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Inhibition of protein glycoxidation and advanced glycation end-product formation by barnyard millet (*Echinochloa frumentacea*) phenolics

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Abstract

Protein glycation plays a vital role in the progression of various diabetes complications. Therefore, inhibition of protein glycation could be a key strategy to prevent these diabetic abnormalities. Evaluation of phenolic compositions and their antiglycation activity revealed that *p*-coumaric and chlorogenic acids were major phenolic acids in barnyard millet. These phenolics exhibited multiple antioxidant activities in various mechanisms and protected the oxidative DNA damage and hydroxyl radical-induced protein fragmentation. Millet phenolics were very effective in scavenging >78% reactive carbonyl intermediates in the reaction and protected protein thiol group oxidation. Furthermore, 68.3% inhibition of protein glycation and reduced formation of protein aggregates were also observed with millet phenolics. Besides, fluorescence intensity measurements indicated a significant decrease in advance glycated end products and protection against glycoxidation-induced protein conformational changes at 100 µg/ml phenolics. These results suggest the potential utility of barnyard millet as an ingredient in functional foods for controlling protein glycation associated diabetic complications.

Keywords: Barnyard millet, Phenolics, Glycoxidation, Protein glycation, Oxidative DNA damage, Protein fragmentation, AGE

Introduction

Protein glycation, a reaction between reducing sugars and amino groups of proteins occurs in various stages. The early-stage represents the formation of a Schiff base and Amadori products. In the latter stages, the Amadori products are oxidized, dehydrated, and condensed to generate advanced glycation products (Vistoli, Maddis, Cipak, Zarkovic, Carini, & Aldini, 2013). This glycation of proteins is associated with glycoxidation and generation of oxygen and reactive carbonyl intermediates. Besides, specific advanced glycation end (AGE) products such as pentosidine, pyrraline, crossline, argpyrimidine, and pentolysine augment the oxidative damage to cells and modify their regular biological functions. *In vivo* accumulation of AGEs also accelerates ageing and causes diabetic complications such as retinopathy, neuropathy, nephropathy, protein denaturation, inflammation, and oxidative stress (Ahmed, 2005; Delgado-Andrade and Fogliano, 2018). Inhibiting the process of protein glycation is one of the key strategies to prevent glycation-mediated diabetic complications. Synthetic inhibitors such as aminoguanidine (AG) (Synvista Therapeutics, Inc.), pyridoxamine (BioStratum, Inc.), N-phenacylthiazolium bromide (Prime Organics, Inc.) and amlodipine (Pfizer, Inc.) are being used clinically for inhibiting protein glycation. Although these synthetic drugs are strong antiglycating agents, they found to have many adverse effects like gastrointestinal disturbances, rare vasculitis, anaemia and flu-like symptoms. Therefore, in recent years, natural phytochemicals from plants that effectively inhibit glycation with fewer side effects have attracted more and more interest from researchers.

Phenolic compounds are secondary metabolites of plants and have been studied for their *in vitro* antioxidant and antiglycation properties. These bioactive compounds may offer protection by scavenging free radicals, chelating transition metals and/or by neutralising carbonyl intermediates during the glycation reaction, thereby inhibiting the formation of AGEs (Meeprom, Sompong, Chan, & Adisakwattana, 2013; Hosseini, Asgary, & Najafi, 2015;

Salami, Rahimmalek & Ehtemam, 2016; Piwowar, Rorbach-Dolata, & Fecka, 2019). Phenolics with strong inhibitory activity on collagen crosslinking and glycation have been isolated from finger and kodo millets (Hegde, Chandrakasan & Chandra, 2002). These millet phenolics are also reported to possess various biological activities such as antioxidative, antihypertensive, anti-tumor activities and inhibit the formation of AGEs (Hou et al., 2018; Xiang, Zhang, Apea-Bah, & Beta, 2019; Liu et al., 2019; Ramadoss & Sivalingam, 2019). In addition, phenolics from small millets (barnyard, foxtail, and proso) exhibited antioxidant capacities in different mechanisms and displayed potent inhibition towards key enzymes associated with postprandial hyperglycaemia (α-amylase and α-glucosidase) (Pradeep & Sreerama, 2015).

Barnyard millet (*Echinochloa frumentacea*) is one of the oldest indigenous millet crops in the semi-arid tropics of Africa and Asia. This millet has a distinct advantage of being a drought-resistant crop and serves as a food for people living in these regions. However, it is underutilised in processed food products and commercial food systems. It is a rich source of sulphur-containing amino acids, protein, fat, vitamins and minerals and nutritionally surpass the staple cereals such as rice, maize and wheat (Bora, Ragaee, & Marcone, 2019). Besides its nutritional importance, bioactive phytochemicals present in this millet may have health-beneficial effects in decreasing the risk of various oxidative stress-mediated complications (Ugare, Chimmad, Naik, Bharati, & Itagi, 2014). Furthermore, barnyard millet grains were reported to possess potent antioxidant activity (Kim et al., 2011). Therefore, these millet antioxidant compounds may be promising agents for the prevention of oxidative stress–induced protein glycation and AGE formation.

Natural inhibitors of protein glycation from barnyard millet could be a more effective strategy to control protein glycoxidation and AGE formation and provide benefits without the side effects associated with synthetic drugs. However, there are no systematic studies on

antiglycation components of barnyard millet. Hence, this study aimed to evaluate the inhibitory effects of barnyard millet phenolics on fructose-mediated protein glycation, AGEs formation and their protective abilities on protein glycoxidation. Furthermore, implications of scavenging reactive intermediates on the different stages of glycation (early, intermediate, and late) and its role in the mitigation of structural changes in protein were also investigated.

Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), D-fructose, sodium dodecyl sulfate (SDS), guanidine hydrochloride, 2,4-dinitrophenylhydrazine (DNPH), 1-deoxy-1-morpholinofructose (DMF), thioflavin T were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Aminoguanidine (AG) from Cayman chemicals, USA, nitroblue tetrazolium (NBT) from Wako pure chemicals, Japan. All other reagents and chemicals were of an analytical or higher grade.

2.2. Sample preparation

Barnyard millet (*Echinochloa frumentacea* Link, *Poaceae*) RAU 11 cultivar (germplasm registered at Genebank, National Bureau of Plant Genetic Resources, Pusa Campus, New Delhi with Indigenous Collection No. 404408 dated 11 June 1978) harvested in 2013, was procured from the University of Agricultural Sciences, Dharwad, Karnataka. Grains were manually cleaned and graded to a uniform size. A portion of the graded grains was ground to a fine powder using a laboratory mill (IKA M20 Labortechnik, Staufen, Germany) and passed through a 250 µm sieve. The flour was defatted with 5 volumes of petroleum ether (w/v) for 6 h at room temperature (2 h × 3 times). The residue was air dried for 12 h. Defatted flour was stored at -20 °C for further analysis.

2.3. Extraction and quantification of total phenolic, total flavonoid and condensed tannins

A portion of defatted flour was extracted with 80% methanol containing 1% HCl (1:50 w/v) in a shaking water bath at 55 °C for 3 h. (3 × times) as reported by Hemalatha, Bomzan,

Rao, and Sreerama (2016). After centrifugation at 3000 ×g for 10 min, supernatants were pooled and concentrated under vacuum. The concentrated extract was freeze-dried and stored at -20 °C until further analysis. The total phenolic content (TPC) of the extract was quantified as described by Chandrasekara and Shahidi (2010) and results were expressed in milligrams of ferulic acid equivalents (FAE) per gram of flour. The total flavonoid content (TFC) was determined by the aluminum chloride colorimetric assay method as described by Chandrasekara and Shahidi (2010). TFC was represented as milligrams of catechin equivalent (CE) per gram of defatted flour. The condensed tannin content (CTC) was evaluated according to the vanillin-HCl method reported by Price, Van-Scoyoc, and Butler (1978) and results were expressed as mg of CE per gram of defatted flour.

2.4. Quantification of phenolic acids by HPLC

Compositional analysis of phenolic acids and flavonoids of barnyard millet were performed by high-performance liquid chromatography using a Shimadzu LC-10A HPLC system (Shimadzu, Kyoto, Japan) equipped with UV/Vis detector. Separations were conducted with Lichrospher C18 reverse phase column (5 µm, 250 × 4.6 mm; Merck, Darmstadt, Germany) with following conditions: flow rate, 1 ml/min, injection volume, 10 µl and column temperature, 25 ºC according to the method of Pradeep and Sreerama (2015). Solvent A, water/ trifluroacetic acid (99.9:0.1; v/v) and solvent B, acetonitrile/ trifluroacetic acid (99.9:0.1; v/v) were used for mobile phase. For phenolic acid elution, linear gradient was applied for the first 24 min with mobile phase B increasing from 0% to 12 %, followed by 20 % and 35 % at 32 and 45 min, respectively. The column was washed with 100% B for 5 min and equilibrated for 5 min at 100% A to initiate the next injection and total run time was 45 min. Quantitative determination of phenolic acids such as gallic, 3,4-dihydroxybenzoic, chlorogenic, vanillic, caffeic, *p*-coumaric and ferulic acids was performed at 254 nm. For flavanoid elution, linear gradient was programmed to increase mobile phase B concentration to 10% in 5 min, 36% in

31 min, 54% in 43 min and 64% in 50 min. The column was washed with 100% B for 5 min and equilibrated for another 5 min at 100% A to initiate the next injection. Detection of myricetin, daidzein, luteolin, naringenin, apigenin and kaempferol was performed at 340 nm. Quantification of phenolic compounds was based on retention time and UV/Vis spectral data. In addition, samples were also spiked with a known amount of authentic standards to identify phenolics in millet extract. The calibration curves were plotted separately for each standard at four different concentrations (1 -5 µg). Results were expressed as microgram per gram of defatted flour.

2.5. Determination antioxidant activity of barnyard millet phenolics

2.5.1. DPPH and ABTS$^{\bullet+}$ radical scavenging activity

The DPPH and ABTS$^{\bullet+}$ radical-scavenging activities were determined using the method of Prior, Wu and Schaich (2005). The DPPH and ABTS$^{\bullet+}$ radical scavenging activity of extracts was calculated from the standard curve of trolox (10-100 µg) and expressed as micromoles of trolox equivalents (TE) per gram of flour.

2.5.2. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay is depended on the reduction of the Fe^{3+}–TPTZ complex to ferrous form at low pH. This reduction was observed by determining the absorption at 595 nm according to the method of Benzie and Strain (1999). Ferric reducing antioxidant power was expressed as micromoles of Fe^{2+} equivalents per g of defatted flour.

2.5.3. Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging ability was determined according to the modified procedure of Sreerama, Takahashi, and Yamaki (2012). The concentration of phenolics used for the assay was 20 µg/ml. The scavenging activity was measured at 230 nm and the hydrogen peroxide scavenging ability was calculated using the following equation:

Hydrogen peroxide scavenging activity (%) = [(H_2O_2) test/ (H_2O_2) control] × 100,

where (H_2O_2) test is the concentration of H_2O_2 in the sample and (H_2O_2) control is the concentration of H_2O_2 in control.

2.5.4. Prevention of hydroxyl radical-mediated DNA damage and protein fragmentation

The prevention of hydroxyl radical-mediated λ-DNA damage and protein fragmentation by barnyard millet phenolics was performed as described by Girish, Vasudevaraju, and Rao (2012). For the prevention of hydroxyl radical-induced DNA damage, λ-DNA (7.5 µg/ml) was incubated with 1 mM $FeSO_4$ and 97.9 mM H_2O_2 for 1 h at 37 °C with or without of barnyard millet phenolics (0.1 mg/ml). EDTA (100 mM) was used as a positive control. Inhibitory effects of millet phenolics against hydroxyl radicals-mediated λ-DNA damage were also examined by determining the changes in fluorescence intensity of ethidium bromide binding to DNA. DNA (7.5 µg/ml) was incubated with 1 mM $FeSO_4$, 25 mM H_2O_2 in 10 mM Tris HCl (pH 7.4) at 37 °C for 1 h in presence or absence of barnyard millet phenolics (0.1 mg/ml). The fluorescence was recorded by emission at 600 nm and exciting at 535 nm. Inhibition of hydroxyl radical-induced protein fragmentation was assessed with bovine serum albumin (BSA). BSA (1 mg/ml) was incubated with 1mM $CuSO_4$ and 97.9 mM H_2O_2 for 24 h at 37 °C with or without of barnyard millet phenolics (0.1 mg/ml) and 100 mM EDTA as positive control. After the reaction, DNA damage and protein fragmentation were assessed by agarose gel electrophoresis and SDS-PAGE, respectively.

2.6. Antiglycation of bovine serum albumin (BSA)

To prepare the glycated protein, BSA (10 mg/ml) was mixed with barnyard millet phenolic extract (75 and 100 µg/ml) in 100 mM potassium buffer saline (pH 7.4) containing 0.02 % sodium azide and incubated for 10 min at 37 °C. Fructose (500 mM) was then added and incubation was continued at 37 °C for a further 12 days (Joglekar, Panaskar, Chougale, Kulkarni, & Arvindekar, 2013). After that, tubes were kept at -20 °C for 1 h to stop the reaction.

The positive control used was 50 mM aminoguanidine. The molecular mass of glycated BSA was determined by SDS-PAGE.

2.6.1. Measurement of fluorescent AGE formation, AGEs products and tryptophan intrinsic fluorescence

The formation of fluorescent AGE was measured as described previously (Wang, Sung, Cao, & Tian, 2009) by evaluating the fluorescence intensity of glycated proteins (0.15mg/ml) using excitation at 370 nm and emission in the range of 400-500 nm (RF5301 PC, spectrofluorimeter; Shimadzu Corporation, Japan). The assessment of fluorescence for specific AGEs was performed at different excitation and emission wavelengths (argpyrimidine-320 and 380; pentosidine-335 and 385; vesperlysine-350 and 405; total AGEs-350 and 440 and crossline-380 and 440, respectively). Results were depicted as percentage inhibition (Awasthi & Saraswathi, 2015). Intrinsic fluorescence of glycation induced changes in the microenvironment of tryptophan residues in BSA (0.45 mg/ml) were measured by excitation at 295 nm and emission in the range of 310–400 nm.

2.6.2. Determination of fructosamine, protein carbonyl content, protein thiol groups and protein aggregation of β amyloid structure

Fructosamine content was quantified by NBT assay adopted from Jariyapamornkoon, Yibchok-anun, and Adisakwattana (2013). Briefly, glycated protein (10 µl) was added to 90 µl of NBT reagent (0.5 mM in 0.1M carbonate, pH 10.8) and kept at 37 °C for 10 min, and the absorbance was read at 530 nm. Deoxy-morpholino-fructose used as a standard to determine the concentration of fructosamine. Quantification of protein carbonyl groups was performed according to the method of Sompong, Meeprom, Cheng, and Adisakwattana (2013). The absorbance was estimated at 370 nm and protein carbonyl content was calculated by using an extinction coefficient of DNPH (Ɛ= 22,000 M-1cm-1). The results were represented as nmol carbonyl/mg protein. The free thiols in glycated protein were evaluated by Ellman's assay with

minor changes (Jariyapamornkoon, Yibchok-anun, and Adisakwattana, 2013). Briefly, 70 µl of native or glycated BSA were incubated with 130 µl of 5 mM DTNB in 100 mM PBS, pH 7.4 for 15 min at 25°C. The absorbance was recorded at 410 nm. The free thiol groups were calculated using L-cysteine calibration curve and expressed as nmol/ mg protein. The amount of protein aggregation of the β amyloid structure was determined according to the method of Bouma et al. (2003). The glycated protein (100 µl) was incubated for 60 min with 32 mM thioflavin T at 25 °C. Fluorescence was recorded at excitation 435 and emission 485 nm.

2.6.3. Determination of altered secondary structural elements in glycated protein

Changes in the content of α-helix and β-sheet were measured by CD spectropolarimeter in the far UV region (200–260 nm) at 25 °C (Jasco J-810; Tokyo, Japan). The concentration of protein used for CD spectra was 0.3 mg/ml in 10 mM PBS (pH 7.4).

2.6.4. Zeta potential

The zeta potential and the electrophoretic mobility of glycated protein were determined with a Zetasizer Nano-ZS (Malvern, Worcestershire, UK). The working temperature was set at 25 °C and 100 repeated runs were accessed for glycated protein.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 software (La Jolla, CA, USA). Data were represented as the mean ± standard deviation of triplicates from three independent experiments. Significant differences ($p \leq 0.05$) were obtained by performing one-way analysis of variance followed by the Duncan test.

3. Results and discussion

3.1. Quantification of phenolics in barnyard millet

Various solvents such as ethanol, methanol, acetone, ethyl acetate, water are generally adopted to extract polyphenol compounds from plant-based foods. Previous experiments in our laboratory indicated that 80% aqueous methanol containing 1% HCl was efficient in extracting

phenolic compounds from barnyard millet at 50 ºC for 3 h in a shaking water bath. Total phenolic, total flavonoids and condensed tannin contents in barnyard millet were found to be 45 ± 3.8 mg FAE/g, 8.7 ± 0.17 mg CE/g and 7.1 ± 0.13 mg CE/g, respectively (Table 1). The composition of phenolics in barnyard millet is shown in Table 1. Gallic, 3, 4-dihydroxybenzoic, vanillic acids (hydroxybenzoic acid derivatives) and chlorogenic, *p*-coumaric, caffieic and ferulic acids (hydroxycinnamic acid derivatives) were detected in barnyard millet (Fig. 1A). Among the phenolic acids, *p*-coumaric, chlorogenic, caffeic and ferulic acids were the major phenolic acids identified in barnyard millet (Table 1). Gallic and 3, 4-dihydroxybenzoic acids were found in low amounts. Kaempferol, myricetin (flavonols), luteolin, apigenin (flavones), naringenin (flavanone) and daidzein (isoflavone) were also identified in barnyard millet (Fig. 1B). Daidzein was the most abundant flavonoid, followed by naringenin and myricetin. Besides, luteolin, kaempferol and apigenin were found in low amounts (Table 1). Polyphenols are considered major contributors to the antioxidant capacities. Flavonoids and tannins scavenge free radicals and exert protective activity against oxidative stress. Pradeep and Sreerama (2015) reported a potent antioxidant activity of caffeic, ferulic, gallic and chlorogenic acids from little and foxtail millets. Also, several reports have described the direct relationship between antioxidant activity of phenolic acids and their capacities to inhibit protein glycation and glycoxidation (Gugliucci, Bastos, Schulze, & Souza, 2009; Sompong et al., 2013). Therefore, the antioxidant abilities of millet phenolics may inhibit the glycoxidation reactions and the formation of protein aggregates.

3.2. Antioxidant properties

3.2.1. Free radical scavenging activity

$DPPH^{\bullet+}$ and $ABTS^{\bullet+}$ radical scavenging assays were used to determine the free-radical scavenging ability of barnyard millet phenolic extracts. $DPPH^{\bullet-}$ and $ABTS^{\bullet+}$ are stable free radicals, widely used in the evaluation of the antioxidant activity of various plant extracts. The

extent of radical scavenging activity was compared to a water-soluble vitamin E equivalent, Trolox (6-hydroxy-2, 5, 7, 8-tetramethychrom an-2-carboxylic acid) and the values were expressed as Trolox equivalent antioxidant capacity (TEAC). Millet extract exhibited 1.95 and 0.97 µmol TEAC for $DPPH^{\bullet+}$ and $ABTS^{\bullet+}$ radicals, respectively (Table 1). Since these reactions are based on hydrogen ion transfer mechanism ($ABTS^{\bullet+}$) or the combination of both hydrogen ion transfer and single electron transfer mechanisms (DPPH) (Prior et al., 2005), various functional groups on phenolic compounds present in millet may have contributed to the observed free radical scavenging activity. It was shown that the inhibitory effect of caffeic, ferulic, chlorogenic acids, myricetin and kaempferol on protein glycation is mainly due of their capacity to scavenge free radicals generated during protein glycation (Piwowar et al., 2019). Therefore, the strong free radicle scavenging activity of barnyard millet phenolics may play a crucial role in the prevention of glycation reactions and subsequent oxidation of protein backbone.

3.2.2. Ferric reducing antioxidant power (FRAP)

The ferric ion reducing antioxidant power assay is a redox-linked spectrophotometric assay frequently used as an indicator for the electron-donating ability of an antioxidant. FRAP value of barnyard millet was found to be 11.64 mmol Fe^{2+} equivalents/g (Table 1). This is in accordance with the results reported for quinoa (Hemalatha et al., 2016) and black gram (Girish et al., 2012). These results indicate that barnyard millet phenolic compounds are capable of donating electrons to reactive radicals, reducing them into more stable and unreactive species.

3.2.3. Hydrogen peroxide scavenging activity

Hydrogen peroxide is a poor oxidizing mediator that mitigate some enzyme activities directly by oxidation of sulfhydryl groups. It can diffuse inside the cell membrane rapidly; when inside the cell, it can react with transition metals such as ferrous ion (Fe^{2+}) and cupric ion (Cu^{2+}) to form hydroxyl ($^{\bullet}OH$) radicals and this might be the initiation of various of its

harmful impacts (Bienert, Schjoerring, & Jahn, 2006). Phenolics can donate electrons to hydrogen peroxide and neutralize it into water. H_2O_2 scavenging activity of barnyard millet phenolics is shown in Table 1. At 20 µg/ml concentration, barnyard millet phenolics showed the strongest effect by scavenging 76 % of the H_2O_2. Hydrogen peroxide scavenging capability of millet phenolics may indicate a biochemical mechanism for contributing some of the health-promoting benefits.

3.3. Effects of millet phenolics on hydroxyl radical-mediated DNA damage and protein fragmentation

The protective effects of barnyard millet phenolics on protein fragmentation and DNA damage induced by hydroxyl radical were studied by electrophoresis. Native lambda DNA (Fig. 2A; Lane 1) and BSA protein (Fig. 2B; Lane 1) resolved as sharp single bands on agarose and SDS-polyacrylamide gels, respectively on electrophoresis. Hydroxyl radicals generated via Fenton reaction with Fe^{2+} or Cu^{2+} (transition metal ions) and hydrogen peroxide led to the degradation of DNA (Lane 2, Fig. 2A) and BSA (Lane2, Fig.2B). EDTA (positive control) was effective in inhibiting DNA breaks (Lane 3, Fig. 2A) and protein fragmentation from hydroxyl radicals (Lane 3, Fig. 2B) by chelating transition metal ions. Barnyard millet phenolics at 0.1 mg/ml prevented the degradation of DNA (Lane 4, Fig. 2A) and protein fragmentation (Lane 4, Fig. 2B). Besides, DNA exposed to Fenton reaction showed a substantial decrease in the fluorescence intensity (>49 %) of ethidium bromide binding to DNA compared to native DNA (Fig. 1A). This decrease in fluorescence intensity could be due to the damage of DNA by hydroxyl radical. Barnyard millet phenolics prevented the DNA damage and restored the fluorescent intensity to >76 % of the native DNA.

3.4. Prevention of protein glycation

3.4.1. Inhibition of fructosamine and protein carbonyl formation

Reorganization of unstable Schiff bases formed during the early stage of glycation between reducing sugars and amino groups of protein results in the production of Amadori products such as fructosamines. Subsequent polymerisation of these products leads to the formation of cross-linked fluorescent AGEs products and various non-fluorescent and non-crosslinking structures (Awasthi, & Saraswathi, 2015; Wu, Tu, & Zhung, 1996; Wang et al, 2016). Besides, reducing sugars under hyperglycaemic conditions could undergo degradation to generate reactive carbonyl intermediates (RCIs). These RCIs play an important role in AGE formation and are well-known biomarkers of biological oxidative stress generated during glycoxidation of proteins (Delgado-Andrade and Fogliano, 2018). In this study, the reaction of fructose with BSA resulted in the generation of 1.55 mM fructosamine and significantly higher levels of protein carbonyls (3.6 nmol/mg) in fructose-induced glycated protein (Table 2). A significant reduction in fructosamine and protein carbonyl contents were observed in a reaction containing a synthetic inhibitor, aminoguanidine (50 mM). Similarly, millet phenolics at 75 and 100 µg/ml were very effective in inhibiting the generation of fructosamine and protein carbonyls (Table 2). Previous studies revealed that scavenging ability of *p*-coumaric, ferulic, vanillic and protocatechuic acids on free radicals derived from the glycoxidation process was associated with the inhibition of AGE formation on BSA (Vlassopoulos, Lean, & Combet, 2014). In addition, it was also reported that the main contributing factors for inhibiting the formation of advanced glycation end products were trapping of reactive carbonyl intermediates by phenolic compounds during initial stages of glycation reaction and blocking of carbonyl groups on sugars and Amadori products by multiple OH groups present on phenolic compounds. Furthermore, Wu, Hsieh, Wang and Chen, (2009) observed strong inhibitory effects of gallic, catechin, epicatechin, *p*-coumaric acid, quercetin and ferulic acids derived from guava leaf extract on the formation of reactive carbonyl contents during glycation process of protein. Therefore, various flavonoids and phenolic acids found in millet highlight their

probable role in preventing the formation of fructosamine in glycation reaction. Moreover, it is to be noted that inhibition of protein glycation and regulating the fructosamine levels during early stages of glycation could deter the formation of various reactive intermediates and fluorescent AGE products.

3.4.2. Prevention of protein thiol group oxidation

Glycation of proteins induces protein oxidation and structural changes in protein resulting in alterations of its biological activity. The thiol group of Cys residues in proteins is susceptible to oxidation leading to the formation of disulfide bonds and protein aggregations. Fructose-induced glycated BSA showed a 59.7% decrease of free –SH groups (12.75 nmol/mg protein) compared to native BSA (31.63 nmol/mg protein) (Table 2). However, the presence of barnyard millet phenolics in glycation reaction prevented the oxidation of free thiol groups. The free -SH content of glycated BSA in the presence of 75 and 100 µg/ml millet phenolics was found to be 16.93 nmol/mg protein and 20.05 nmol/mg protein, respectively. Several studies have suggested a strong positive correlation between the antioxidant activity of phenolic compounds and the capacities to inhibit protein glycoxidation. Flavonoids having tetrahydroxyl and hexahydroxyl groups in their structures are reported to possess strong ability to protect the oxidation of free thiol groups in protein (Fujimoto & Masuda, 2012; Abdallah, El-Bassossy, Mohamed, El-Halawany, Alshali, & Banja, 2016). Therefore, flavonoids with numerous OH groups in their structures such as kaempferol & luteolin (tetrahydroxyl) and myricetin (hexahydroxyl) found in barnyard millet (Table 1) may have prevented the oxidation of free thiol groups in BSA.

3.4.3. Inhibition of protein crosslinking

Reducing sugars readily react with lysine and arginine residues of proteins to form intermolecular cross-linked and high molecular mass aggregates (Delgado-Andrade & Fogliano, 2018). SDS-PAGE evaluation of protein crosslinking and adduct formation in

fructose-induced protein glycation is shown in Figure 3A. Native BSA separated as a single band of molecular weight ≈ 67000Da (Lane 1, Fig. 2A). However, a high molecular weight band and low-intensity band corresponding to native BSA were found in glycated BSA (Lane 2, Fig. 3A). Aminoguanidine, a well-known inhibitor for AGE, prevented BSA adduct formation resulting in a single band corresponding to native BSA (Lane 3, Fig. 3A). Millet phenolics markedly suppressed the loss of native protein and inhibited the protein cross-linking or protein aggregate formation (Lane 4 and 5, Fig. 3A). The strong ability of millet phenolics to scavenge free radicals, RCIs and their protective capabilities on protein thiol group oxidation may have contributed to the prevention of protein cross-linking and aggregate formation.

3.4.4. Fluorescence quenching on AGEs formation and intrinsic tryptophan fluorescence

The initial stages of glycation are more susceptible to the progression of Schiff's or Amadori products and generate oxidation stress. Further, this undergoes a late-stage irreversible cascade of reactions involving dehydration, hydrolysis and rearrangement leading to the formation of AGEs (Awasthi, & Saraswathi, 2015; Delgado-Andrade and Fogliano, 2018). Figure 3A shows the fluorescence intensities of native BSA and AGE fluorescence of glycated BSA in the absence and presence of established glycation inhibitor, aminoguanidine or millet phenolics. In the absence of inhibitor, native BSA exhibited a fluorescence intensity of 8 a.u. However, AGE fluorescence of glycated BSA was about 4.5 fold higher than the native BSA. Aminoguanidine was very effective in reducing the fluorescent AGEs in glycated BSA. Similar to aminoguanidine, millet phenolics also showed strong ability in reducing the fluorescent AGEs (>3-fold reduction).

The fluorescence intensity (I_{max}) of intrinsic tryptophan (Trp) residues in BSA was investigated to get an insight into the changes in the micro-environment in glycated protein (Figure 3B). Intrinsic tryptophan fluorescence is commonly used to study the tertiary structural changes upon glycation in proteins (Szkudlarek, Sulkowska, Maciazek-Jurczyk, & Rownicka-

Zubik, 2016). Aromatic amino acids, particularly tryptophan, act as an important fluorophore and give evidence of intermolecular interaction and conformational changes in the protein. As shown in Figure 3B, the maximum fluorescence intensity of native BSA (507 a.u) was observed at 340 nm, characteristic of Trp. Fructose-induced glycation resulted in a considerable decrease of I_{max} of BSA (231 a.u). Aminoguanidine and millet phenolics at different concentrations restored the I_{max} to 484-520 a.u (Figure 3B). These results indicate the ability of millet phenolics to prevent the glycation enforced conformational changes in BSA. The aromatic amino acids in proteins are prone to oxidation by reactive intermediates. Millet phenolics with a strong capacity to scavenge RCIs (Table 2) may have prevented the glycation-induced modification of hydrophobic microenvironment of BSA. In addition to total AGEs fluorescence, quantification of four specific biomarkers of AGE products such as crossline, vesperlysine, argpyrimidine and pentolysine, were also carried out. These specific AGEs products play a crucial role in the development of diabetes complications and are characterised by their well-defined fluorescence properties (Awasthi, & Saraswathi, 2015; Delgado-Andrade & Fogliano, 2018). These AGEs products are highly reactive and are vital in the progression of hyperglycaemic complications such as nephropathy, neuropathy and retinopathy. Results presented in Figure 3C suggest that barnyard millet phenolics (100 µg/ml) effectively reduced the fluorescence intensity of all the four AGEs products. The maximum reduction of fluorescence intensity was observed in argpyrimidine (42.3 %) and total AGEs (73%). Besides, AGEs inhibitory activity of barnyard millet phenolics was found to be similar to the aminoguanidine.

Noticeably, chlorogenic, caffeic and ferulic acids, which are predominant in barnyard millet (Table 1) were reported to possess strong ability to prevent protein glycation in BSA-glucose model system (Salami et al, 2016; Piwowar et al., 2019). In addition, previous computational analysis and mass spectrophotometric studies reported the interaction of Lys 93

and Arg 194 residues of BSA to chlorogenic acid and Arg-185 and Try-137 residues to caffeic acid through hydrogen bonds and block protein glycation (Vlassopoulos et al., 2014; Bhattacherjee & Datta, 2015). It is to be noted that in addition to phenolic acids, daidzein, naringenin and myricetin were the principal flavonoids found in barnyard millet (Table 1). These flavonoids may protect against glycation-derived free-radical-mediated oxidation by acting as divalent metal ion chelators and inhibiting the self-oxidation of reducing sugars, Amadori products and reactive carbonyl species (Silvan, Srey, Ames, & Castillo, 2014). It is reported that anti-glycative mechanism of daidzein, an isoflavone may be related to its conjugation to glycation sites on protein structure (free amino groups), its antioxidant ability and trapping of dicarbonyl intermediates. (Hosseini et al., 2015). Likewise, multispectroscopic and molecular docking studies on the inhibition of AGE formation in BSA–methylglyoxal model, naringenin displayed strong inhibition on the formation of advanced glycation end products (Liu et al., 2019). Besides, Chen et al, (2010) showed that myricetin with multiple hydroxyl groups in its structure could decrease LDL glycation and electrophoretic mobility of glycated LDL in a dose-dependent manner. Therefore, direct interaction of chlorogenic, caffeic and ferulic acids of barnyard millet with amino groups of Lys and Arg residues of BSA and protective capacity of millet flavonoids against glycation-induced free-radical-mediated oxidation of protein along with their potential ability to conjugate glycation sites via hydrogen bonds may have contributed to the inhibition of protein glycation and AGE formation

3.4.5. Effect of barnyard millet phenolics on amyloid fibrillation

Glycation can also induce protein crosslinking and aggregation, which leads to β-amyloid fibrillation. Accumulation of β-amyloid fibrillation adducts may contribute to amyloidosis, a contributing factor for the pathological progression in diabetes and neurodegenerative disorders (Bouma et al., 2003; Vidal & Ghetti, 2011). Higher accumulation of β-amyloid structure (> 4.7 fold) was observed in fructose-induced glycated protein than native BSA

(Table 2). However, aminoguanidine reduced the formation of aggregated amyloid structure to about 2.5 fold. A significant reduction was also found with barnyard millet at 75 and 100 µg/ml (3.2 and 2.9 folds, respectively). Previously, reduction in the levels of amyloid cross β-structure in a BSA/fructose model system by cinnamic acid-derived phenolic acids were reported in the literature (Adisakwattana, Sompong, Meeprom, Ngamukote, & Yibchok-anun, 2012). Therefore, barnyard millet having hydroxyl cinnamic acid derivatives such as *p*-coumaric, chlorogenic, caffeic, ferulic acids as major phenolic acids may prevent AGE-mediated diabetic complications and decrease the risks of neurodegenerative diseases.

3.5. Protective effects of barnyard millet phenolics on protein structure

3.5.1. Circular dichroism

The covalent attachment of sugar to the ε-amino groups of lysine residues in proteins during protein glycation results in the alterations of their secondary structural elements (Sreerama & Woody, 1993; Awasthi & Saraswathi, 2015). Far-UV CD spectroscopy was used to extract quantitative information on the transition of secondary structural elements of BSA upon glycation. Figure 4 shows the typical far-UV CD spectra of native and glycated BSA in the absence and presence of millet phenolics at different concentrations. Significant alteration in the secondary structure of BSA was noticed upon glycation, which was diminished by adding millet phenolics. Native BSA showed two major negative ellipticity at 222 and 208 nm, which is a characteristic feature of the α-helical structure. In addition, the minor negative ellipticity observed at 218 nm is attributed to the β -sheet. These negative bands were found to be decreased in glycated BSA, where reduction of α-helix (27%) and β -sheet (13%) contents were observed compared to native BSA. These results suggest that fructose-induced glycation of BSA altered its secondary structure by disrupting the intramolecular forces. Presence of millet phenolics in glycation reaction resulted in >30% improvement in α-helix and β -sheet contents (Figure 4). However, in the presence of millet phenolics, the increase of the magnitude of

negative ellipticity was smaller when compared to aminoguanidine. These results indicate that inhibitory effects of millet phenolics on protein cross-linking and protein glycoxidation may have prevented the structural alterations in BSA.

3.5.2. Zeta potential

Zeta potential measurement is key to understand surface hydrophobicity, aggregation and conformational changes of a macromolecule by determining the electrophoretic mobility. Zeta potential and electrophoretic mobility of non-glycated BSA was found to be -8.38 mV and -0.65 μmcm/Vs, respectively (Table 2). However, glycated BSA exhibited lower values for these parameters (-10.59 mV and -0.73 μmcm/Vs for zeta potential and electrophoretic mobility, respectively). Noticeably, the presence of millet phenolics or aminoguanidine in glycation reaction showed zeta potential and electrophoretic mobility values closer to non-glycated BSA (Table 2). Generally, proteins altered by glycoxidation reaction exhibit modifications in their surface hydrophobicity, electrophoretic mobility and isoelectric point (Joglekar et al., 2013). Moreover, glycation causes partial unfolding of proteins, which alter the electrophoretic properties. Similar to preventing the loss of secondary structural elements upon glycation, the ability of millet phenolics to scavenge free radicals and reactive carbonyls and their potent inhibitory effects on protein glycation may have contributed to the protection of surface hydrophobicity and structural modifications in protein.

4. Conclusion

Barnyard millet phenolics possess potent radical scavenging and metal chelation activities. These phenolic compounds inhibited protein glycation and AGE formation at several stages of the glycation reaction in BSA model system. Besides, reduced generation of oxygen and reactive carbonyl intermediates, protein aggregated products and AGE levels were also observed with millet phenolics. Furthermore, these phenolic compounds also prevented thiol group oxidation, conformational changes and structural modifications in BSA. Moreover,

scavenging of reactive carbonyl intermediates and free radicals by millet phenolics may also have contributed to their antiglycation ability. The results suggest that barnyard millet phenolics may mitigate various diabetic abnormalities and could be used as functional food ingredients for controlling protein glycation associated diabetic complications. However, further studies are necessary to assess its protective effects on diabetes in animal models.

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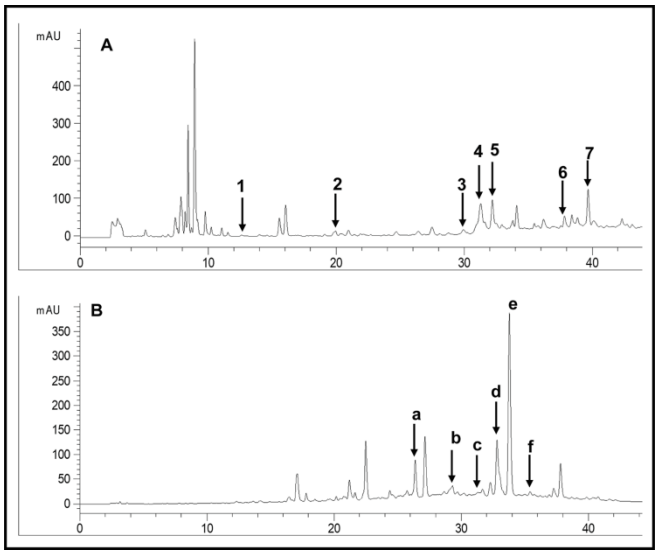
Legend to figures

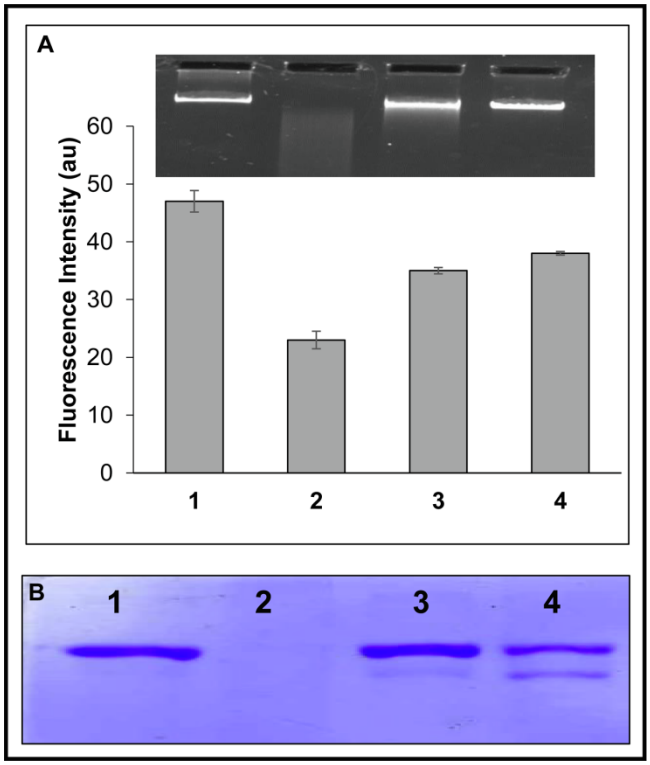
Figure 1. HPLC profiles of phenolic compounds of barnyard millet. A. Phenolic acids: 1-Gallic acid; 2-Dihydroxybenzoic acid; 3-Chlorogenic acid; 4-Vanillic acid; 5-Caffeic acid; 6-*p*-Coumaric acid; 7-Ferulic acid. B. flavonoids: a-Myricetin; b-Daidzein; c-Luteolin; d-Naringenin; e-Apigenin; f-Kaempferol.

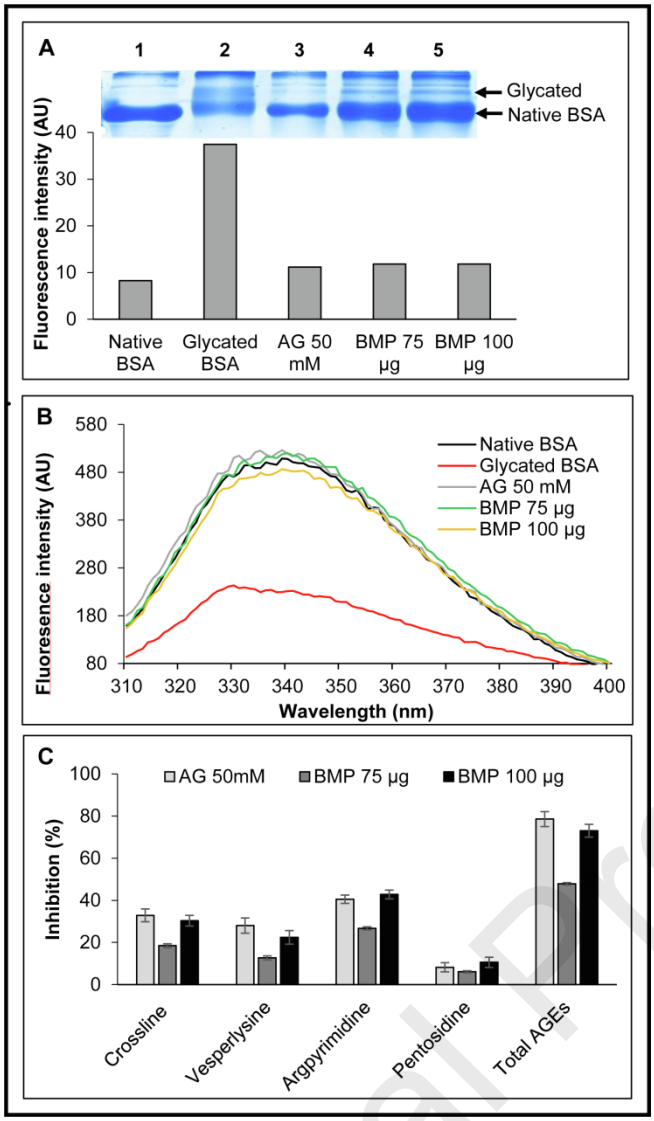
Figure 2. Prevention of hydroxyl radicals-mediated λ-DNA damage and protein fragmentation by barnyard millet phenolics. A. Fluorescence intensity and agarose gel electrophoresis (inset) of λ-DNA. Lanes 1, λ-DNA; 2, λ-DNA + $FeSO_4$ + H_2O_2; 3, λ-DNA + $FeSO_4$ + H_2O_2 + EDTA; 4, λ-DNA + $FeSO_4$ + barnyard millet phenolics. B. SDS-PAGE of native and fragmented BSA. Lanes 1, BSA; 2, BSA + $CuSO_4$ + H_2O_2; 3, BSA+ $CuSO_4$ + H_2O_2 + EDTA; 4, BSA+ $CuSO_4$ + H_2O_2 + barnyard millet phenolics.

Figure 3. Inhibitory effect of barnyard millet phenolics (BMP) on protein glycation. Fluorescence intensity of advanced glycation end products (AGEs) formation in fructose-mediated glycated BSA. A. Inset shows the modulation of high molecular weight adducts formation in BSA by barnyard millet phenolics in SDS-PAGE. B. Intrinsic tryptophan fluorescence emission profiles of native and modified BSA. C. Inhibition of specific biomarkers of AGE products by millet phenolics.

Figure 4. Far UV-CD spectra for secondary structural elements of native and glycated BSA.







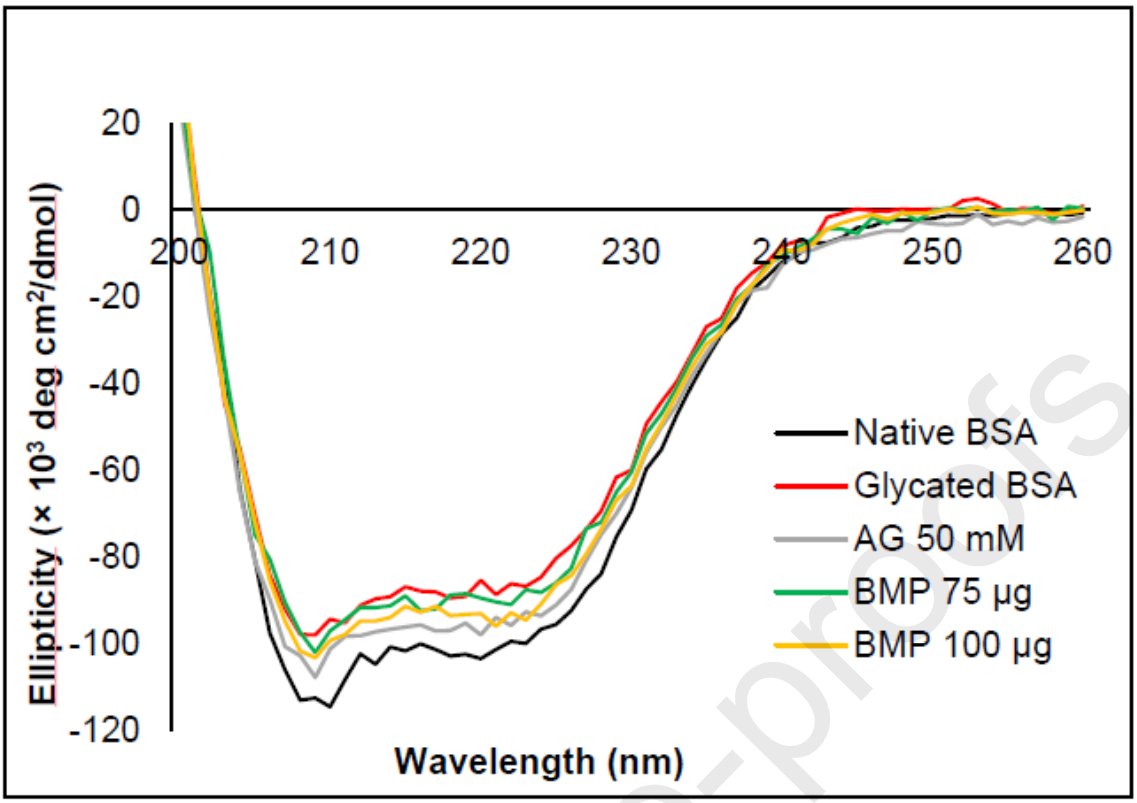


Table 1. Phenolic indices, composition and antioxidant activity of barnyard millet.

Phenolic indices (mg/g)			
Total phenolic content[a]	Total flavonoid content[a]	Condensed tannin content[a]	
45 ± 3.8	8.7 ± 0.17	7.1 ± 0.13	
Phenolic composition (µg/g defatted flour)			
Hydroxybenzoic acid derivatives			
Gallic	31.6 ± 2.2		
3,4-Dihydroxy benzoic	42.2 ± 0.7		
Vanillic	104.8 ± 1.5		
Hydroxycinnamic acid derivatives			
Caffeic	203.82 ± 4.5		
Chlorogenic	317.3 ± 4.6		
Ferulic	143.3 ± 18.1		
p-Coumaric	797.4 ± 14.9		
Flavanoids			
Myricetin	21.9 ± 0.7		
Daidzein	33.6 ± 0.4		
Luteolin	10.8 ± 0.3		
Naringenin	32.2 ± 1.6		
Apigenin	3.8 ± 0.1		
Kaempferol	6.3 ± 0.2		
Antioxidant activity			
DPPH (µmol trolox/g)	ABTS (µmol trolox/g)	FRAP (µmol/g)	H_2O_2 [b] (%)
1.95 ± 0.14	0.97 ± 0.05	11.64 ± 0.18	76.85 ± 3.49

[a]Total phenolic content was expressed as mg of ferulic acid equivalents/g, whereas total flavonoid and proanthocyanidin contents were expressed as mg of catechin equivalents/g.
[b]Concentration of phenolics used for H_2O_2 scavenging activity was 20 µg/ml ferulic acid equivalents.
Results are expressed as mean ± standard deviation (n = 3) of defatted flour.

Table 2. Modulation of barnyard millet phenolics on reactive intermediates, amyloid aggregation and surface hydrophobicity of BSA

	Fructosamine (mM)	Carbonyl content (nmol/mg protein)	Thiol group (nmol/mg protein)	Amyloid fibrils (Arbitrary unit)
Native BSA	0.37 ± 0.01	0.37 ± 0.09	31.63 ± 1.12	347.9 ± 28
Glycated BSA	1.55 ± 0.03	3.76 ± 0.10	12.75 ± 0.78	1661.3 ± 63
Aminoguanidine (50mM)	0.91 ± 0.08	1.67 ± 0.10	27.14 ± 0.85	889.5 ± 73
Millet phenolics (75 µg/ml)	1.16 ± 0.06	0.89 ± 0.01	16.93 ± 1.9	1114.3 ± 107
Millet phenolics (100 µg/ml)	1.03 ± 0.08	0.81 ± 0.01	20.05 ± 0.87	1015.9 ± 69

Credit Author Statement

MA was responsible for performing experiments, made significant contributions to development of protein glycation reaction, analysis of glycated products, acquisition and analysis of data and writing article.

YNS conceived the project, designed experiments, supervised execution of the project, analysed, interpreted the data, and critically revised the article.

Both the authors analysed the results, discussed and approved the final version of the manuscript.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- *p*-Coumaric and chlorogenic acids were predominant phenolic acids in barnyard millet
- Barnyard millet phenolics prevented oxidative DNA damage and protein fragmentation
- Millet phenolics inhibited protein glycation, crosslinks and AGEs formation
- Reactive carbonyl intermediates were effectively scavenged by millet phenolics
- Millet phenolics protected glycoxidation-induced protein conformational changes