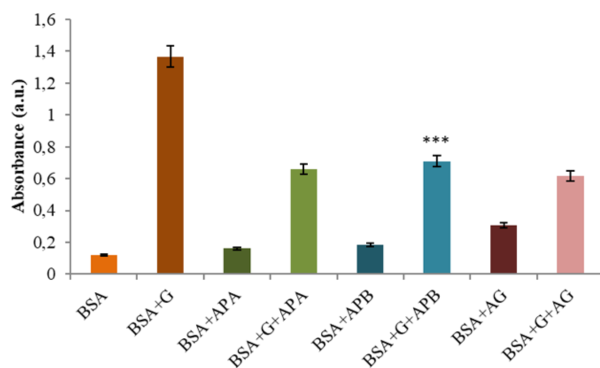

Figure 3. Estimation of browning in glycated protein sample (n = 3 and *** = p < 0.001). BSA: bovine serum albumin; G: glucose; APA: aqueous extract of *A. platensis*; APB: buffered extract of *A. platensis*; AG: aminoguanidine

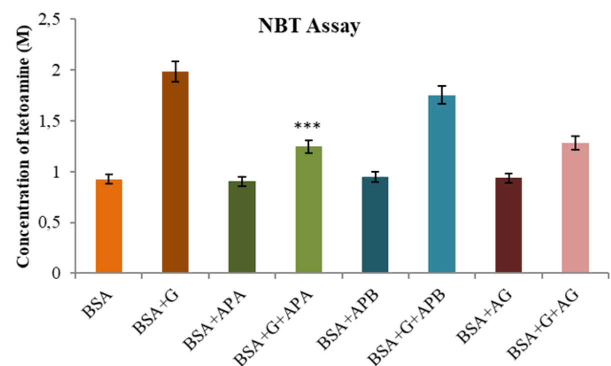

Figure 4. Estimation of fructosamine (ketoamine) content in glycated samples (n = 3, and *** = p < 0.001). A lower concentration of ketoamines suggests a greater ability of the sample to inhibit the formation of early glycated products such as fructosamine. BSA: bovine serum albumin; G: glucose; APA: aqueous extract of *A. platensis*; APB: buffered extract of *A. platensis*; AG: aminoguanidine

Table 2. Determination of IC_{50} values of both extracts

	APA			APB		
Antioxidant assay	ABTS ecolorization	NO radical scavenging	DPPH decolorization	ABTS decolorization	NO radical scavenging	DPPH decolorization
Equation from graph	y = 0.4959x + 18.292	y = 0.6998x - 8.3007	y = 0.3874x + 41.234	y = 0.4773x + 25.971	y = 0.7172x - 1.2693	y = 0.3671x + 42.596
R^2	0.9825	0.9572	0.9852	0.9797	0.9514	0.8391
IC_{50} (µg/ml)	63.9403	83.3105	22.627	50.3435	71.4853	20.1688

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); APA: *A. platensis* aqueous extract; APB: *A. platensis* buffered extract; DPPH: 2,2-diphenyl-1-picrylhydrazyl; IC_{50}: half-maximal inhibitory concentration; NO: nitric oxide.

a slightly higher reduction potential compared to APB.

Fructosamine assays

The Maillard reaction leads to the formation of ketoamines, which were measured using the NBT assay. In this study, the standard sample BSA+G showed the highest level of ketoamine formation, with a concentration of 1.979 M, serving as a reference against which other test samples were compared. All test samples, however, demonstrated a reduction in ketoamine formation, indicating their potential to inhibit the production of ketoamine products. Notably, sample APA displayed a concentration of 1.245 M, proving to be as effective as the positive control AG, which had a concentration of 1.275 M. In contrast, sample APB showed a lower reduction potential, with a concentration of 1.75 M, compared to the standard sample.

Determination of Total AGEs

The fluorometric determination of AGEs relies on the principle of measuring the intrinsic fluorescence emitted by these compounds. AGEs exhibit fluorescence when excited by a specific wavelength of light. The emitted fluorescence provides a direct correlation to the concentration of AGEs in the sample. The graphical representation in Fig. 5 illustrates the absorbance against wavelength, showing a clear decrease in absorbance for both extracts, especially in comparison to the standard (BSA+G). The reduction is more pronounced in the presence of the APB extract, suggesting that APB exhibits greater potency as an antiglycating agent compared to APA.

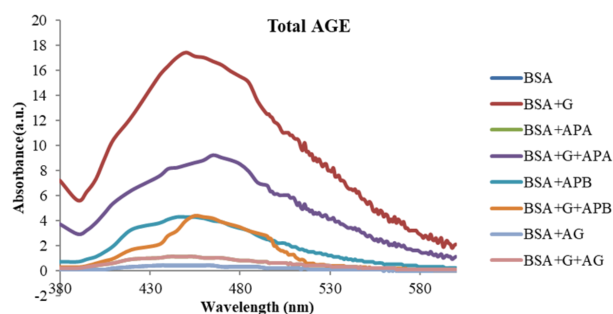

Figure 5. Determination of Total AGEs through fluorescence spectrum study (n = 3). BSA: bovine serum albumin; G: glucose; APA: aqueous extract of *A. platensis*; APB: buffered extract of *A. platensis*; AG: aminoguanidine

Investigating presence of Individual AGEs

The determination of advanced glycation end-products (AGEs) such as argpyrimidine, pentosidine, vesperlysine, and crossline is achieved through fluorimetry. The principle involves exploiting the intrinsic fluorescence properties exhibited by certain AGEs. Figure 6. demonstrates that both APA and APB possess the capability to inhibit all four advanced glycation end-products (AGEs). Notably, APB exhibits a higher level of inhibition compared to APA. Specifically, APA inhibits argpyrimidine by 23.33%, while APB achieves a greater inhibition of 31.26%. In the case of pentosidine, APA inhibits its formation by 29.65%, whereas APB inhibits it by 30.12%. Furthermore, APA inhibits vesperlysine and crossline by 13.35% and 43.36%, respectively, while APB inhibits them by 18.23% and 51.28%, respectively. These findings underscore the more pronounced inhibitory effect of APB on glycation compared to APA.

Discussions

The study aimed to evaluate the antioxidant and antiglycation potential of *A. platensis* extracts, namely APA (prepared in water) and APB (prepared in buffered solution). The antioxidant potential was determined using 3 assays namely, ABTS, DPPH and NO radical Scavenging Assay. The IC50 values of both extracts were consistent with our previous publication (Paramanya et al. 2023). The antiglycation analysis involved various parameters, including browning, fructosamine formation, total ad-

vanced glycation end-products (AGEs), and the inhibition of individual AGEs (Kumar and Ali 2023).

The evaluation of browning demonstrated the effective reduction of browning reactions by both APA and APB, with APA showing a slightly higher reduction potential (51.67 ± 2.15%) compared to APB (48.07 ± 1.83%). These findings indicate that both extracts harbor properties capable of mitigating the browning process, with APA demonstrating a slightly higher efficiency. Interestingly, these results align with a study by Zhang et al. in 2023, where the combination of *A. platensis* with maltodextrin was investigated to enhance its efficacy in preventing browning. The consistency between our findings and the referenced study supports the notion that *A. platensis*, when appropriately formulated, exhibits promising potential in inhibiting browning reactions.

Fructosamine assays demonstrated the extracts' ability to reduce ketoamine formation, indicative of their antiglycation potential. Both APA and APB exhibited substantial reductions, with APA displaying a concentration of 1.245 M, comparable to the positive control AG (1.275 M). APB, however, showed a lower reduction potential with a concentration of 1.75 M, indicating differences in their antiglycation effectiveness.

The fluorometric determination of total AGEs revealed a significant reduction in absorbance for both extracts, with APB demonstrating a more pronounced effect. This suggests its superior potency as an antiglycating agent compared to APA. This observation aligns with the results from the browning and fructosamine assays, indicating a consistent pattern in the inhibitory effects of APB over APA across various assays. Further analysis of individual AGEs corroborated these findings, with APB showing more robust inhibitory effects on glycation compared to APA. Specifically, APB exhibited higher inhibition percentages for argpyrimidine, pentosidine, vesperlysine, and crossline when compared to APA. The consistency across multiple assays strengthens the evidence (Paramanya et al. 2023) supporting APB's antiglycating potential, emphasizing its effectiveness in attenuating glycation processes.

The preparation of the APB extract in a buffered environment, which reflects extraction under controlled pH conditions, is likely a key factor in its enhanced effectiveness. The buffering agent not only stabilizes the extraction process but also maintains a constant pH during the subsequent incubation period (Janská et al. 2021). This stability is crucial for preserving the integrity and functionality of bioactive compounds within the extract (Acevedo-Fani et al. 2020). In contrast, pH fluctuations in unbuffered conditions can negatively affect the activity of these compounds (Getachew et al. 2020). The buffering capacity of APB ensures a consistent and stable environ-

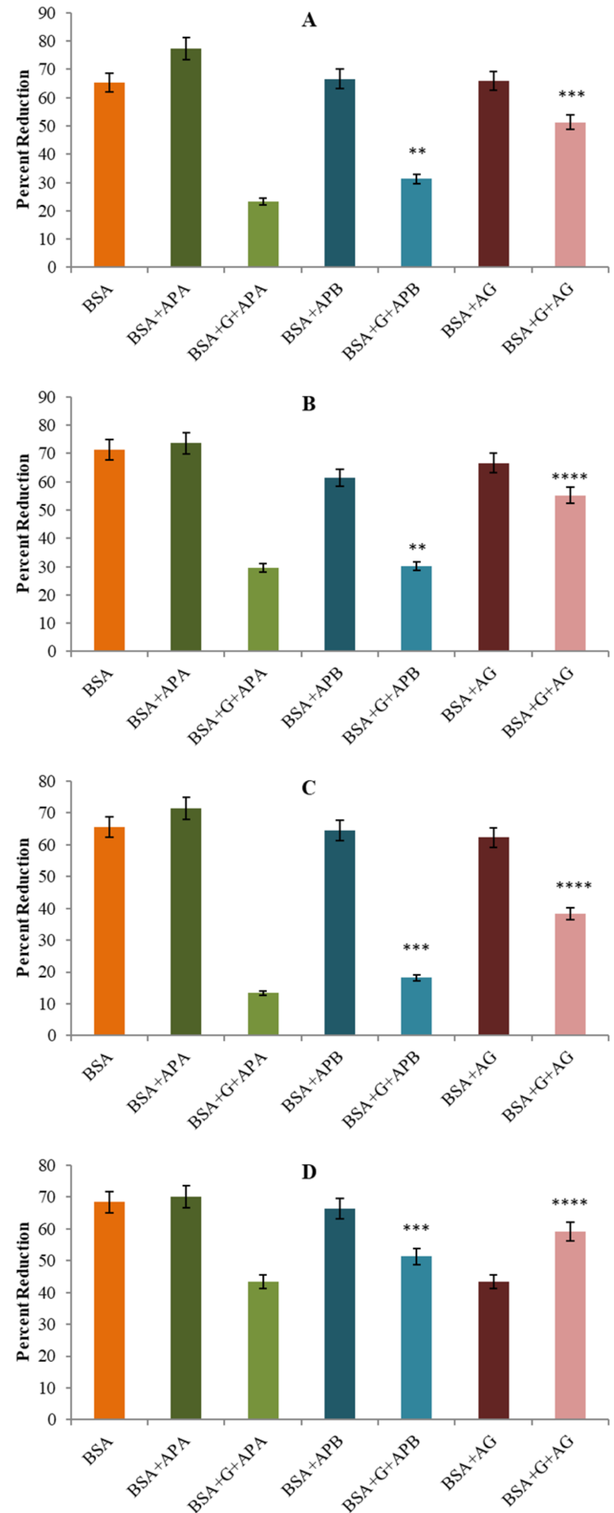

Figure 6. Evaluation of reduction of individual AGEs in glycated samples (** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$). (A) argpyrimidine; (B) pentosidine, (C) vesperlysine, and (D) crossline. BSA: bovine serum albumin; G: glucose; APA: aqueous extract of *A. platensis*; APB: buffered extract of *A. platensis*; AG: aminoguanidine

ment, protecting the bioactive constituents and enhancing their potential antiglycation and anti-aggregation effects. This methodical preparation approach underscores the importance of maintaining precise pH conditions to improve the efficacy of natural extracts, which explains why APB outperformed APA in our study.

Conclusion

This study highlights the differences in antiglycating potential between aqueous (APA) and buffered (APB) extracts of *Arthrospira platensis*. The buffered extract (APB) showed significantly higher efficacy in inhibiting browning and the formation of AGEs, such as argpyrimidine, pentosidine, vesperlysine, and crossline, likely due to the stable pH conditions maintained during extraction, which preserved the bioactivity of its constituents.

These findings provide a basis for future research to explore the molecular mechanisms behind APB's superior activity and its therapeutic potential in managing glycation-related conditions. The study underscores the importance of extraction methods in optimizing the biological activity of *A. platensis* extracts, offering valuable insights for the development of new treatments targeting glycation disorders.

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