Synergistic Interaction between Bambusa arundinacea and Euphorbia hirta Leaf Extract on Antioxidant, Antiglycation and DNA Protective Properties.

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Abstract
Oxidative stress and enhanced non-enzymatic protein glycation result in the pathogenesis of various disorders in humans, including diabetes mellitus. This study examined the synergistic action of Bambusa arundinacea with Euphorbia hirta leaf extract for anti-oxidant potential, antiglycation effect on fructose-mediated proteins, and DNA-protective properties. The methanolic extract shows significantly (P<0.01) higher total phenolic and flavonoid content when compared with ethyl acetate and hexane extract in both plants. The combined methanolic extract (CME) of both plants exhibits higher antioxidant potential, as indicated by the lower IC50 values obtained for DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity (4.82 µg/ml) and NO (nitric oxide) scavenging activity (3.94 µg/ml) with stronger Trolox equivalent antioxidant capacity when compared to B. arundinacea and E. hirta individually. The antiglycation analysis revealed that CME is more potent to inhibit fructose-mediated glycation of human serum albumin (HSA) at the early and middle stages. In addition to this, CME more effectively inhibits protein glycation in the late phase, which was confirmed by declining fluorescence intensity. Furthermore, a high degree of positive correlation was obtained between antioxidant and antiglycation capacities through Pearson’s correlation coefficient. The results also demonstrated that CME displays a synergistic effect in protecting DNA from oxidative damage and preventing the formation of glycation of DNA.

Keywords: Bambusa arundinacea, Euphorbia hirta, Combined methanolic extract, Antioxidant, Antiglycation

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INTRODUCTION
Diabetes mellitus is a worldwide problem that affects millions of people. It is characterised by an increase in blood glucose levels known as hyperglycemia, the development of oxidative stress due to the generation of free radicals, and a variety of diabetes-related complications. Free radicals imply ageing and cause damage to three important biological molecules, including nucleic acids, proteins, and lipids [1]. One of the most serious consequences of
hyperglycemia is non-enzymatic protein glycation, which includes the formation of advanced glycation end products (AGEs). This entire chain of reactions has three major phases. The reaction begins with the formation of Schiff bases via the reversible condensation of a carbonyl group from a reducing sugar, such as glucose, with a free amino group of protein. In the second stage, amadori products result from the irreversible and stable rearrangement of Schiff bases. Amadori products are prone to generating reactive dicarbonyl compounds, which are then rearranged to produce stable reactive products known as advanced glycation end products (AGEs) [2].

Under normal physiological conditions, the rate of AGEs formation is slow, but it accelerates under hyperglycemic conditions. When blood glucose levels are high for extended periods, as in untreated diabetes, the glycation process occurs primarily to produce compounds known as AGEs. These accumulated AGEs have the potential to alter the structural and functional properties of proteins. Glycated proteins are more likely to increase the production of free radicals [3]. Human serum albumin (HSA), a short-lived protein, undergoes structural modifications and unfolding events as a result of glycation. In diabetes mellitus type 2 (T2DM) patients, the effects of glycation reduce albumin's ability to act as a scavenger and transporter protein [4]. AGEs are involved in pathogenesis such as diabetes-related complications, cardiovascular complications, kidney malfunctions, osteoporosis, cancer, neurodegenerative diseases, and liver disorders [5,6]. The detrimental effects of AGEs were demonstrated by either directly trapping and cross-linking proteins or binding via AGE receptors (RAGEs) on the cell surface. Thus, AGEs can result in altered chemotaxis, angiogenesis, oxidative stress, cell proliferation, and programmed cell death [7, 8]. In addition to this, glycation-induced reactive oxygen species cause glycoxidative damage to nucleic acid [9]. Glycation of nucleic acid causes strand breaks, the unwinding of a double helix, mutations, and nucleotide-nucleotide cross-links in the structure of DNA [10]. AGEs are linked to an increase in mutations, DNA strand breakage, and cytotoxicity [11].

To treat these conditions, some inhibitors derived from natural sources, such as medicinal plants, are required. Many natural and synthetic compounds have been identified as being capable of preventing glycation and glycation-mediated ROS generation [12]. A plant-based component with antioxidant and antiglycation properties could be one of the approaches for glycation and its related pathogenesis. Bambusa arundinacea and Euphorbia hirta are widely distributed in Asian regions and have been used as medicines due to their various beneficial effects. Bamboo is a very good source of bioactive components, vitamins, minerals, and amino acids. Bamboo leaves contain pharmacologically active components such as polyphenols, saponins, general glycosides,
glycosylated flavones, betain, and cholin, which contribute to a variety of biological activities [13]. Bamboo leaf extract has a high biological and therapeutic potential, including antioxidant, anti-microbial, anti-inflammatory, anti-diabetic, and anti-ulcer properties [14]. The phytochemical screening showed that E. hirta contains tannins, flavonoids, alkaloids, glycosides, and saponins [15]. E. hirta has shown a broad range of biological properties, including antimicrobial, antifungal, anti-inflammatory, antioxidant, anti-cancer, and antidiabetic activities [16]. In recent years, phytotherapy has shifted toward the use of combination therapies, which employ multiple active components from different herbal plants. Current research evidence suggests that combination therapy may provide greater therapeutic benefits for cancer, atherosclerosis, and diabetes. The complex interactions between the multiple bioactive components found in Bambusa arundinacea and Euphorbia hirta that result in synergistic therapeutic effects could increase efficiency to reduce oxidative stress and glycation of human serum albumin (HSA). The current investigation is carried out to study the synergistic effects of B. arundinacea with E. hirta on antioxidant capacities, antiglycation, and DNA protective properties.

**MATERIALS AND METHODS**

**Collection of plant leaves and extraction**

Bambusa arundinacea and Euphorbia hirta leaves were collected from the Botanical Garden of Sardar Patel University and the local surroundings of Anand, respectively. The collected leaves were washed well with distilled water and allowed to air dry. The dried sample was ground into coarse powder and passed through a 60-mesh sieve. Leaf powders of B. arundinacea and E. hirta were separately taken for the extraction procedure with solvents hexane, ethyl acetate, and methanol through a Soxhlet apparatus. Plant extracts were collected and allowed to evaporate in a rotary evaporator. The obtained residues were weighed, dissolved in DMSO, and used for the experiments.

**Phytochemical analysis**

**Estimation of total phenolic content (TPC) and total flavonoid content (TFC)**

The total phenolic content of extracts from various solvents was determined using the Folin-Ciocalteu method [17] with some modifications. The total phenolic compound was calculated as mg/g dry powder GAE (Gallic acid equivalents) using the gallic acid calibration curve. The total flavonoid content of all the extracts from different solvents was measured according to a previously described method [18] and expressed as mg quercetin equivalent /g dry powder.

**Antioxidant analysis**

The combined methanolic extract (CME) was prepared by mixing residue in a 1:1 ratio of methanolic extract of both plants. Methanolic extracts of B. arundinacea (BME), E. hirta (EME), and a combined methanolic extract (CME) were used at different concentrations to determine antioxidant activity using the DPPH free radical scavenging assay, nitric oxide...
scavenging assay, and Trolox equivalent antioxidant capacity.

**Determination of DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay**

The assessment of DPPH free radical scavenging activity was carried out as per an earlier method [19]. In short, each of the extracts (100 μl) was added to 3 ml of DPPH reagent (2.4 mg%/ml) and incubated in the dark at room temperature for 30 min, and the absorbance was measured at 517 nm on a UV-Visible spectrophotometer. Ascorbic acid was used as a reference, and the results were expressed as percentage inhibition by using the following formula, where DPPH solution was used as a control.

\[
\%\text{Inhibition} = \frac{([\text{Absorbance of Control-absorbance of sample}) / \text{absorbance of control}] \times 100}{1}
\]

**Nitric oxide scavenging assay**

The Griess reagent was used to measure nitric oxide scavenging activity, as described by the prior method [20]. The extracts (100 μl) were treated with 0.5ml of sodium nitroprusside (50mM) and incubated at room temperature for 1 hour in this method. The absorbance at 540nm was measured immediately after the addition of 0.5ml Griess reagent [1% sulfanilamide in 5% H3PO4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (NED)]. Ascorbic acid was used as a control, and NO scavenging activity was calculated using a formula.

\[
\text{Scavenging activity (}) = [1-(A1 -A2)/A0] \times 100%,
\]

Where A0 was the absorbance of the control (the mixture without extract), A1 was the absorbance of the mixture in the presence of the extract and A2 was the absorbance without the Griess reagent.

**Trolox equivalent antioxidant capacity (TEAC) assay**

The TEAC assay was performed according to a previously described procedure with slight changes [21]. A stock solution of 7 mM ABTS (2,2’-azinobis (3-ethylbenzothiazoline 6-sulfonic acid) was prepared with 2.45 mM of potassium persulfate. This mixture was kept in the dark for 12-24 hours until a stable absorption (in the range of 0.700 ± 0.030) was obtained at 734 nm. To this ABTS mixture, 100 μl of extracts were added separately, and after 7 minutes of incubation, the absorbance of each reaction mixture was measured at 734 nm. In this experiment, an ABTS solution was used as a control and the results were expressed as μM TEAC/ g of extract.

**Preparation of glycated human serum albumin (HSA) and nucleic acid (DNA)**

A previous experiment described a procedure for preparing glycated HSA with minor modifications [22]. In brief, HSA (40 mg/ml) was incubated with fructose (500 mM) in phosphate buffer saline (200 mM, pH 7.4, containing 0.02% sodium azide). These reaction mixtures were kept at 37 °C for four weeks and studied using the HSA-Fructose model. A human genomic DNA was isolated from human blood according to a procedure described by an earlier study [23]. For the preparation of glycated DNA, the isolated
DNA (100 µg/ml) was incubated with fructose (500 mM), under a similar condition maintained for glycated HSA preparation. This glycated mixture is referred to as DNA-fructose.

Assessment of antiglycation activity
BME, EME, and CME were added at a concentration of 10 µg/ml to HSA-Fructose and DNA-Fructose separately. As a standard, aminoguanidine (AG) was incubated with HSA-Fructose and DNA-Fructose at the same concentration. For positive control, HSA-Fructose and DNA-Fructose (without extracts or AG) were taken. Aliquots from the various reaction mixtures were assessed at time intervals of 7 days, 14 days, 21 days, and 28 days for antiglycation activities. The level of fructosamine protein thiol content, amyloid - aggregation by Congo red binding, and fluorescence intensity of AGEs formation (late phase, at the 28th day only) were used to determine inhibition of HSA glycation. The inhibition of DNA glycation was measured through fluorescence studies.

Fructosamine assay
The level of fructosamine in different aliquots was determined using the nitro blue tetrazolium (NBT) assay, with some modifications suggested by Ardestani and Yazdanparast [24]. For this, 0.2 ml of aliquots (from both glycated HSA and DNA) were treated with NBT reagent (0.3 mM, 0.8 ml) prepared in sodium carbonate buffer (100 mM, pH 10.35) and incubated at 37°C for 15 minutes. The absorbance was taken at 530 nm and the results were represented as % inhibition of fructosamine as per the given formula:

% Inhibition = \[\frac{(\text{Absorbance of A1} - \text{absorbance of A2})}{\text{absorbance of A1}}\] x 100

Where, A1= Absorbance of the positive control, A2= Absorbance of the mixture with extracts or AG

For the DNA-Fructose model, the level of fructosamine was calculated using an extinction coefficient of 12640 cm⁻¹m⁻¹ for monoformazan. The amount of fructosamine formed at the end of the 28th day was represented as nmol/mg DNA.

Determination of protein thiols
The determination of thiol groups was carried out by a method as per Ellman’s assay [25]. The amount of thiol content was expressed as nmol/mg of protein.

Binding of Congo red
The Congo red binding ability was tested using a method described by Klunk et al [26]. At room temperature, 0.2 ml of aliquots were treated with 0.1 ml of Congo red (100 M in phosphate buffer saline (PBS) with ethanol (10% v/v)). Following a 20-minute incubation period, the intensity of the reaction mixtures was measured at 530 nm and the results were expressed as % inhibition using the same formula as the fructosamine assay.

Determination of fluorescence intensity of AGEs
The formation of AGEs in the glycated samples with plant extracts and AG was measured on the 28th day of incubation using fluorescence intensity at an excitation wavelength of 370 nm and an emission
wavelength of 440 nm using a spectrofluorometer (PerkinElmer) [27]. The percentage of inhibition was calculated using the following formula.

\[
\% \text{ Inhibition of AGEs formation} = \left( \frac{\text{fluorescence intensities of A1 - fluorescence intensities of A2}}{\text{fluorescence intensities of A1}} \right) \times 100
\]

Where A1= Glycated BSA or DNA, A2= Glycated BSA or DNA treated with extract or AG

**DNA-damage protection assay**

With minor modifications, the ability of plant extract to protect genomic DNA from the harmful effects of reactive oxygen species produced by Fenton's reagent was evaluated [28]. Isolated human genomic DNA (3.0 μL) was mixed with Fenton’s reagent (30 mM H\textsubscript{2}O\textsubscript{2}, 50 mM Ascorbic acid, and 80 mM FeCl\textsubscript{3}) followed by the addition of each extract (10 μg/mL) and the total volume of the mixture was brought up to 20 μL using double-distilled water. The reaction mixtures were allowed to incubate for 30 min at 37°C. The reaction mixtures (20 μL) were loaded on 0.8% agarose gel (prepared by dissolving 0.4 g of agarose in 50 mL of 1 x TBE Buffer) and electrophoresed at 90 V for 1 hour before staining with ethidium bromide.

**Results**

**Total phenolic and flavonoid content**

Total phenolic and total flavonoid content were calculated using a gallic acid and quercetin calibration curve, and the results were expressed in gallic acid equivalents (GAE) per g of extract and mg Quercetin equivalent/g of extract, respectively (Fig.1). The current findings show that methanolic extract of *B. arundinacea* (BME) contains significantly higher total phenolics (43.89 ± 1.39 mg GAE/g) and flavonoids (25.40 ± 1.28 mg QE/g) than extract from ethyl acetate and hexane. The concentration of phenolics (28.93 ± 1.08 mg GAE/g) and flavonoids (26.50 ± 0.89 mg QE/g) was found to be highest for the methanolic extract of *E. hirta* (EHE), followed by ethyl acetate and hexane. *B. arundinacea* is reported to have a higher phenolic content, whereas *E. hirta* (EHE) has the highest flavonoid content. It is critical to note that the polarity of the solvent influences the extraction content. Methanol is a good solvent for extracting active phytochemicals such as phenolics and flavonoid compounds. Based on the amount of extracted phenolics and flavonoids, a combined methanolic extract (CME) was made by combining equal parts of *B. arundinacea* methanolic extract (BME) and *E. hirta* methanolic extract (EME).
Figure 1: Total phenolic and flavonoid content of *Bambusa arundinacea* and *Euphorbia hirta* leaf extracts in different solvents. Values as mean ± standard deviation (n = 3) Lowercase letters a and b indicate significantly (P<0.01) higher total phenolic and flavonoid content for methanolic extract when compared to ethyl acetate and hexane extract, respectively.

### Antioxidant analysis

At various concentrations (from 2g/ml to 10g/ml), antioxidant activity was measured using antioxidant parameters such as DPPH radical scavenging activity, NO scavenging activity, and Trolox equivalent antioxidant capacity. *In vitro* DPPH radical scavenging activities of *B. arundinacea*, *E. hirta*, and combined methanolic extract (CME) were remarkable in a dose-dependent trend. (Fig. 2). The result showed that combined methanolic extract (CME) exhibits higher DPPH radical scavenging activities (99.36 ± 7.35% with IC50 value 4.82 μg/ml) when compared to individual extracts. (BME IC50 = 7.11 μg/ml and EME IC50 = 6.57 μg/ml). The NO scavenging capacity was measured by the decrease in a pink colour complex resulting from a reduction of NO production presented in Fig. 3. Combined methanolic extract showed its greatest NO scavenging effect with a lower IC50 value of 3.94 μg/ml at all studied concentrations of extract, as compared with individual *B. arundinacea* and *E. hirta* methanolic extract. CME has a much greater ability to scavenge NO radicals than individual extracts. Fig.4 represents the Trolox equivalent antioxidant capacity (TEAC) of extracts. In examining the results of the TEAC assay, CME has the greatest antioxidant capacity (60.41 ± 3.98 μM TEAC/ g of extract), followed by *B. arundinacea* (4 ± 3.42 μM TEAC/ g of extract) and *E. hirta* methanolic extract (30.31 ± 2.78 μM TEAC/ g of extract). The lower IC50 values of CME in comparison to BME and EME in Table 1 demonstrate its greater potency as antioxidant capacities. With respect to the result of total antioxidant potential, the CME is more potent...
than an individual methanolic extracts of plants.

Figure 2: DPPH radical scavenging activity of individual and combined methanolic extracts of *Bambusa arundinacea* and *Euphorbia hirta*. The data are presented as mean ± SD of independent experiments where n=3.

Figure 3: Nitric Oxide (NO) radical scavenging activity of *Bambusa arundinacea*, *Euphorbia hirta* and combined methanolic extract (CME). The data are presented as mean ± SD of independent experiments where n=3.
Figure 4: TEAC of individual and combined methanolic extracts of Bambusa arundinacea and Euphorbia hirta. Values are the mean ± SD of triplicates analysis. Values with the letters a and b indicates significantly different at p < 0.05.

Table 1: Antioxidant potential of BME, EME and CME based on IC50 values (µg/ml).

<table>
<thead>
<tr>
<th>Antioxidant activity</th>
<th>IC50 Concentration (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>BME</td>
</tr>
<tr>
<td>DPPH radical scavenging activity</td>
<td>7.11</td>
</tr>
<tr>
<td>Nitric oxide scavenging activity</td>
<td>6.19</td>
</tr>
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</table>

IC50 value represents the concentration of each extract that scavenge DPPH radical by 50%

**Antiglycation analysis**

The investigation for antiglycation using the HSA-fructose model includes the estimation of biochemical parameters of antiglycation, such as fructosamine content, protein thiol content, and Congo red dye binding capacity at different time intervals ranging from the 7th to the 28th day, and the formation of AGEs was estimated based on intrinsic fluorescence intensity by at the 28th day as an indicator of a late phase of glycation. When compared to the positive control, aminoguanidine, all three extracts (BME, EHE, and CHE) were found to be ineffective at inhibiting glycation of human serum albumin after the initial incubation period (7th day, Fig.5). The ability of the extracts to suppress glycation was dose-dependent as incubation time progressed, as shown in Fig.5, while the combined methanolic extract (CME) exhibiting the strongest anti-glycation activity, providing nearly 85% inhibition of NBT reduction similar to the positive control aminoguanidine. The effect of all three extracts (BME, EHE, and CHE) on the oxidation of thiol groups is shown in Fig.6. During the experimental period, the thiol groups in the HSA/fructose system decreased. When BME and EHE were used in an HSA/fructose system, a significant increase in thiol groups was observed in a
time-dependent manner when compared to glycated HSA. Again, CME displayed the greatest effect on protein thiol content (5.21 ± 0.19 nmol/mg of protein) in comparison with AG and individual extracts.

Figure 5: The inhibitory effects of BME, EME, CME, and AG on fructosamine content in the HSA-F model of glycation at different time intervals. Results are expressed as mean ± standard deviation (SD). Letter “a” indicates significantly different (p < 0.05) when compared to BME, EME and CME (on the 7th day), whereas “b” when compared to BME and EME (on the 14th, 21st, and 28th day).

Figure 7 depicts the inhibition of Congo red binding as an amyloid formation at the latter phases in glycation. The results showed that CME (71.24 %) inhibited Congo red binding in a time-dependent manner compared to BME and EME and almost similar to positive control AG. The fluorescence intensity of the HSA-fructose solution attributed to the formation of glycoprotein was measured to monitor the formation of AGE (Fig.8).

According to the spectrofluorometric analysis of the HSA-fructose model, CME is the most significant at the late phase, with the greatest inhibition (57.14%) compared to positive controls AG (52.04%), BME (39.80%), and EME (30.61%). The inhibitory action of extracts on DNA glycation was measured through fructosamine content and intensity of fluorescence on the 28th day.
Figure 6: Measurement of protein thiol content of BME, EME, CME and AG in the HSA-F model of glycation at different time intervals. Each value represents the mean ± SD (n = 3). Lowercase letter “a” values indicate significantly different (P<0.01) when compared to HSA + fructose (at 7th day), whereas “b” when compared to BME, EME and HSA + fructose (at 14th, 21st, and 28th day).

Figure 7: The inhibitory effects of BME, EME, CME and AG on the binding of Congo red in the HSA-F model of glycation at a different time interval. Values with the letters a and b indicates significantly different at p <0.05.
Figure 8: Fluorescence intensity (an excitation wavelength of 370 nm and an emission wavelength of 440 nm) for the detection of AGEs formation on the 28th day by HSA, glycated HAS, and glycated HSA treated with BME, EME, CME, and AG.

Figure 9: Fluorescence intensity (an excitation wavelength of 370 nm and an emission wavelength of 440 nm) for the detection of AGEs formation on the 28th day by DNA, glycated DNA, and glycated DNA treated with BME, EME, CME, and AG.
The level of fructosamine obtained using the DNA-fructose model is shown in Table 2. The fluorescence analysis revealed that CME had 50% inhibition on the late stage of DNA glycation, which was significantly higher than that of BME (30.10%) and EME (28.57%) (Fig.9). Table 3 shows the Pearson's correlation coefficient ‘r’ for the correlation between two variables, antioxidant and antiglycation potential of BME, EME, and CME. The current study found a high degree of positive correlation (r > 0.8) for various antioxidation and antiglycation parameters.

Figure 10: The protective effects of BME, EME and CME on Fenton-induced DNA damage. Lane description: Lane 1- Control human genomic DNA, Lane 2- Fenton (hydroxyl radical)-induced DNA damage, Lane 3-CME treated DNA, Lane 4- BME treated DNA and Lane 5- EME treated DNA. Fig.10 depicts the protection provided by BME, EME and CME against Fenton (hydroxyl radical) induced DNA damage on isolated human genomic DNA integrity. Control human genomic DNA (Lane 1), Fenton (hydroxyl radical) induced DNA damage (shearing effect) (Lane 2), as well as the DNA integrity, is slightly affected when treated with BME (Lane 4) and EME (Lane 5) separately. CME preserved the DNA integrity more efficiently compared to BME, EME indicated from Lane 3. DNA protection imparted by CME to Fenton induced DNA damage correlates well with the radical scavenging potential of CME.

<table>
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<tr>
<th>DNA-Fructose model</th>
<th>Fructosamine content (nmol/mg DNA)</th>
<th>% Inhibition of fructosamine</th>
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<tbody>
<tr>
<td>Native DNA</td>
<td>2.8 ± 0.32</td>
<td>-</td>
</tr>
<tr>
<td>Glycated DNA</td>
<td>14.11 ± 1.14</td>
<td>-</td>
</tr>
<tr>
<td>AG</td>
<td>7.82 ± 0.57</td>
<td>44.58</td>
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<tr>
<td>BME</td>
<td>9.47 ± 0.72</td>
<td>32.88</td>
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<tr>
<td>EME</td>
<td>8.97 ± 0.66</td>
<td>36.43</td>
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<tr>
<td>CME</td>
<td>6.68 ± 0.54</td>
<td>52.66</td>
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</tbody>
</table>

Table 3: Correlation coefficient ‘r’ between antioxidant and antiglycation parameters of BME, EME and CME.
### Discussion

Traditional Indian medicine (Ayurveda) has grown in popularity in recent years due to its beneficial effects on a variety of chronic conditions like diabetes mellitus [29]. Diabetes mellitus is a clinical condition in which hyperglycemia persists for an extended period, facilitating nonenzymatic protein glycation and ultimately increasing the accumulation of advanced glycation end products (AGEs) [30]. Therefore, AGEs may be therapeutic targets for the treatment of type 2 diabetes and its complications [31]. It is critical to identify interventions that use therapeutic plant extracts to prevent AGE formation or inhibit their activity. We approached combinational therapy and compared it to monotherapy of two important antidiabetic plants, *B. arundinacea* and *E. hirta*, in this study. The presence of a variety of bioactive constituents is critical in enhancing the medicinal properties of plants, which is dependent on the solvent used to extract the plant material. Methanolic extracts of both plants had the highest levels of phytochemical content when compared to hexane and ethyl acetate, indicating that methanol is the most effective solvent for bioactive compound extraction. Our findings are consistent with those of other researchers, who discovered that methanolic extracts contained the most TPC and TFC when compared to other solvents [32, 33, 34]. Mono-herbal therapy nowadays has been replaced in modern practice by herb-herb combinations or polyherbal therapy. It would pave the way for new approaches to therapeutic advancement and pharmaceutical product development in the future. In this study, methanolic extracts of *B. arundinacea* and *E. hirta* are combined to investigate their synergetic effect on oxidative stress and glycation of human serum albumin (HSA). According to our findings, the combined methanolic extract (CME) is rich in phytochemical components, which accounts for its increased antioxidant capacity indicated by DPPH radical scavenging property [35]. It is well established that phenolic and flavonoid compounds are important antioxidant constituents in medicinal plants and have direct relationships with antioxidant capacities [36, 37]. As revealed in our current study, *B. arundinacea* and *E. hirta* derived components can scavenge nitrogen oxide radicals. CME significantly reduced the production of

<table>
<thead>
<tr>
<th>Plant extract</th>
<th>Antioxidant activity</th>
<th>Fructosamine content (for glycated BSA)</th>
<th>Protein thiol content</th>
<th>Congo red binding</th>
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<tbody>
<tr>
<td>BME</td>
<td>DPPH</td>
<td>0.98</td>
<td>0.86</td>
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<td></td>
<td>NO</td>
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<td>0.91</td>
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<tr>
<td>EME</td>
<td>DPPH</td>
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<td>CME</td>
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</table>
reactive nitrogen species more effectively than individual extracts. For a long time, phenolic compounds were thought to have the ability to inhibit NO and peroxynitrite production [38]. TEAC assay was developed based on the ability of antioxidants to scavenge the ABTS*+ free radicals [39]. These results suggested that the combined methanolic extract (CME) exhibits higher ABTS free radical-scavenging capacity when compared to separate B. arundinacea and E. hirta extract. The higher antioxidant potential of CME is thought to be a synergistic effect of a diverse group of polyphenols compounds found in the combination of B. arundinacea and E. hirta in the current study. The synergetic effect of CME produces because the interaction of phytochemicals from different plant extracts often provides multi-target (polyvalent) treatment [40]. Furthermore, the concentration of phytoconstituents in duel herbal preparation is directly proportional to the concentration of antioxidant capacity noted previously [41]. Nonetheless, extensive research has been conducted on protein glycation inhibition using mono-herbal strategies [42]. Our findings show that polyphenol-enriched CHE is more effective than individual plant extracts at inhibiting the formation of AGEs and glycation of human serum albumin (HSA).. This study discovered that BME and EME inhibit non-enzymatic protein glycation at the early (fructosamine formation), intermediate (reducing protein thiol group), and late phases (Congo red binding ability), but CHE inhibits protein glycation and AGE formation more effectively. Polyphenols (flavonoids, phenolic acid) prevent the formation of AGEs through various mechanisms such as ROS inhibition, dicarbonyl trapping, antioxidant activation, and protein crosslinking breakdown [43]. CHE protects human serum albumin damage from glycation, by preventing the loss of protein thiol groups [44]. According to our experiment the potency for antiglycation by three various extracts has been found from lower to higher as follows EME< BME < CME. This protein glycation inhibitory effect of CME could be attributed to E. hirta's dedquercetrin, dimethoxyquercetrin, hirta coumaro flavonoside, and hirta flavonoside-B, as well as bamboo leaf extract's chlorogenic acid, caffeic acid, ferulic acid, p-coumaric acid, orientin, homoorientin, vitexin, and (BME) [45,46]. According to a previous experiment it was revealed that polyphenols exhibit a strong correlation with the inhibition of protein glycation [47]. A characteristic fluorescence is a marker for the development of AGEs, which is remarkably reduced by the combined plant extract. Hence, the synergistic effect of the active principles present in B. arundinacea and E. hirta might be accountable for the antioxidant activity and inhibitory action on the glycation of the combined extract. In support of this enhanced activity, there is an early report which suggested that supplementation of a mixed herbal extract (MHE) significantly inhibits the generation of d Nε-(carboxymethyl) lysine (CML), one of AGEs, in individuals with abnormal sugar metabolism [48]. The protection ability of
each plant extract against the Fenton reaction on isolated human genomic DNA was studied using agarose gel electrophoresis. The Fenton reaction generates hydroxyl radicals causing oxidative DNA strand breaks, resulting in a shearing effect on DNA agarose gel electrophoresis [49]. The DNA protective effect of *E. hirta* on Fenton’s reaction-mediated degradation of DNA has been previously established [50]. The current study confirms that a combination of plant extract (CME) contains a higher anti-oxidative potential, which could contribute significantly to its DNA protective effects.

**Conclusion**

The findings of this study showed that a combination of a bioactive constituent from BME and EHE is more potent in preventing the glycation of human serum albumin (HSA) by fructose in a dose-dependent manner after week 2 and that the inhibition was stronger on 3rd and 4th week. As a result, combining herbal preparations should provide more synergistic biological effects for medicinal needs than single herbal preparations. This newly discovered herbal combination is thought to have therapeutic potential in patients with diabetes or age-related diseases due to its antioxidant and anti-glycation properties, as well as its ability to protect DNA. Our findings justified further research into the molecular mechanism of the antiglycation potency of two herbal combinations.

**Conflicts of interest:** The authors declare no conflicts of interest relevant to this article.

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