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Beta vulgaris var. cicla methanol extract prevents the formation of advanced glycation end products and protein oxidation against -glucose, and -fructose-induced protein glycation in vitro

Research Article

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Abstract

Beta vulgaris is an edible plant and herbal medicine traditionally used to treat diabetes and breast cancer and protect against liver damage induced by an HF diet. The aim of this investigation was evaluating the inhibitory activity of B. vulgaris (CH) extract on the formation of advanced glycation end products (AGEs) against fluorescent and Non-fluorescent AGEs, protein oxidation, and protein aggregation in an in vitro model of glucose and fructose protein glycation and Congo red test. Our study provides evidence that CH significantly reduced fluorescent and no fluorescent (Nε-CML) and fructosamine levels in AGEs-BSA systems during four weeks of study. It also avoided protein oxidation, which was shown by the depletion of protein thiol and reduced protein carbonyl, preventing structural loss of protein and AGEs formation by inhibiting protein oxidation and protein glycation. Furthermore, CH extract also inhibited amyloid cross β-structure and increased GPx enzymatic activity. This study demonstrated that CH has a noticeable antiglycation effect in a varied glycation modification of albumin and can retard or forewarn AGEs-related diabetes type 2.

Key Words: *Beta vulgaris, G*lycation, Glucose, Fructose, Protein oxidation.

Introduction

Diabetes is related to chronic complications such as cardiovascular, nephropathy and retinopathy, and nephropathy diseases. Glycation is a non-enzymatic process that is accelerated by chronically elevated plasma glucose levels which can modify protein structures affecting their functions. The formation of covalent adducts is accelerated by hyperglycemia affecting proteins such as albumin and collagen and cells such as renal mesangial cells and vascular endothelial cells. Accumulated AGEs are related to the pathogenesis of diabetes and other diseases (1). After time glycation products produce a heterogeneous, stable group of compounds known as advanced glycation end-products (AGEs) provoking irreversible functional and structural injury to the protein molecules. The reaction of free amino groups of the protein with carbonyl groups in reducing sugars leads to the formation of advanced glycation end products (AGEs) (2). The non-enzymatic glycation reaction of the proteins is carried out in three stages. The base of Shiff is formed in the early stage by reaction of reducing sugars with free amino groups of protein, produced after Amadori products. In the second stage, the Amadori products are dehydrated and oxidized to generate dicarbonyl compounds such as glyoxal, glycolaldehyde, and methylglyoxal. In the last stage, are produced forms stable AGEs due to the abilities of the tricarbonyl compounds to cross-link with amino groups of the proteins (3).

Thereby, there is considerable interest in the investigation of plants with the antiglycation effect that has the ability to prevent AGE formation resulting in preventing and delaying the commencement of diabetic complications (4).

The edible plant Beta *vulgaris* subspecies cicla L. var. flavescens has been used in traditional medicine for its antibacterial and hypoglycemic properties (5). Contains phytochemicals that have beneficial activities including anti-inflammatory, antioxidant, and antiproliferative effects (6) The antidiabetic potential of *B. vulgaris* leaves was reported in diabetic Wistar rats induced with streptozotocin (STZ) and a high-fat diet (HFD) demonstrated that prevents it liver damage and hepatic steatosis by its insulin-sensitizing, and hypoglycemic properties and their capacity to upregulate antioxidants and PPARα (7). According to the literature, there is no report of in vitro anti-glycation activity of *B. vulgaris* leaves thus, our investigation aimed to determine the anti-glycation effect of the

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leaves extracts of *B. vulgaris* by using a battery of in-vitro assays.

Materials and methods

Chemicals

Aminoguanidine hydrochloride (AG), bovine serum albumin (BSA, fraction V), l-cysteine, 2,4-dinitrophenylhydrazine (DNPH), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), guanidine hydrochloride, sodium azide (NaN3), methyl glyoxal (MGO), trichloroacetic acid (TCA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other solvents used in this study were of analytical grade purchased from Fermont (CDM, Mexico). Human hair was provided from local barbershops in Ciudad de Mexico (CDMX) Mexico.

Plant material

Beta vulgaris var. cicla was collected in the vicinity of Amecameca Mexico State, Mexico. The plant was identified and authenticated by Biol. Aurora Chamal, Department of Botany, National School of Biological Sciences, National Polytechnic Institute, where a voucher specimen (No. 18113) has been deposited for further reference.

Extraction

Powdered air-dried leaves of chard (550 g) were macerated with methanol (MeOH; 2 L) for 5 days at room temperature and dried using a rotatory evaporator to yield green gum. The chard extract efficiency was 36.13% (w/w). Chard extract (CH) dissolved in distilled water was used in in vitro experiments.

Evaluation of AGES in vitro

In vitro glycation of bovine serum albumin (BSA)

BSA (10 mg/mL) was incubated with fructose or glucose (0.5 M) in 0.1 M PBS, pH7.4 containing sodium azide (NaN_3; 0.02 %) under sterile conditions at 37 °C for 4 weeks with or without CH extract (1.0, 2.0 and 3.0 mg/mL) and aminoguanidine (AG, 2.0 mg/mL) was included as the positive control at 37 °C for 4 weeks [8]. Every week were collected samples of 0.5mL. After samples were undergoing dialysis for 48 h in PBS with a one-time exchange of PBS in 24 hours. The level of AGEs was determined by fluorimetry with a specific fluorescence (Ex 370 nm/Em 440 nm) by using a spectrofluorometer Perkin-Elmer LS50B.

Evaluation of fructosamine and nε-(carboxymethyl) lysine (cml)

The levels of fructosamine and Non-fluorescent AGEs, Nε-(carboxymethyl) lysine (Nε-CML), were evaluated by commercially available ELISA kit Assay (BioVision, CA, USA and Cell Biolabs, CA, USA)

Measurement of protein carbonyl

2,4-dinitrophenylhydrazine (DNPH; 10 mM) in 400 µL of 2.5 M HCl was incorporated into 100 µL of glycated BSA and incubated at room temperature for 60 min in the dark. After, was added 500 µL of trichloroacetic acid (20 % (w/v) and kept on ice for 5 min [9]. Then, was centrifuged at 4000 rpm for 15 min and the protein pellet was after washed with ethyl acetate/ethanol (1:1 v/v) and dissolved in guanidine hydrochloride (6 M). The absorbance was evaluated at 370 nm. The level of protein carbonyl was calculated using an absorption coefficient of 22,000 $M^{-1}cm^{-1}$. The date was expressed as nmol carbonyl/mg protein.

Measurement of protein thiol groups

5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB; 6 mM) was incubated with glycated BSA (10 µL) at room temperature for 15 min. The absorbance was evaluated at 410 nm. The free thiol level was calculated for the standard curve of l-cysteine (0.4–11 µM) and expressed as nmol/mg protein [10].

Advanced oxidation protein products of BSA (AOPP)

BSA was incubated with PBS alone (negative control C-) or chloramine T (20 mM, positive control C+) or vitamin C (10 mM; used as active control due it is an inhibitor of protein oxidation C*) for 60 minutes at 37°C. CH extract was added to a final concentration of 0.8/8 (v/v). The level of AOPP was evaluated to determine the degree of albumin oxidation by the Witko-Sarsat method [11]. The determination of AOPP is based on the reaction with potassium iodide (KI) in the presence of acetic acid. The derivatives were determined spectrophotometrically at 340 nm. A calibration curve should be generated using increasing concentrations of chloramine-T solution as a reagent for iodination.

Evaluation of protein aggregation

Amyloid cross β-structure is an important indicator for protein aggregation. Using a Congo red test was measured the effect of CH on protein aggregation [12]. Briefly, the glycated BSA (glucose; 100 µL) was incubated with 100 µL of 100 µM Congo red in 10% (v/v) ethanol/PBS for 20 min at 25°C. The absorbance was measured at 530 nm.

Measure on glycated GPx solution

We prepared a solution of GPx (10 mg mL^{-1}) in phosphate-buffered saline (PBS) at pH 7.4. Glucose solution (50 mMl^{-1}) was prepared. After an aliquot of GPx is taken and mixed with glucose solution and incubated for 16 weeks at 37 °C analyzed by fluorometry assay [13]. Measurement of GPx Function was performed by related activity assay kit (GPx (AB-102530; ABCAM, USA), enzymatic colorimetric method, The enzyme activity was measured as U mL−1.

Statistical analyses

Results from three independent experiments were analyzed and presented as mean ± SD For each set of experiments, statistical analysis was carried out by analysis of variance (one-way ANOVA) and using Tukey's post hoc test to evaluate significance. Data with $p < 0.05$ were considered significant.

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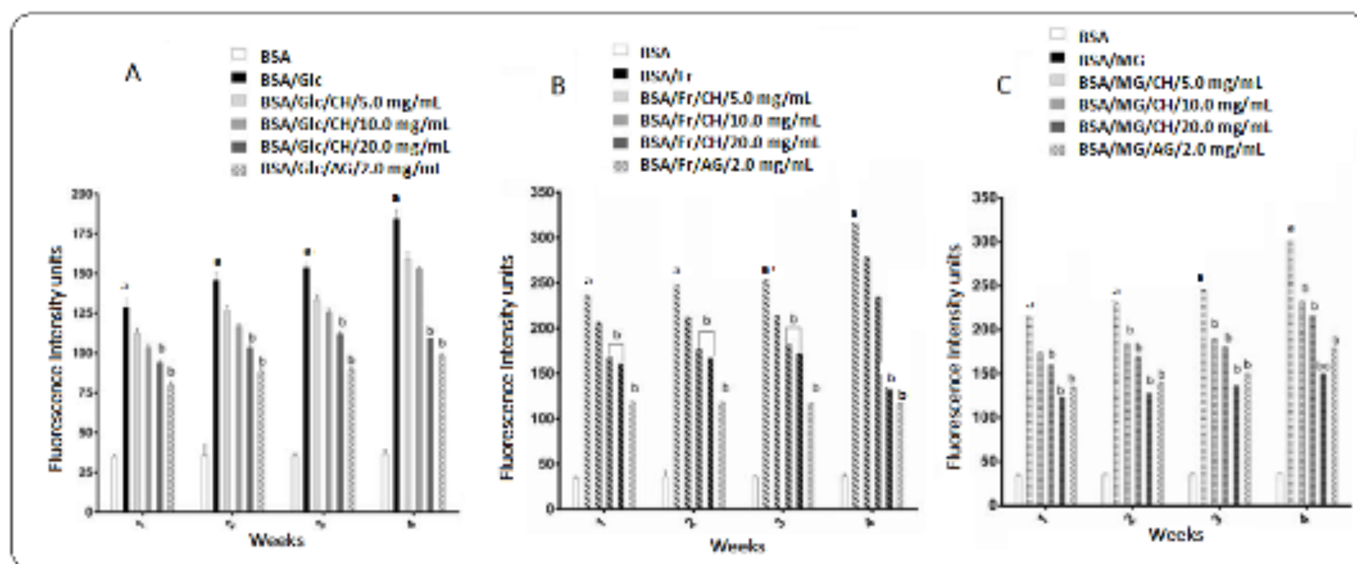
Results and Discussion

Several studies have been demonstrating the reactivity of glucose, fructose, and methylglyoxal with proteins, finding indicating the accumulation of faster cross-linking products, dicarbonyl compounds, and protein-bound fluorescence [14]. Thus, the formation of AGEs is crucial in diabetic complications [15]. resulting in scientific investigations focused on the development of novel agents with anti-glycation effects with the ability to prevent and alleviate diabetes complications. Due to the traditional usage of chard as antidiabetic and supported by in vivo experiments [7]. we studied CH extract as a potential agent against BSA glycation.

The fluorescent AGE formation was evaluated in BSA–glucose, BSA–fructose, and BSA–methylglyoxal systems used as antiglycation models and monitored throughout 4 weeks of incubation. As shown in Fig. 1A-1C was observed a significant ($p< 0.05$) increment in fluorescent intensity in BSA incubated with glucose, fructose, and methylglyoxal. The finding indicated that the fluorescent AGE formation was increased by 4.83-fold, 7.83-fold, and 8.10-fold respectively. Nevertheless, CH (5.0, 10.0, 20.0 mg/mL) decreased in a dose-dependent manner the formation of AGEs through the 4 weeks of incubation with a percentage inhibition of ranged from 28–36% in the BSA/glucose system, 36–45%, in BSA/fructose system, and 45–48% in BSA/methylglyoxal system. CH extract (20 mg/ml) in BSA/glucose system, and BSA/fructose system, have similar potent in the reduction of AGE formation when compared with AG standard at 2 mg/ml while in BSA/methylglyoxal antiglycation model CH extract showed a remarkable antiglycation effect, to those in the aminoguanidine.

Glycated proteins are mainly formed by the interaction of a nonenzymatic reaction between reducing sugars (glucose and fructose) and amino groups contained in the protein through a nucleophilic addition producing Schiff bases [16]. which spontaneously rearranges itself into an Amadori product. After a series of reactions take place such as fragmentation, dehydrations, new rearrangements, and oxidation-reduction reactions leading to the formation of AGE that forms a covalent crosslink between vessel-wall components, and cellular matrix proteins altering their function and structure. In addition, interacts with RAGE receptors, present on the surface of a diversity of cells forming endocytosis, pro-inflammatory events, prooxidant, cellular activation, or degradation cellular [17]. is known that not only ages are derived from glucose and fructose, but also reactive carbonyl species including methylglyoxal are important intermediates generated throughout the glycation of proteins by the glucose [18]. thereby the MG was included as a target in this investigation. According to the data obtained, CH extract inhibited the reaction between glucose, fructose, and methylglyoxal with BSA. The results demonstrated that CH extract efficiently inhibited fluorescent AGE formation. Nε-CML is reported to be the most comprehensively studied in AGE formation, in relation to biological and chemical properties. Schiff's base or Amadori product in the presence of reactive oxygen species can be fragmented to produce carbohydrate intermediates of short-chain to generate AGEs formation. After cleavage Amadori product into Nε-CML by endogenous H_2O_2 is carried out. This reaction is a defense response against an attack of the free radical on the proteins [19].

Fig. 1. The effects of CH (5.0, 10, and 20 mg/mL) and aminoguanidine (AG, 2 mM) on fluorescent AGEs formation in the BSA/glc, BSA/fr, and BSA/MG systems. Each value represents the mean ± DS (n=3). $^{a}p< 0.05$ when compared to BSA alone, $^{b}p< 0.05$ when compared to BSA incubated with glc, fr, and MG, at the same week of incubation.



The effect of CH extract on the level of fructosamine which is an early glycation product is shown in Fig. 2A to 2C. After 4 weeks of the experiment, the level of fructosamine incubated with glucose, fructose, and methylglyoxal systems produced a 28.3-fold, 4.5-fold, and 2.33-fold respectively increase compared to BSA. Findings demonstrated that CH extract (20 mg/mL) reduces the level of fructosamine

by about 26%, 35.5%, and 55.7% respectively. Fig. 3A to 3C displayed the effect of CH extract on protein-bound CML formation. Findings indicated that the formation of CML in BSA incubated with glucose, fructose, and methylglyoxal was significantly 2.90-times, 5.05-times, and 1.78-times respectively higher than only BSA. The addition of CH extract could decrease CML-derived AGE by approximately 47.4%, 62%, and 42.37%, when compared to BSA, incubated with glucose, fructose, and methylglyoxal respectively. However, the Inhibitory effect of CH extract was found to be slightly more efficient than GA used as a positive control in all cases.

The measure of fructosamine is mainly used to determine the accumulation of early glycation products (Amadori). The data indicated that the inhibition of fructosamine levels by CH extract is due to the reduction of the formation of AGEs. In addition, CH by inhibiting Amadori production, consequently, prevents conversion into AGEs. The Amadori products are capable of fragmenting producing superoxide anion to form non-fluorescent and fluorescent AGEs in the early stage of the glycation [20]. Clinically fructosamine is used in diabetic patients, as an indicator for short-term control of blood glucose [20]. While the generation of CML is mainly a characteristic compound of AGEs produced from the oxidative breakdown of Amadori products mediated by 3-deoxyglucosone, methylglyoxal, and glyoxal, among others [21]. The results demonstrated that CH extract efficiently inhibited non-fluorescent AGE formation.

Fig. 2. The antiglycative activity of CH (5.0, 10, and 20 mg/mL) on the fructosamine formation in BSA/glc, and BSA/fr, BSA/MG systems; AG, (positive control). Different superscript letters within each monitoring time indicate significant differences ($p<0.05$) among BSA and BSA/glc or BSA/fr or BSA/MG at the different tested concentrations

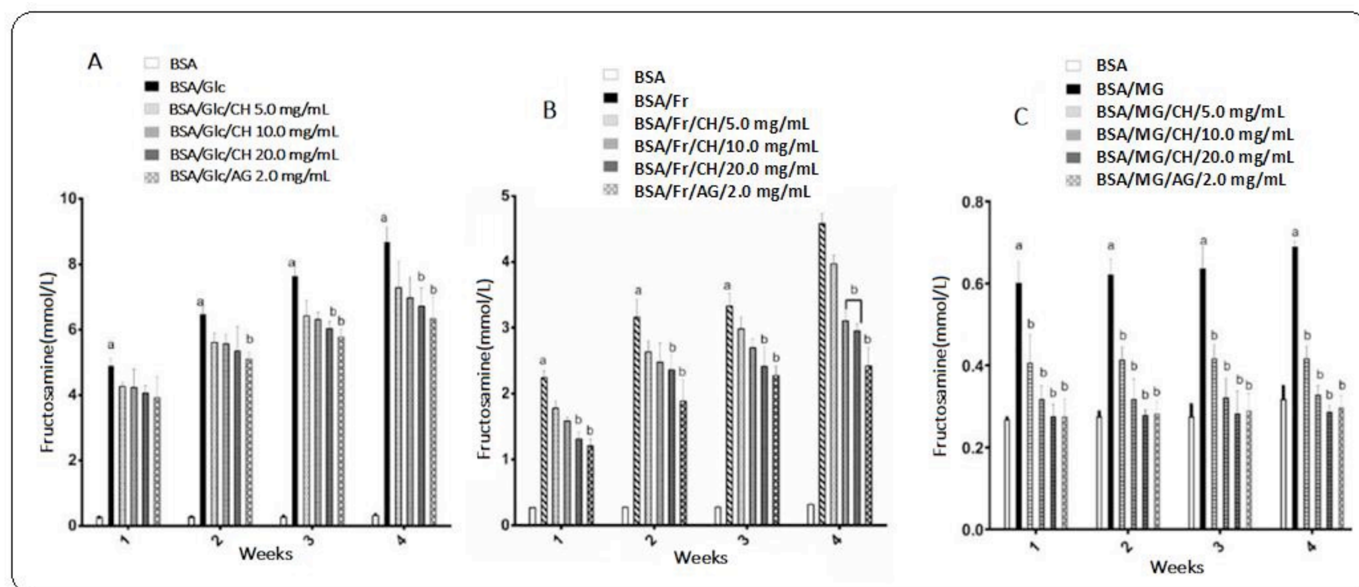
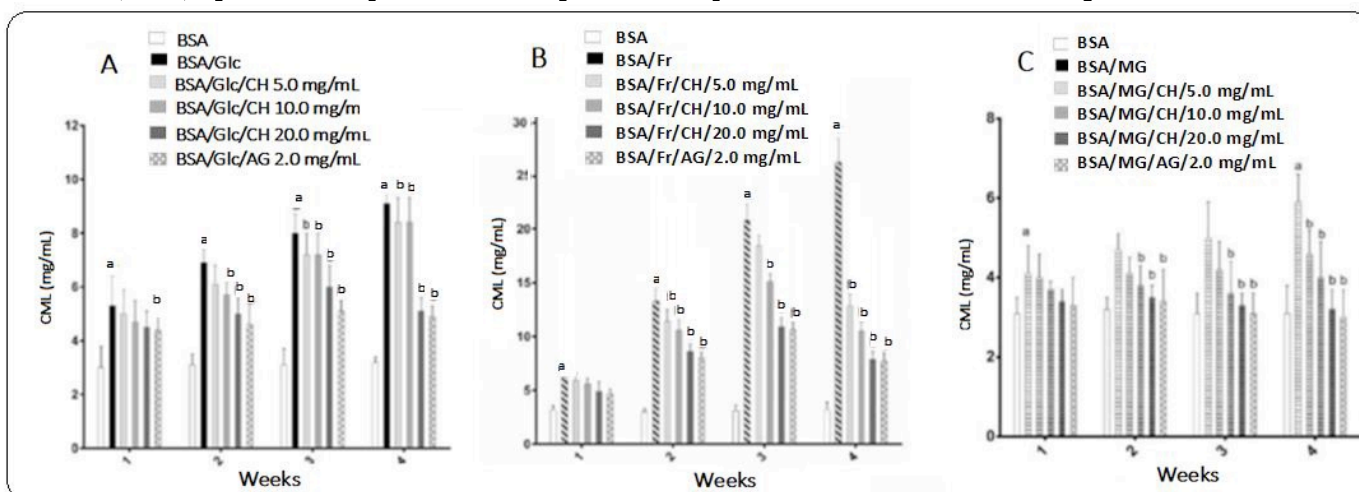


Fig. 3. The effects of CH (5.0, 10, and 20 mg/mL) and AG (2 mg/mL) on the level of Nε-(carboxymethyl) lysine (CML) in the BSA/glucose, BSA/fructose, and BSA/methylglyoxal system. Each value represents the mean ± DS (n = 3). [a]***p< 0.05*** **compared to BSA,** [b]***p***< **0.05 compared to BSA incubated with glc, fr, and MG**



Chronic generation of ROS causes oxidative injury to proteins resulting in depletion of the thiol group of protein and the introduction of carbonyl groups on the side chain of proteins [21]. The effect of CH on protein oxidation is shown in Tables 1-2. As indicated the Table 1, the carbonyl group content in glucose-glycated BSA, fructose-glycated BSA, and methylglyoxal-glycated BSA significantly increased 8.03-times, 14.5-times, and 16.38-times respectively throughout the experimental period, whereas CH extract (5–20 mg/mL) significantly repress an increase in protein carbonyl content in BSA incubated with glucose, fructose, and methylglyoxal systems to those in the glucose-glycated BSA. The percentage decrease of carbonyl content by CH extract (5–20 mg/mL) in the BSA-glucose system at week 4 ranged from 16.7% to 32.05%, while in the BSA-fructose system 17% to 39.78%, and 44.57% to 58.96% in BSA-methylglyoxal system. However, significant inhibition of protein carbonyl content was observed in the presence of AG (2 mg/mL; 45.93%, 50.66%, and 49.76% respectively) at week 4 of experimentation.

Table 1. The effects of CH extract and AG on protein carbonyl formation of glucose, fructose, and methylglyoxal modified BSA.

Carbonyl content [nmol/mg protein]				
Group (mg/mL)	Week 1	Week 2	Week 3	Week 4
BSA	0.23 ±0.016	0.25 ±0.001	0.25 ±0.002	0.26 ±0.001
BSA/Glc	1.28 ±0.108 [a]	1.58 ±0.137 [a]	1.83 ±0.150 [a]	2.09 ±0.150 [a]
BSA/Glc/CH/5.0	1.10 ±0.241 [a]	1.35 ±0.198 [a]	1.55 ±0.164 [a]	1.74 ±0.129 [a]
BSA/Glc/ CH/10.0	1.04 ±0.262 [a]	1.26 ±0.185 [a]	1.44 ±0.135 [a]	1.61 ±0.242 [a]
BSA/Glc/ CH/20.0	0.90 ±0.184 [a, b]	1.09 ±0.205 [a, b]	1.25 ±0.292 [a, b]	1.42 ±0.177 [a, b]
BSA/Glc/AG/2.0	0.77 ±0.28 [a, b]	0.89 ±0.137 [a, b]	1.02 ±0.139 [a, b]	1.13 ±0.131 [a, b]
Carbonyl content [nmol/mg protein]				
Group (mg/mL)	Week 1	Week 2	Week 3	Week 4
BSA	0.23 ±0.016	0.25±0.001	0.25±0.002	0.26±0.001
BSA/Fr	2.06 ±0.106 [a]	2.65 ±0.160 [a]	3.06 ±0.134 [a]	3.77 ±0.113 [a]
BSA/Fr/CH/5.0	1.79 ±0.108 [a]	2.27 ±0.145 [a]	2.59 ±0.236 [a]	3.13 ±0.275 [a]
BSA/Fr/ CH/10.0	1.53 ±0.141 [a]	1.91 ±0.158 [a]	2.17 ±0.27 [a]	2.60 ±0.213 [a, b]
BSA/Fr/ CH/20.0	1.42 ±0.142 [a]	1.72 ±0.130 [a, b]	1.90 ±0.255 [a, b]	2.27 ±0.494 [a, b]
BSA/Fr/AG/2.0	1.11 ±0.057 [a, b]	1.42 ±0.102 [a, b]	1.62 ±0.118 [a, b]	1.86 ±0.109 [a, b]
Carbonyl content [nmol/mg protein]				
Group (mg/mL)	Week 1	Week 2	Week 3	Week 4
BSA	0.23 ±0.016	0.25±0.001	0.25±0.002	0.26±0.001
BSA/MG	2.97 ±0.148 [a]	3.10 ±0.128 [a]	3.76 ±0.148 [a]	4.24 ±0.080 [a]
BSA/ MG / CH/5.0	2.05 ±0.106 [a, b]	2.11 ±0.146 [a, b]	2.23 ±0.152 [a, b]	2.35 ±0.140 [a, b]
BSA/ MG/ CH/10.0	1.61 ±0.135 [a, b]	1.68 ±0.132 [a, b]	1.87 ±0.152 [a, b]	1.95 ±0.152 [a, b]
BSA/ MG/ Fr/ CH/20.0	1.46 ±0.149 [a, b]	1.48 ±0.149 [a, b]	1.60 ±0.149 [a, b]	1.74 ±0.150 [a, b, c]
BSA/ MG/ AG/2.0	1.54 ±0.171 [a, b]	1.60 ±0.152 [a, b]	1.92 ±0.153 [a, b]	2.13 ±0.101 [a, b]
ap < 0.05 when compared to BSA alone and bp < 0.05, when compared to BSA incubated with glucose (BSA/Glc), fructose (BSA/Fr), or methylglyoxal (BSA/MG)				

Structural alteration of glycated BSA can be evaluated by depleting the thiol group. The activity of CH extract on the oxidation of protein thiols is shown in Table 2. When incubated BSA with glucose or fructose or methylglyoxal the level of thiol groups had continually reduced throughout the experimental 4 weeks. Then the addition of CH extract (5–20 mg/mL) and AG a significant increase in the level of thiol groups was observed. Results indicated in Table 2 the percentage of prevention of depleting thiol group ranged from 1.2-fold to 1.23-fold in BSA incubated with glucose 1.04-fold to 1.30-fold in BSA incubated with fructose and ranged from 1.10-fold to 1.36-fold in BSA incubated with methylglyoxal.

Table 2. The effects of CH extract and AG on thiol group content in glucose, fructose, and methylglyoxal modified BSA

Thiol groups [nmol/mg protein]				
Group (mg/mL)	Week 1	Week 2	Week 3	Week 4
BSA	1.19 ±0.012	1.17 ±0.031	1.00 ±0.014	0.98 ±0.013
BSA/Glc	0.87 ±0.016 [a]	0.81 ±0.015 [a]	0.75 ±0.014 [a]	0.72 ±0.004 [a]
BSA/Glc/CH/5.0	0.91 ±0.049 [a]	0.86 ±0.038 [a]	0.77 ±0.062 [a]	0.74 ±0.048 [a]
BSA/Glc/ CH/10.0	0.99 ±0.023 [a]	0.96 ±0.022 [a]	0.88 ±0.034 [a]	0.85 ±0.054 [a]
BSA/Glc/ CH/20.0	1.07 ±0.082 [a, b]	1.08 ±0.027 [a, b]	0.91 ±0.033 [a, b, c]	0.89 ±0.043 [a, b]
BSA/Glc/AG/2.0	1.10 ±0.036 [a, b]	1.11 ±0.075 [a, b]	0.94 ±0.069 [a, b]	0.91 ±0.025 [a, b]
Thiol groups [nmol/mg protein]				
Group (mg/mL)	Week 1	Week 2	Week 3	Week 4
BSA	1.19 ±0.012	1.17 ±0.031	1.00 ±0.014	0.98 ±0.013
BSA/Fr	0.82 ±0.018 [a]	0.77 ±0.012 [a]	0.72 ±0.012 [a]	0.69 ±0.008 [a]
BSA/Fr/CH/5.0	0.89 ±0.011 [a]	0.80 ±0.062 [a]	0.79 ±0.12 [a]	0.72 ±0.042 [a]
BSA/Fr/ CH/10.0	0.95 ±0.097 [a]	0.91 ±0.083 [a]	0.82 ±0.107 [a]	0.80 ±0.082 [a]
BSA/Fr/ CH/20.0	1.03 ±0.039 [a, b]	0.99 ±0.093 [a, b,]	0.90 ±0.052 [a, b]	0.90 ±0.053 [a, b]
BSA/Fr/AG/2.0	1.11 ±0.011 [a, b]	1.06 ±0.07 [a, b]	0.96 ±0.122 [a, b]	0.93 ±0.075 [a, b]
Thiol groups [nmol/mg protein]				
Group (mg/mL)	Week 1	Week 2	Week 3	Week 4
BSA	1.19 ±0.012	1.17 ±0.031	1.00 ±0.014	0.98 ±0.013
BSA/MG	0.80 ±0.013 [a]	0.77 ±0.084 [a]	0.71 ±0.072 [a]	0.69 ±0.082 [a]
BSA/ MG / CH/5.0	0.88 ±0.148 [a]	0.87 ±0.126 [a]	0.77 ±0.020 [a]	0.76 ±0.069 [a]
BSA/ MG/ CH/10.0	0.96 ±0.083 [a]	0.96 ±0.098 [a]	0.87 ±0.069 [a]	0.87 ±0.026 [a]
BSA/ MG/ Fr/ CH/20.0	1.13 ±0.113 [a, b]	1.00 ±0.134 [a, b]	0.98 ±0.090 [a, b]	0.94 ±0.038 [a, b]
BSA/ MG/ AG/2.0	1.06 ±0.106 [a, b]	0.98 ±0.127 [a, b]	0.96 ±0.098 [a, b]	0.88 ±0.169 [a, b]

[a]$p < 0.05$ when compared to BSA alone and [b]$p < 0.05$, when compared to BSA incubated with glucose (BSA/Glc), fructose (BSA/Fr), or methylglyoxal (BSA/MG

The process of oxidative degradation in Amadori products produces free radicals, causing injury due to oxidation of the proteins, in consequence, protein carbonyl content and thiol groups are considered an oxidative modification of BSA and were used for evaluating protein oxidation in the glycation process [22]. The reactive oxygen species are produced during glycoxidation and glycation and oxidize side chains of the amino acid [23]. In this investigation, a significant increase in the oxidation of thiols and protein carbonyl formation in BSA incubated with glucose, fructose, and methylglyoxal was observed. Results demonstrated that the addition of CH extract significantly reduced the oxidation of sulphydryl and protein carbonyl formation suggesting that antioxidant phytochemicals content in CH extract scavenging free radicals and reactive carbonyl group formation.

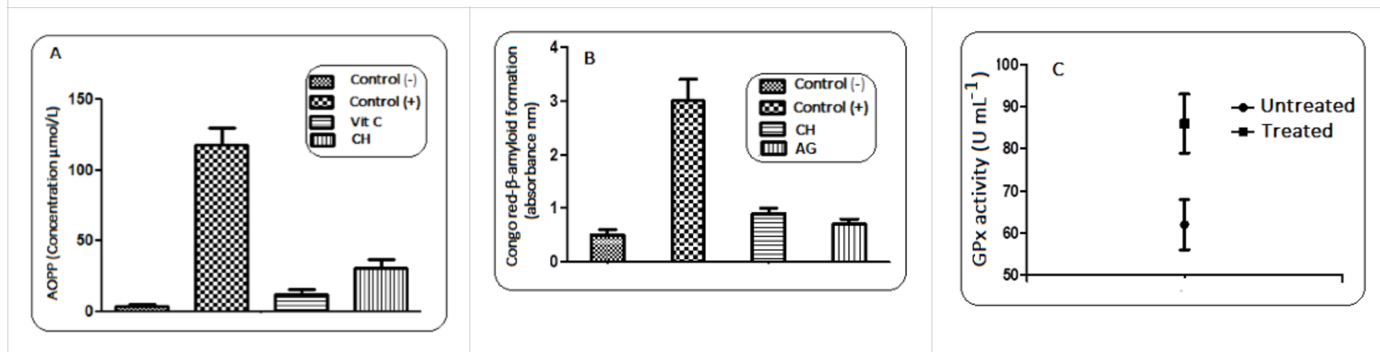
The level of AOPP as evaluated with spectrophotometry was in a positive control C (+) 28.5-fold higher than in the negative control C (-) after 60 minutes of incubation. While CH extract and vit C inhibits the oxidation of BSA by 73.7% and 89.83% then 60 minutes of incubation respectively.

The difference between CH and vitamin C was statistically significant at 60 minutes of incubation time ($p < 0.001$). The formation of AOPP and AGEs resulting from oxidative stress and hyperglycemia chronic trigger metabolic disturbances including diabetes. Both are the main protagonists in the complications of diabetes and contribute to the DVLC including inflammation, dyslipidemia, and hypertension [24]. The results demonstrated that CH extracts displayed strong reduction effects on the oxidation process, while the reduction effect of vitamin C after 60 minutes of incubation was more pronounced.

In glycated BSA the fluorescence intensity in Congo red test was significantly increased compared with the negative control, indicating the formation of amyloid-β structure in BSA. Findings obtained in this investigation demonstrated that CH extract showed the strongest inhibition of 70% amyloid-β aggregation like AG (76.6%), results are displayed in Fig. 4A-B. The findings obtained for inhibition of amyloid-β structure formation agree with the results obtained from the early and late stages of glycation resulting in inhibition in the amyloid-β formation [25].

Fig. 4C shows the effect of CH extract on glycated GPx structure using fluorimetry analysis. Considering that protein denaturation is a process of enzyme glycation, CH extract treatment improves this process by 26% compared with the untreated group. The decrease in the fluorescence intensity produced by CH extract treatment could possibly be caused by a change in amino acids and modifications in the secondary and tertiary structures neighboring these amino acids. These variations changed the denatured protein (GPx) towards their native structure and as consequence, the enzymatic activity is increased.

Fig 4. (A) The effects of CH on the concentration of AOPP after 60 minutes of oxidation were determined spectrophotometrically. (B) The effect of CH extracts on the in vitro formation of amyloid-β products in BSA glycation using Congo red test. Each value represents the mean ± DS (n = 3). All results were statistically significant at $p < 0.001$ compared to control; (C) effect of CH extract on glycated GPx structure.



Conclusion

Beta vulgaris var. cicla methanol leaf extract effectively inhibits monosaccharide-induced AGE formation, methylglyoxal-induced AGE formation, BSA-carbonylation, and oxidation of sulphydryl group. In addition, findings demonstrated that CH extract is effective in decreasing CML formation related to reduced levels of fructosamine and displaying potent inhibition of amyloid-β aggregation when compared to aminoguanidine, the results suggested that the mechanism of CH extract for inhibiting protein glycation may be associated with their antioxidant properties. However, this is not the only reason to explain the anti-glycation mechanism of CH extract. Also can be proposed, a breaking the cross-linking structures in AGEs blocking Schiff bases, the carbonyl groups in reducing sugars, and inhibiting the formation of late-stage Amadori products. CH extract demonstrated a high antiglycation effect that can be useful as a preventive treatment of AGEs in diabetic complications

CRediT authorship contribution statement

RMPG: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. JMMF: Resources, Writing - review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

References

1. Monnier VM. Glycation products as markers and predictors of the progression of diabeticcomplications. Ann. NY. Acad. Sci. 2005;1043(1):567–581.
2. Katayama S, Haga Y, Saeki H. Loss of filament-forming ability of myosin by non- enzymatic glycosylation and its molecular mechanism. FEBS Lett. 2004;575(1-3):9-13
3. Bourajjaj, M, Stehouwer C.D, Van Hinsbergh V.W, Schalkwijk C.G. Role of methylglyoxal adducts in the development of vascular complications in diabetes mellitus. Biochemi. Soci. Trans. 2003; 31(6):1400-1402.
4. Adisakwattana S, Jiphimai P, Prutanopajai P, Chanathong B, Sapwarobol S, Ariyapitipan, T. Evaluation of α-glucosidase, α-amylase and protein glycation inhibitory activity of edible plants. International Journal Food Sci. Nutr. 2010;61(3):295–305.
5. Mohammed HS, Abdel-Aziz MM, Abu-Baker MS, Saad AM, Mohamed MA, Ghareeb MA. Antibacterial and potential antidiabetic activities of flavone c-glycosides isolated from *Beta vulgaris* subspecies cicla L. var. Flavescens (Amaranthaceae) cultívate in Egypt. Curr. Pharm. Biotech. 2019;20(7):595-604. doi: 10.2174/1389201020666190613161212.
6. Ninfali P, Antonini E, Frati A, Scarpa ES. C-Glycosyl Flavonoids from *Beta vulgaris* Cicla and betalains from *Beta vulgaris* rubra: antioxidant, anticancer and anti-inflammatory activities-A Review. Phytother. Res. 2017;31(6):871-884. doi: 10.1002/ptr.5819.
7. Al-Harbi LN, Alshammari GM, Al-Dossari AM, Subash-Babu, P, Binobead MA, Alhussain MH, et al. *Beta vulgaris* L. (Beetroot) Methanolic Extract prevents hepatic steatosis and liver damage in t2dm rats by hypoglycemic, insulin- sensitizing, antioxidant effects, and upregulation of PPARα. Biology (Basel). 2021;10(12):1306-1311. doi: 10.3390/biology10121306.
8. Povichit N, Phrutivorapongkul A, Suttajit M, Chaiyasut C, Leelapornpisid P. Phenolic content and in vitro inhibitory effects on oxidation and protein glycation of some Thai medicinal plants. Pakistan J. Pharm. Sci. 2010;23(4):403–408.
9. Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol.1990;186:464–478.
10. Ellman GL. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 1959;82:70–77
11. Witko-Sarsat V, Friedlander M, Capeillère-Blandin C, Nguyen-Khoa T, Nguyen AT, Zingraff J, et al. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. Kidney Intern. 1996;9(5):1304–1313.

12. Bouma B, Kroon-Batenburg LM, Wu YP, Brünjes B, Posthuma G, Kranenburg O de Groot PG, Voest EE, Gebbink MF. Glycation induces the formation of amyloid cross-β structure in albumin. J. Biol. Chem. 2003;278(43):41810–41819
13. Rowan S, Bejarano E, Taylor A. Mechanistic targeting of advanced glycation end-products in age-related diseases. Biochim. Biophys. Acta Mol. Basis Dis. 2018; 1864(12):3631-3643. doi: 10.1016/j.bbadis.2018.08.036.
14. Meeprom A, Sompong W, Chan CB, Adisakwattana S. Isoferulic acid, a new anti-glycation agent inhibits fructose- and glucose-mediated protein glycation in vitro. Molecules. 2013;18(6):6439–6454
15. Singh VP, Bali A, Singh, N, Jaggi AS. Advanced glycation end products and diabetic complications. Korean J. Physiol. Pharm. 2014;18(1):1–14. doi. 10.4196/kjpp.2014.18.1.1.
16. Rondeau, P., Bourdon, E. The glycation of albumin: structural and functional impacts. Biochimie. 2011; 93(4):645–658.
17. Grzebyk E, Knapik-Kordecka M, Piwowar A. Advanced glycation endproducts and cathepsin cysteine protease in type 2 diabetic patients. Polska Arch. Med. Wewn. 2013;123(3):364–370.
18. Nohara Y, Ususi T, Kinoshita T, Watanabe M. Generation of superoxide anions during the reaction of guanidine compounds with methylglyoxal. Chem. Pharm. Bull. 2002;50(1):179–184.
19. Wu CH, Huang SM, Lin JA, Yen GC. Inhibition of advanced glycation endproduct formation by foodstuffs. Food Funct. 2011;2:24–234. doi. 10.1039/C1FO10026B
20. Fujiwara Y, Kiyota N, Motomura K, Mera K, Takeya M, Ikeda T, Nagai R. Some natural compounds enhance Nε-(carboxymethyl)lysine formation. Ann. NY. Acad. Sci. 2008;1126:52–154
21. Ardestani A, Yazdanparast R. *Cyperus rotundus* suppresses AGE formation and protein oxidation in a model of fructose-mediated protein glycoxidation. Intern. J. Biol. Macromol. 2007;41(5):572–578.
22. Simó-Servat O, Planas A, Ciudin A, Simó R, Hernández C. Assessment of advanced glycation end-products as a biomarker of diabetic outcomes. Endocrinol. Diabetes Nutr. (England). 2018;65(9):540-545. doi: 10.1016/j.endinu.2018.06.003
23. Shen Y, Xu Z, Sheng Z. Ability of resveratrol to inhibit advanced glycation end-product formation and carbohydrate hydrolyzing enzyme activity, and to conjugate methylglyoxal. Food Chem. 2017; 216:153–160
24. Peppa M, Uribarri J, Vlassara H. Glucose, advanced glycation end products, and diabetes complications: What is new and what works. Clinical Diabetes. 2003, 21,186–187.
25. Rahmanifar E, Miroliaei M. Differential effect of biophenols on attenuation of AGE-induced hemoglobin aggregation. Intern. J. Biol. Macromol. 2020;151:97– 805.

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