

Polyphenols of leaves of *Apium graveolens* inhibit in vitro protein glycation and protect RINm5F cells against methylglyoxal-induced cytotoxicity

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ABSTRACT

Background: The health benefits of edible plants have been widely investigated and disseminated. However, only polyphenols have been found to have sufficient therapeutic potential to be considered in clinical trials. Fewer manuscripts have other applications such as prospective health benefits and disease treatment. Other components of edible plants are responsible for a range of other benefits including antimalarial, burns, flu, cancer, inflammation, diabetes, glycation, antimicrobial, prevention of neurodegeneration, analgesic, antimigraine activity, sedative activities, etc. Accordingly, the public needs to be informed of the potential edible plants have to act on different targets and maintain better control over diabetes compared to commercial drugs which can be toxic, have side effects, do not have the capacity to maintain blood glucose at normal levels, and do not protect the patient from the complications of diabetes over time. Consequently, edible plants, such as *Apium graveolen*, which have therapeutic targets on AGEs formation, are potentially a better alternative treatment for diabetes.

Aim: To demonstrate if CM is capable of suppressing the formation of AGEs and protein oxidation *in vitro*. Also to investigate if *A. graveolens* is a potential functional food that can be used to prevent the development of diabetic complications.

Methods: The leaves of celery were extracted with methanol (CM). Polyphenols contents in CM were investigated by liquid chromatography-electrospray ionization mass. The ability of the compounds to inhibit formation of AGEs was evaluated in vitro models using formation of AGE fluorescence intensity, level of fructosamine, N ϵ -(carboxymethyl)lysine (CML), methylglyoxal (MG)-derived protein, and formation of amyloid cross β structure. Protein-oxidation was determined by thiol group and protein carbonyl content. Inhibition of MG-derived AGEs and MG-trapping ability were also measured. Additionally, insulin production was determined in methylglyoxal-treated pancreatic RINm5F cells assay.

Results: Apigenin, kaempferol, apiin, rutin, caffeic acid, ferulic acid, chlorogenic acid, coumaroylquinic acid, and p-coumaric acid were the major polyphenols contained in CM. In all the model tests CM displayed potent AGE inhibitory activity, suggesting that CM delayed the three stages of glycation. Accordingly, the mechanisms of action of celery involving dicarbonyl trapping and breaking the crosslink structure in the AGEs formed may contribute to the protection of pancreatic RINm5F cells against MG conditions.

Conclusion: These findings indicate that CM have an excellent anti-glycation effect which may be beneficial for future development of antiglycating agents for the treatment of diabetes.

Keywords: *Apium graveolens*, anti-glycation, polyphenols methylglyoxal, insulin, pancreatic cells

BACKGROUND

Advanced glycation end products (AGEs) are irreversible and stable compounds produced in the glycation process, which is carried out by non-enzymatic reactions between free amino groups and reducing sugars. Then, AGEs react with other free amino groups that generate protein modification which are produced in order to change both their biochemical and physiochemical characteristics [1]. The accumulation of AGEs plays an important role in the pathology of diabetes complications [2]. The interaction of the receptor of advanced glycation endproducts (RAGE) with AGE form reactive oxygen species (ROS) generating oxidative stress and thereby causing vascular inflammation [3] and DNA damage with the induction of cell apoptosis [3]. Fructose and glucose are the most common reducing sugar in blood circulation which reacts with amino groups of proteins to produced AGEs. For example, in a fructose experiment, protein glycation reacted more quickly than glucose [4]. Due to the finding about the adverse effects of AGEs in diabetes, the use of anti-glycating agents have recently been associated with significant approaches in preventing age-related symptoms of this disease.

An important dicarbonyl metabolite derived from glucose metabolism is methylglyoxal (MG), which forms advanced glycation end products (AGEs) after reacting with proteins, all of which are implicated in the pathogenesis of diabetes complications [5]. From previous studies, incubation of insulin *in vitro* with MG produced changes in the insulin molecule and impaired insulin-

mediated glucose uptake in adipocytes [6]. The high concentration of carbohydrate caused production of MG to induce cumulative changes which increase development of type 2 diabetes and insulin resistance.

Apium graveolens L. (Celery) is a biennial plant and a commercially important spice belonging to the family Apiaceae. Celery is used as a fresh plant, seeds, stalk, oleoresin, and oil to flavor foods. Celery extracts are reported to possess many pharmacological properties, such as attenuating liver enzymes and improving the lipid profile in cholesterol-fed diets [7]. In another study, the methanolic extract of *A. graveolens* seed demonstrated a protective effect on liver damage [8] and also inhibited the oxidant process due to the constituents derivatives of methoxyphenyl chromenone and L-tryptophan [9]. Nonpolar extract of root and bulbs of *A. graveolens* demonstrated a significant cytotoxicity effect [10]. Apiuman, a pectic polysaccharide isolated from *A. graveolens*, increased interleukin-10 production, decreased the interleukin-1 β , and diminished the neutrophils migration, causing anti-inflammatory activity [11]. The antidiabetic, lipid profile, and antiglycation effect of the chloroform extract of the celery leaves was determined in previous studies [12]. In another study, we discovered that the hydroalcoholic extract of celery decreased low-density lipoprotein, triglycerides, and cholesterol [13]. The mechanism of this hypocholesterolemic effect is due to the content of compounds with sugar/amino acid moiety in the extract which causes an effect on bile acid secretion [14]. In the present study, we investigated the effects of CM on the production of AGEs through three stages: the early, intermediate, and late of formation of AGEs [15].

METHODS

Chemicals and reagents

Cloruro de nitroblue tetrazolium (NBT), bovine serum albumin (BSA), aminoguanidine, glucose, methylglyoxal (MG), apigenin, luteolin, kaempferol, apiin, rutin, caffeic acid, ferulic acid, p-coumaric acid, dimethylsulfoxide (DMSO), trichloroacetic acid (TCA), guanidine hydrochloride, 2,4-dinitrophenylhydrazine (DNPH), and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were obtained from Sigma (St. Louis, MO, USA). OxiSelect™ N ϵ -(carboxymethyl) lysine (CML) ELISA kit was purchased from Cell Biolabs (San Diego, CA, USA). Insulin-secreting β -cells line (RINm5F) were acquired from the American type culture collection (ATCC). RPMI médium (Difco, Mexico). All other chemicals used were of the analytical grade from Fermont (Los Angeles, CA, USA).

Plant material

Fresh celery was collected in Amecameca, Mexico State. Voucher specimen (6543) is kept in the Herbarium of Escuela Nacional de Ciencias Biologicas-IPN. Fresh materials for the celery (stalk and leaves) were cut into small pieces and dried at 50 °C for 4 h and then plant materials were finely powdered and passed through a mesh #40-60 sieve prior to extraction [16].

Plant extraction

1 kg of the sample's powder was transferred into a 10-L reaction vessel and then 4 l of methanol was added. The mixture was refluxed for 3 h and filtered using Whatman filter paper [17, 18]. The

extraction was repeated twice. The filtrates were combined and concentrated into dryness using a rotary evaporator at 40-50°C. The crude extract (CM) was weighed, kept in tubes wrapped with aluminium foil to reduce the risk of oxidation, and stored in deep freeze.

Characterization of phenolic by liquid chromatography-electrospray ionization mass (LC/ESI-MS)

The methanol extract was subjected to Ultimate 3000 C18 semi prepared column (10 mm x 150 mm; DIONEX, USA), and eluted with a gradient mobile phase of methanol:ethyl acetate(1: 9) → methanol:ethyl acetate (1:1) → methanol at a flow rate of 30 ml h⁻¹, in the period of 60 minutes, being monitored at 215 nm. Fractions were checked by silica gel thin-layer chromatography (TLC) and eluted with ethyl acetate : methanol (9 : 1) after the TLC was visualized at UV 365 nm and sprayed with a solution of AlCl₃ in ethanol.

LC/ESI-MS (Agilent 1100 HPLC) was used for phenolic compounds identification using a reversed-phase BDS Hypersil C18 (3 urn particle size; 150 x 2.1 mm, i.d.) column. The solvent system was a mixture of water, acetonitrile (A), and formic acid (B), with the following gradient of A: 0 min, 20%; 10 min, 25%; 15 min, 100%. Elution was carried out with the solvent flow rate of 0.3 ml min⁻¹. Mass spectra were acquired using electrospray ionization in the positive (PI) mode, at low (70 V) and high fragmentation voltages (250 V) for ionization mode. The mass spectra were recorded for the range of *m/z* 100-800.

In Vitro Glycation of BSA

The inhibition of protein glycation was measured by a spectrofluorometer (BIO-TEK, Synergy HT, USA). Before the assay, all the solutions were filters in sterile plastic-capped vials through 0.22 µm membrane filters to maintain sterility and strict asepsis during the entire process. Glycated samples were prepared by incubating 10 mg/ml of bovine serum albumin (BSA) with glucose (250 mM/ml) in 1 ml of phosphate buffer (PBS), pH 7.4 containing 0.02% sodium azide (NaN₃) for 4 weeks at 37 °C in the absence or presence of CM (1.0, 1.5, 2.0, 2.5, 3.0 mg/ml) or aminoguanidine (AG, 1.0 mg/ml). Positive control (BSA + glucose) was maintained under similar conditions and all the incubations were performed in triplicates. Dimethylsulfoxide (DMSO, 4%) was used as a solvent for this study. At the end of the incubation period, all the samples were verified to be free of microbiological contamination. The appraisal of the anti-bacterial effect was performed using the Agar well diffusion method [19]. The unbound glucose from all of the samples was removed by dialysis against the phosphate buffer (200 mM, pH 7.4). Aliquots of the reaction mixtures were then assayed on a spectrofluorometric detector at excitation and emission wavelengths of 350 and 450 nm respectively [20]. The antiglycation potential of CM was determined by estimation of seven parameters from dialysates such as fructosamines, AGEs, protein carbonyls, Nε-(carboxymethyl) Lysine (CML), Thioflavin T (Th, T), binding of congo red, and protein thiols.

Formation of Fructosamine Adduct

The levels of fructosamine adduct was measured by nitro blue tetrazolium (NBT) assay [21]. For this purpose, 10 µl of glycated BSA and positive control in carbonate buffer (pH 10.3) was added to 90 µl of 2.5 mM NBT reagent. After 30 min of incubation at 37°C, the mixture was

measured at 530 nm. Pyridoxamine (Pyr) was used as a positive standard. The concentration of fructosamine was calculated using the following equation:

$$\text{Inhibitory activity (\%)} = [(A_0 - A_1)/A_0] \times 100$$

A_0 is the absorbance value of the positive control at 530 nm

A_1 is absorbance of the glycated albumin samples coincubated with triterpenes at 530 nm.

Determination of N ϵ -(carboxymethyl) lysine (CML)

N ϵ -(carboxymethyl) lysine (CML), a major antigenic AGE structure, was determined using an enzyme linked immunosorbant assay ELISA kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's protocol. The absorbance of dialysates samples was compared with the CML-BSA standard provided in the assay kit.

Determination of Protein Carbonyl Content

The carbonyl group in glycated BSA, a marker for protein oxidative damage, was estimated according to the method of Levine *et al.* [22]. Briefly, 400 μ l of 10 mM DNPH in 2.5 M HCl was added to 100 μ L of glycated samples. After 1 h incubation in the dark, 500 μ l of trichloroacetic acid (TCA) to the 20% (w/v) was used for protein precipitation (5 min on ice) and then centrifuged at 10,000 g for 10 min at 4 °C. The protein pellet was washed with 500 μ l of ethanol/ethyl acetate (1:1) mixture three times and resuspended in 250 μ l of 6 M guanidine hydrochloride. The absorbance was measured at 370 nm. The carbonyl content of each sample was calculated based on the extinction coefficient for DNPH ($\epsilon = 22,000 \text{ M}^{-1}\text{cm}^{-1}$). The results were expressed as nmol carbonyl/mg protein.

Thiol Group Estimation

The free thiols in glycated albumin samples were measured according to Ellman's assay using DTNB [23]. Briefly, 70 μ l of glycated samples were incubated with 5 mM of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in 0.1 M PBS, pH 7.4, at 25 °C for 15 min. The absorbance of samples was measured at 410 nm. The free thiol concentration was measured using a standard curve carried out with standard BSA concentrations (0.8 to 4 mg/ml, corresponding to 19–96 nmol total thiols). The results were expressed as % protection to protein thiols by comparison with the positive control.

Thioflavin T (Th, T) Assay

Thioflavin T, a marker for the amyloid cross- β structure, was assayed according to the method of Tupe *et al.* [24]. For this purpose, glycated samples (10 μ l) was added to 100 μ l of 64 μ M thioflavin T in 0.1 M PBS, pH 7.4 and incubated at 25 °C for 1 h at room temperature. The fluorescence intensity was measured at excitation wavelength of 435 nm and emission wavelength of 485 nm with correction for back ground signals without Th.T. The results were expressed as % inhibition measured by the formula:

$$\text{Inhibition \%} = [(F_0 - F_1) / F_0] \times 100$$

F₀ is the florescence of the positive control

F₁ is the florescence of the glycated albumin samples coincubated with MC

Binding of Congo Red

Congo red assay is a marker for the amyloid cross β structure that was also estimated by measuring absorbance at 530 nm [25]. Briefly, 500 μ l of glycated samples were incubated with 100 μ l of Congo red (100 μ M in phosphate buffer saline (PBS) in ethanol (10 % v/v) for 20 min at 25 °C. Absorbance was recorded for the congo red incubated samples in addition to the congo red background. The data were expressed as % inhibition calculated by the following formula:

$$\text{Inhibition \%} = [(A_0 - A_1) / A_0] \times 100$$

A₀ is the absorbance at 530 nm of positive control

A₁ is the absorbance at 530 nm of the glycated albumin samples coincubated with MC

The Formation of Methylglyoxal-derived AGE

In the investigation of an inhibitor on the middle stage of the glycation of protein, the MG trapping capacity was estimated according to the method described by Peng *et al.* [26]. For this purpose, 0.5 ml of BSA (20 mg/ml) was incubated with 0.4 ml of 2.5 mM MG and 0.5 ml of CM (0.5-2.0 mg/ml) or AG (1.0 mg/ml) as a positive control in 0.1 M phosphate buffer saline (PBS), pH 7.4 at 37°C for 7 days. After incubation, the fluorescence intensity was measured at the excitation wavelength of 370 nm and an emission wavelength of 420 nm. The percentage inhibition of MG-derived AGE formation was calculated as the following formula:

$$\text{Inhibition of MG-derived AGEs (\%)} = [((FC - FCB) - FS - FSB) / (FC - FCB)] \times 100$$

FCB were the fluorescent intensity of control with MG and blank of control without MG.

FS and FSB were the fluorescent intensity of sample with MGO and blank of sample without MG.

Assay for methylglyoxal-trapping ability by high performance liquid chromatography (HPLC)

MG (1 mM) was mixed with CM (1 mg/mL) or aminoguanidine (1 mg/mL) in the phosphate buffer solution (pH 7.4) and was incubated at 37 °C for 1 day. Quantification of MG was founded on the measure of its derivative compound, 2-methylquinoxaline (2-MQ) using 5-methylquinoxaline (5-MQ) as the internal standard in HPLC. The mixture containing 5 mM 5-MQ (100 μ L) and 20 mM o-phenylenediamine (100 μ L) was added immediately after incubation into the sample vials. After 30 min, the samples were filtered at room temperature and ready for HPLC analysis. The remaining MG in the samples was quantified by HPLC (Shimadzu Corp., Kyoto, Japan) equipped with a SPD-10A UV-vis detector, LC-10 AD pump, and LC-Solution software. AC 18 column (250 \times 4.6 mm i.d.; 5- μ m particle size) (Inertsil ODS 3 V) was used for 2-MQ analysis. The column temperature was kept at room temperature. The mobile phase for the HPLC consisted of HPLC

grade methanol (solvent A) and water (solvent B) with a constant flow rate set at 1.3 mL/min. Subsequently, aliquots of 10 μ L were subjected to HPLC assay. An isocratic program was performed with 70 % solvent A and 12-min running time per sample. The 2 and 5-MQ was monitored at 315 nm. Peak integrality ratios of 2-MQ to 5-MQ were used for quantitative analysis. Using the standard curve of 2-MQ/5-MQ was calculated the amount of MG [27]. The percentage of MG trapping was determined using the equation:

$$\% \text{ MG trapping} = \frac{\text{Amount of MG in control} - \text{MG in CM}}{\text{Amount of MG in control}} \times 100$$

Cell culture

RINm5F cells were grown under standard cell culture conditions humidified atmosphere with 5% CO₂ at 37 °C) in a RPMI medium containing 100 μ g/mL streptomycin, 100 U/ml penicillin, and 10% fetal bovine serum (FBS).

Assay MG on cell viability of RINm5F cells

RINm5F cells (3 x 10⁴ cells/well) were seeded on 24-well plates and treated with CM (100, 250, and 500 μ g/mL) or MG (10 μ g/ml) for 24 h. Afterwards, the medium was removed, washed with phosphate-buffered saline (PBS), and stained with crystal violet (200 mg/ 100 ml) for 20 min in phosphate-buffered formaldehyde. The crystal violet bound to the cells was dissolved in 20 g/l sodium dodecylsulfate (SDS) solution, and its absorbance was measured at 570 nm [28].

Insulin production in methylglyoxal-treated pancreatic RINm5F cells

RINm5F cells were treated MG (10 μ M) without or with CM (100, 250, 500 μ g/ml) and stained using an anti-insulin antibody for 1 h, followed by incubation with a secondary anti-rabbit FITC antibody for another 1 h. The insulin levels in RINm5F cells were then measured using flow cytometry.

Statistical Analysis

Data were expressed as means \pm standard error of mean (S.E.M), N=3. Data were analyzed using one way ANOVA followed by Tukey's HSD post hoc test. A *p*-value <0.05 was considered to be statistically significant.

RESULTS

Identification of phenolic compounds

Data from the LC-DAD-ESI/MS were used to identify the five phenolic compounds and five flavonoids that were the most abundant in CM . A comparison of the UV, Rt, and MS data with those known standards established that apigenin, luteolin, kaempferol, apiin, rutin, caffeic acid, ferulic acid, chlorogenic acid, coumaroylquinic acid, and p-coumaric acid are the major phenolic acids and flavonoids contained in celery [29]. After, verification was carried out by spiking each of the standards with the extract. The identification was verified in each case. The date obtained are similar to those compounds from celery [30].

AGEs inhibition activity

The formation of AGEs was measured by the fluorescence intensity of the BSA-glucose solutions. As shown in Figure 1, when concentrations of CM at 1.0, 1.5, 2.0, 2.5, 3.0 mg/ml was added to the BSA/glucose system, a significant reduction in the fluorescence intensity to concentration-dependent was observed at the end of week 4 of incubation. The percentage inhibition of AGEs formation by CM at the concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 mg/ml was 40, 48.9, 56.2, 66, 79.3 % respectively. However, aminoguanidine (1 mg/ml) inhibited the formation of AGEs in BSA/glucose system by 79.9%.

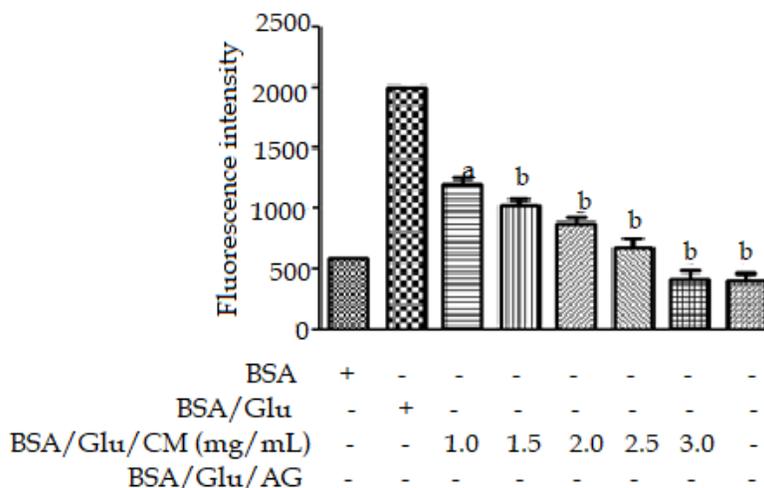


Figure 1. The effects of CM at concentrations of 1-3.0 mg/ml on formation of fluorescent AGE at 4 weeks of incubation. The results are expressed as mean ± SEM (n = 4). ^ap < 0.01 when compared to BSA and ^bp < 0.05 when compared to BSA/Glu. Aminoguanidine (AG).

Effect of CM on formation of fructosamine

Schiff’s bases are formed in early stages of glycation and transformed to Amadori products as fructosamine. Glycation of BSA with a monosaccharide significantly increased the level of fructosamine after four weeks of study. At concentrations of CM at 1.0, 1.5, 2.0, 2.5, and 3 mg/mL the formation of fructosamine was inhibited in BSA/Glucose by 52%, 60%, 68%, 72% and 77%, respectively, compared with pyridoxamine as the positive control (58.3%). Phenolic compound like pyridoxamine, may inhibit multiple stages of AGEs formation. This is in contrast with other inhibitors, such as aminoguanidine, which completely inhibited AGEs but not Amadori formation. Inhibition of the formation of Amadori product is shown in Figure 2.

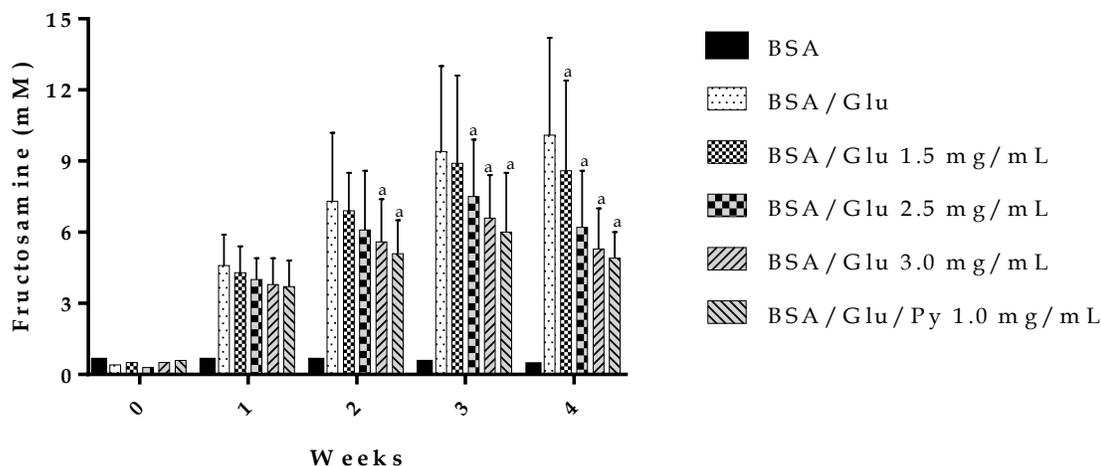


Figure 2. The effects of CM at concentrations of 1-3.0 mg/ml on formation of fructosamine at 4 weeks of incubation. The results are expressed as mean ± SEM (n = 4). ^a*p* < 0.01 when compared to BSA and ^b*p* < 0.05 when compared to BSA/Glu. Pyridoxamine (Pyr)

Effect of CM extract on the level of Nε-(carboxymethyl) lysine (CML)

The level of Nε-(carboxymethyl)lysine (Nε-CML) has been used as a indicator for the formation of non-fluorescent AGEs generated from the oxidative breakdown of Amodori product [31]. Nε-CML is a glyco-oxidation product which is not fluorescent and not reactive, and is also an indicator of the advanced stages of the Maillard reaction. Glucose-induced glycated BSA demonstrated an increase in Nε-CML formation, when compared to non-glycated BSA. The results revealed that CM at a concentration of 1, 1.5, 2.0, 2.5, and 3 mg/mL significantly (*p* < 0.05) inhibited the level of Nε-CML in glycated BSA with 26.6, 33.89, 39.85, 45.59, and 51.54% respectively after 4 weeks of incubation. In contrast, AG (1.0 mg/mL) inhibited Nε-CML formation about 45.1% in glucose-glycated BSA. These results of the level of Nε-CML are presented in Fig 3.

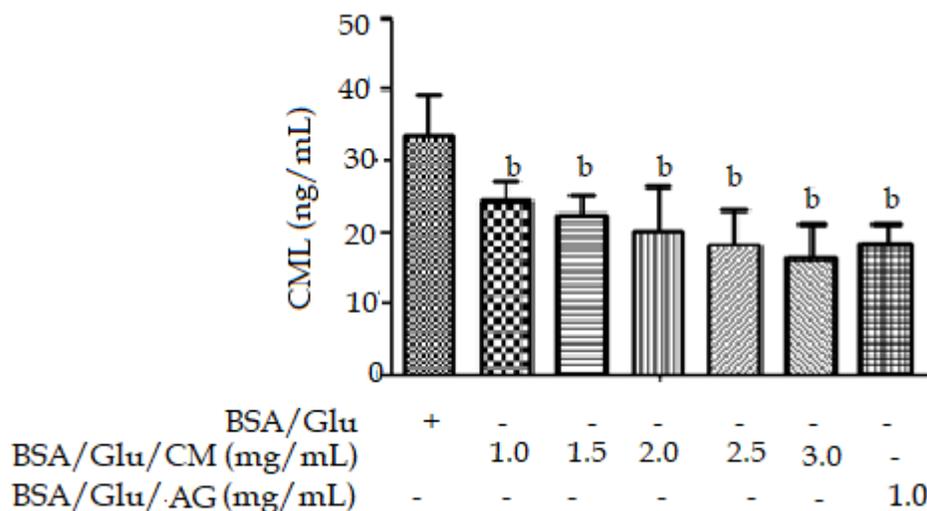


Figure 3. The effects of CM at concentrations of 1-3.0 mg/ml on level of Nε-(carboxymethyl)lysine (CML) at 4 weeks of incubation. The results are expressed as mean ± SEM (n = 4). ^a*p* < 0.01 when compared to BSA and ^b*p* < 0.05 when compared to BSA/Glu. Aminoguanidine (AG).

Effect of CM on the level of protein thiol groups and protein carbonyl content

The level of thiol groups and carbonyl content were used to determine the protein oxidation generated by glycation process. Data indicated that in the glucose-glycated BSA system, the level of thiol groups had been reduced at week 4. The level of thiol groups were significantly increased after the addition of CM (1–3.0 mg/mL) as well as AG (1.0 mg/mL). The results displayed in Figure 4 that the percentage of prevention of depleting thiol group by CM were in the rank of 64.4 to 86.2% in glucose-glycated BSA. However, AG (1.0 mg/mL) only prevents the depletion of protein thiol groups in glucose-glycated BSA by 8.5% after 4 weeks of incubation. Data indicated that in glucose-glycated BSA system the carbonyl content significantly increased after 4 weeks of incubation. As shown in Figure 4, at week 4 a significant decrease in carbonyl content was observed in glycated BSA plus CM. Our findings demonstrated that CM at concentrations of 1, 1.5, 2.0, 2.5, and 3 mg/mL significantly ($p < 0.05$) inhibited the protein carbonyl formation in a range of 21.4 to 64.3% as compared with glycated BSA. Additionally, a significant reduction of AG (1 mg/mL on protein carbonyl content of 88% for glucose glycated BSA) was observed at the same week.

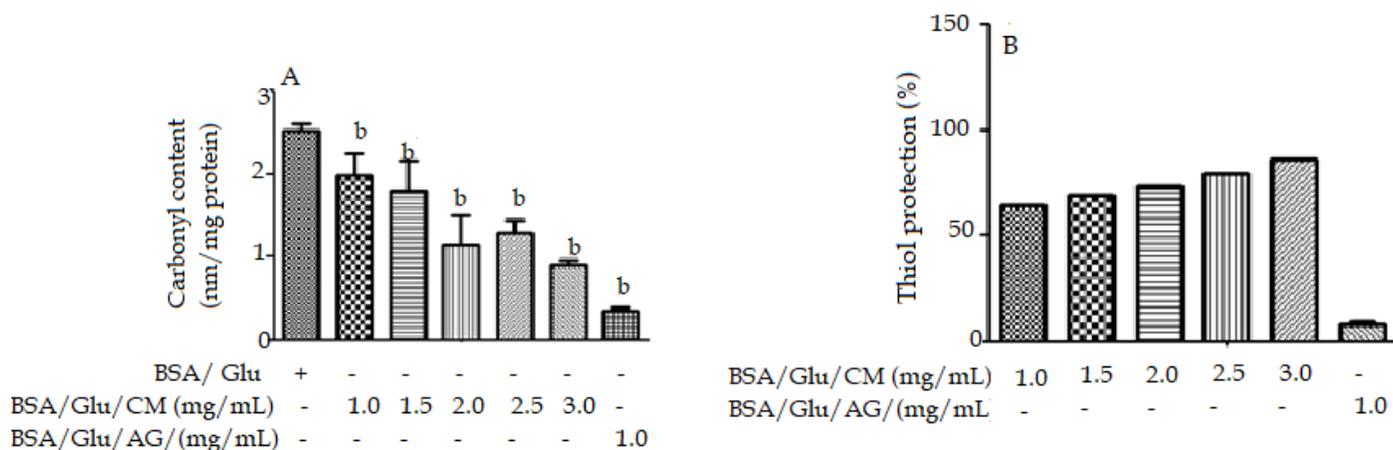


Figure 4. The effects of CM at concentrations of 1-3.0 mg/ml on carbonyl content and (A) and on thiol group (B), at 4 weeks. The results are expressed as mean ± SEM (n = 4). ^a $p < 0.01$ when compared to BSA and ^b $p < 0.05$ when compared to BSA/Glu. Aminoguanidine (AG).

Effect of CM on amyloid cross β structure

The ability of CM to reduce aggregation of glycated albumin was investigated by using two amyloid markers, i.e. Th. T. and congo Red (Fig 5). BSA incubated with glucose throughout four weeks of period increase the level of amyloid cross β structure. In glycated albumin samples (Th. T. and congo Red) co-incubated with CM (1.0, 1.5, 2.0, 2.5, and 3.0 mg/mL) similar results were exhibited demonstrated a significant attenuation to the elevated level of amyloid cross β structure in both assays.

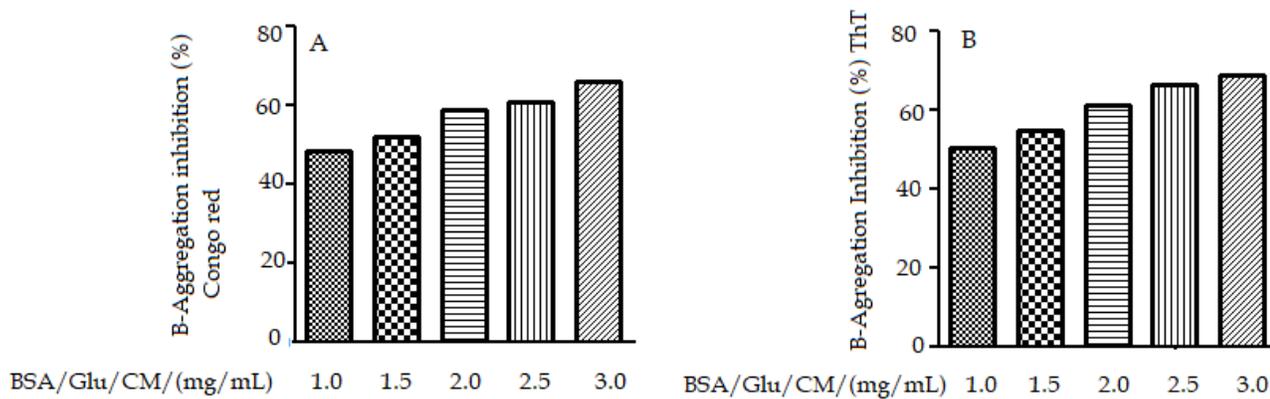


Figure 5. The effect of CM on level of β aggregation on Congo red (A) and ThT assays (B) in glucose-glycated BSA system are expressed in percent (n = 4).

Inhibition of MG-derived AGEs

AGEs are well-known to be produced from carbohydrates like glucose and fructose leading to the formation of carbonyl species, such as MG, which is a crucial intermediary formed during the glycation of proteins by glucose. Consequently, MG should be considered a target. CM was evaluated for their inhibitory effects on the MG-derived AGEs formation by using BSA–MG antiglycation model. MC was added in various concentrations (0.5, 1.0, 1.5, 2.0, 3.0 mg/ml) for incubation with MG and BSA (Fig. 6). All concentrations of CM used markedly inhibited the formation of MG-derived AGEs in BSA with a percentage inhibition in a range of 18.6 to 58.7% (Fig 6). However, a significant reduction of AG (1 mg/mL) on the formation of MG-derived AGEs of 75.12% in BSA was observed at the same week.

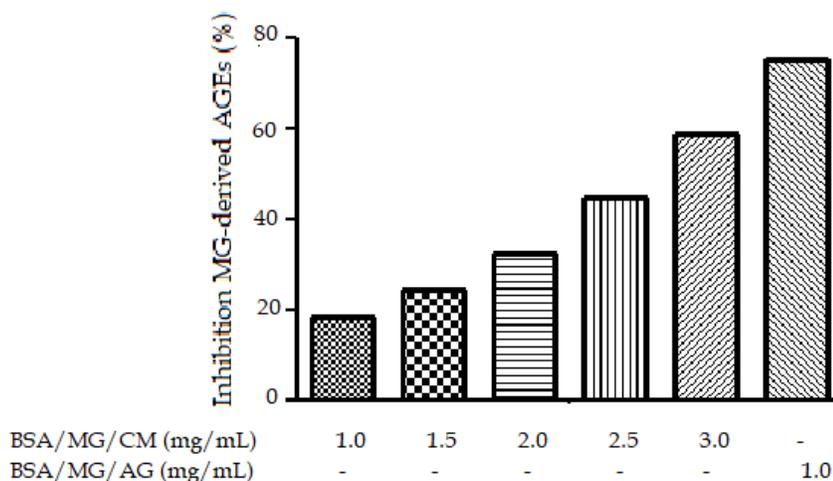


Figure 6. Percentage of inhibition of CM at concentrations of 1.0-3.0 mg/ml on the formation of MGO-derived AGEs in BSA. Results are represented as mean±SEM (n=3).

Methylglyoxal-trapping capacity

MG-trapping ability was carried out in order to investigate whether CM could directly scavenge MG. At the concentration of 1 mg/ mL, CM was significant scavenge MG with a percentage inhibition of 49.10%. Whereas AG had the highest potent MG-trapping ability with a percentage inhibition of 98.51%. However, CM was less potent than AG when compared at same concentration of 1 mg/mL.

RINm5F cell viability and insulin secretion

The results shown in Fig. 7A indicate that treating cells with MG at 10 μ M for 24 h decreased RINm5F cell viability. However, CM treatment at concentrations of 100 to 500 μ g/mL 12 h before the addition of MG at 10 μ M significantly protected against MG induced cell death to recover insulin production. Without MG treatment, CM did not affect cell viability in RINm5F (Fig. 7A). However, when RINm5F cells were pretreated with CM at concentrations of 100, 250, and 500 μ g/mL 12 h before the addition of MG at 10 μ M founding that CM stimulating insulin secretion protecting against MG-induced cell death (Fig 7B).

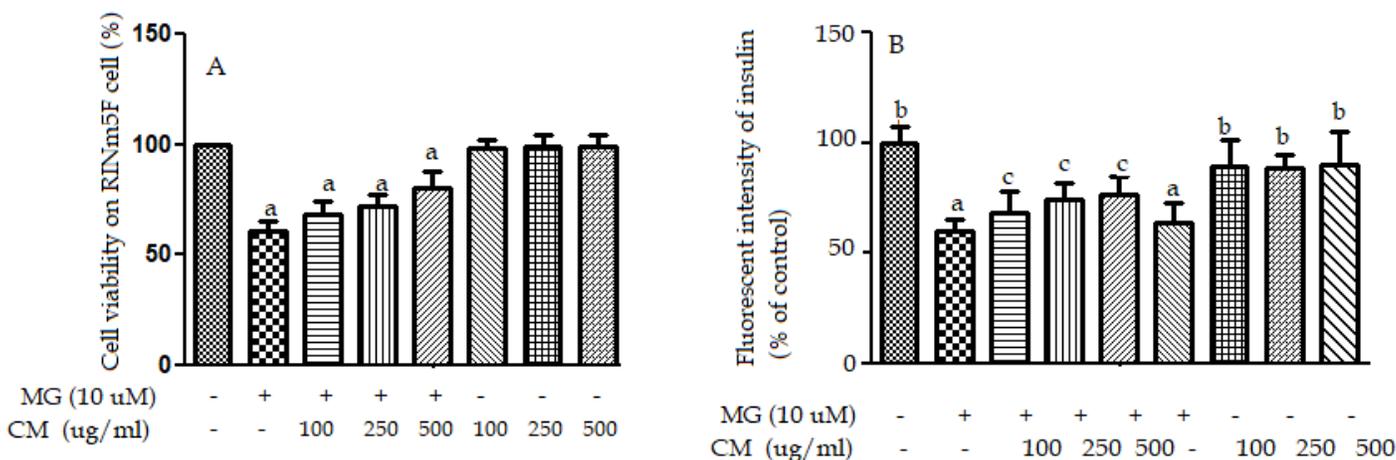


Figure 7. The effects of cell viability on RINm5F cells with CM (100, 250, and 500 μ g/mL), with or without MG (10 μ M) for 24 h (A). Insulin production was measured in RINm5F cells which were co-treated with MG (10 μ M) with or without CM (100, 250, and 500 μ g/mL), for 24 h (B). Data were shown as mean \pm SD (n = 3). Different letter was shown significant difference $p < 0.05$.

DISCUSSION

The results indicated that the content of polyphenolic compounds in MC extract using LC-DAD-ESI/MS was consistent with previous studies [32]. Numerous studies have been published evaluating the antioxidant capacity of flavonoids, which have the ability to chelate transition metals, quench free radicals through the donation of hydrogen atoms and electrons, or inhibit lipid peroxidation [33]. Glycation is a major source of oxygen species and of reactive carbonyl which are generated by non-oxidative and oxidative pathways [34]. The increase of free radical production is associated with AGE formation. Therefore, compounds with an antioxidant effect may be able to inhibit the formation of AGE [35]. Celery revealed the presence of bioactive

compounds such as apigenin [35] lutein [36, 37], kaempferol [37], and rutin [38] are associated with antiglycation agents for having a double bond between C-2 and C-3, an OH group at C-5 on the A ring [39]. Additionally, polyphenol acid as caffeic acid [40], chlorogenic acid [40], ferulic acid [41] and p-coumaric acid [42] demonstrates a high antiglycation effect. Previous findings on celery revealed that these polyphenols have a high antioxidant effect [43]. Furthermore, our findings suggested that these compounds, the major flavonoids and phenolic acids contained in celery, contribute to the anti-glycation activity of celery.

Some polyphenolic compounds and their conjugated metabolites were quantified in the blood samples of adults consuming freeze dried fruits powder for 90 days. Results indicated that anthocyanins were present in the highest concentration (85.7 ± 9.0 nmol/L, $t=2$ h, day 90) [44]. However, some phenolic acid metabolites in circulation were persistently in lower concentrations (25.7 ± 5.0 μ mol/L), which suggests they are extensively metabolized and can persist in circulation [45]. This may be the result of metabolic recycling (enteric and enterohepatic) of anthocyanins, which can prolong systemic exposure to various metabolites [46] or changes in the activities of efflux transporters to maintain equilibrium and changes in the gut microbiome or changes in metabolic processes which are altered with prolonged exposure to polyphenols, such as the upregulation of key metabolizing enzymes (phase I and II) [47]. Polyphenol degradation is carried out in fruits, vegetables, and in certain microorganisms by polyphenol oxidase, while being scarce in animal tissues and not producing degradation of polyphenolic compounds in animals blood.

Protein glycation is a spontaneous modification of amino acids and proteins by reducing monosaccharides. This reaction generates AGEs which are implicated in the development of diabetes complications among others [34]. The formation of AGEs contains three stages: early, intermediate, and late. In the early stage of glycation, reducing sugars reacts with proteins, generating the formation of Schiff base and eventually a rearrangement producing an Amadori product [48]. In the intermediate stage of glycation degradation of Amadori products and autoxidation of glucose generated reactive dicarbonyls as methylglyoxal and 3-deoxyglucosone. Consequently, in the late stage of glycation AGEs are formed by degradation of Schiff bases, and Amadori products, dicarbonyl compounds also produce protein modification (crosslinking) and reactions between AGE precursors and Amadori products.

According to our findings, CM prevented the formation of AGEs in BSA-glucose system at the initial stage of glycation delaying conversion of the initial glycated product to AGEs. Glycation-induced modifications in BSA which are related to alteration in its conformation. The different degree of CM activities against various glycation products can reveal their mechanism of action.

Fructosamines in the BSA/glucose system are early glycation products which were inhibited by CM. Similar to pyridoxamine, fructosamines may inhibit multiple stages of AGEs formation. In contrast, other inhibitors like aminoguanidine completely inhibited AGEs but not Amadori albumin formation. Consequently, in the early stages of glycation, after the reaction between the free amino group from protein and carbonyl group from reducing sugar, the reversible Schiff's bases are formed and transformed to more stable Amadori products/fructosamine. Subsequently, through reactive oxygen the Amadori product undergoes fragmentation to generate carbohydrate intermediates of short-chain which alter arginine and lysine residues to produce AGEs. As a result, the inhibition of fructosamine plays an important role in reducing vascular complication [49]. The addition of CM has inhibitory effects on the early stages of glycation. However, these results

demonstrated that the antiglycation actions of CM during the early stage of glycation is not entirely due to the inhibition of Amadori products.

The level of N ϵ -(carboxymethyl)lysine (N ϵ -CML), has been used as a indicator for the formation of the non-fluorescent AGEs generated from oxidative breakdown of Amadori product [50]. N ϵ -CML is a glyco-oxidation product which is not fluorescent and not reactive, and is also an indicator of the advanced stages of the Maillard reaction. Glucose-induced glycated BSA demonstrated an increase in N ϵ -CML formation when compared to non-glycated BSA. The Amadori products react with amino acids to produce N ϵ -CML, which is the most abundant in AGEs. Additionally, the degradation of Amadori products led to reduced N ϵ -CML, dicarbonyl intermediate formation, and protein oxidation. CM significantly reduced the formation of N ϵ -CML in glycated BSA with a decrease in the formation of AGEs. These results suggested CM inhibited AGEs production predominantly by inhibiting dicarbonyl generation, while only one part was inhibited by fructosamine adduct formation. According to our findings, compounds may be inhibited at multiple stages, as they may be involved in these three stages.

Glycation is an important mechanism to generate conformational changes of proteins by increasing the level of amyloid cross β structure, which plays a important role in protein aggregation [51]. Amyloid cross- β structure is aggregated of protein which can accumulate in the brain, pancreas, and other organs. Significantly, the accumulation of amyloid cross- β structure aggregation produces pancreatic islet amyloidosis, which impairs insulin secretion and damages β -cell [52]. Our results demonstrate CM can reduce the formation of amyloid cross- β structure, thereby decreasing the risk of the development of pathologic lesions in pancreatic β -cells of type 2 diabetic patients.

The albumin in plasma is a major source for thiol groups being able to form intermolecular aggregates and disulfide bonds. Glycation produces a change in the structure of protein, leading to a loss of protein thiol groups, which is reflected in the generation of free radicals [53]. Fructose and glucose increased significantly oxidized thiol groups and protein carbonyl formation than in the native BSA, which formed under the glycooxidation process. Additionally, CM is able to reduce the content of protein carbonyl and protected thiol groups from oxidation, demonstrating strong reduction potential.

MG is a reactive carbonyl species which is a crucial intermediary formed during the glycation of proteins by glucose. Consequently, MG should be considered a target. The reaction of lysine residues with MG form MG-derived lysine dimer and N ϵ -(1-carboxyethyl)lysine. Furthermore, protein glycation by MG reacts with arginine residues to produce methylglyoxal-derived hydroimidazolone which is the most abundant MG-derived AGE in human blood that contributes to several diseases, including diabetes [54]. The processes of glucose autoxidation and protein glycation may produce reactive carbonyl species including glyoxal and MG. Subsequently, both protein carbonyl derivatives and reactive carbonyl intermediaries in AGE formation are susceptible to oxidative reaction to amino acids as cysteine (thiol side chain). During glycooxidation and glycation, reactive nitrogen species and reactive oxygen species are generated which are able to oxidize chains of amino acid to form a carbonyl derivative and to the eliminate thiols groups decreases the oxidative defense of protein. In the present study, it has been demonstrated that CM has the ability inhibit MG-derived AGEs and the MG-trapping ability which may be used in the prevention and management of AGE-mediated diabetic complication.

Rutin contained in the CM extract forming the mono-MG adduct had the MG-trapping ability, which was reported by Yoon et al. [55], a study that demonstrated how neighboring hydroxyl groups in the rutin are an active site which forms mono or di-MG adducts for the trapping of MG. Several studies have demonstrated the structure-activity relationships of flavonoids in trapping MG, indicating that the presence of more hydroxyl groups on the phenyl ring result in an increase in the trapping MG [56]. Consequently, the inhibition of MG-derived AGEs could be an important strategy for treatment of diabetes.

The results demonstrated total insulin levels within RINm5F cells decreased under MG conditions and increased in cells treated with MC. These results suggest that CM contains anti-AGEs effects which protect pancreatic β -cells against the increased mitochondrial ROS and the loss of insulin in MG conditions.

CM significant decrease in glucose-BSA system, it significantly delay these processes. The blockage of the carbonyls during glycation, the carbonyl group in reducing sugars in addition to breaking the crosslinking structure which plays an important role in the development of antiglycating agents [57].

Celery is a promising antiglycation agent worthy of further studies for their *in vivo* antiglycation effect in STZ-induced diabetic mice to determine AGEs/RAGE/ROS interaction. The characterization of protein glycation was accomplished using electrophoresis and Tandem mass spectroscopy.

CONCLUSION

In this study, using different *in vitro* glycation models, we find that CM extract from *Apium graveolens* have inhibitory activity at multiple stages of glycation as Amadori products, AGEs-specific fluorescence, late glycation products and protein-AGEs crosslinking. Additionally, CM preventing mainly carbonyl formation, inhibit the formation of MG-derived AGEs and MG-trapping ability, oxidative protein damages and thiol oxidation which are formed under the glycooxidation process. Furthermore, CM protects the function and survival of pancreatic RINm5F cells lines under MG condition. These results demonstrate that CM is capable of suppressing the formation of AGEs and protein oxidation *in vitro*. As a result, *A. graveolens* is a potential functional food in preventing the development of diabetic complications.

List of Abbreviations: AG, aminoguanidine; BSA, bovine serum albumin; CM, MeOH extract; CML, N ϵ -(carboxymethyl) lysine DMSO, dimethylsulfoxide; DNPH, guanidine hydrochloride, 2,4-dinitrophenylhydrazine; glu, glucose; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); LC/ESI-MS, liquid chromatography-electrospray ionization mass, MG, methylglyoxal; NBT, cloruro de nitroblue tetrazolium; PBS, phosphate-buffered saline; Pyr, Pyridoxamine; RINm5F, Insulin-secreting β -cells line; RPMI, medium; SDS, sodium dodecylsulfate; TCA, trichloroacetic acid.

Authors' Contributions: Dr. RMPG design the study, analyzed the data, participate in the characterization of phenolic by LC/ESI-MS and drafting of the manuscript. Dr. JFE and M.Sc. VAJ performed glycation of BSA, Formation of fructosamine adduct. determination of N ϵ -(carboxymethyl) lysine (CML), determination of protein carbonyl content, thiol group estimation, thioflavin T and binding of Congo red assays. Dr. JFE design RINm5F cell studies. Dr. AMR design characterization of phenolic and determination of methylglyoxal-trapping capacity by

LC/ESI-MS. All authors contributed to this work. The final manuscript was reviewed and approved by all.

Competing Interests: The authors declare that they have no conflicts of interest.

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REFERENCES

1. Vanessa FT, Prioletta A, Zuo P, Folli F: Hyperglycemia-induced oxidative Stress and its role in diabetes mellitus related cardiovascular diseases. *Curr Pharm Des* 2013, 19: 5695–5703.
2. Ahmed N: Advanced glycation end products-role in pathology of diabetes complications. *Diabetes Res Clin Pract* 2007, 67: 3-21.
3. Kang JH: Oxidative damage of DNA induced by methylglyoxal in vitro. *Toxicol Lett* 2003, 145:181–187.
4. Semchyshyn HM, Miedzobrodzki J, Bayliak MM, Lozinska LM, Homza BV: Fructose compared with glucose is more a potent glycooxidation agent in vitro, but not under carbohydrate-induced stress in vivo: potential role of antioxidant and antiglycation enzymes. *Carbohydr Res* 2014,384: 61–69.
5. Desai K, Wu L: Methylglyoxal and advanced glycation end products: new therapeutic horizons?. *Recent Pat Cardiovas Drug Discov* 2007, 2: 89–99.
6. Dhar A, Desai K, Kazachmov M, Yu P, Wu L: Methylglyoxal production in vascular smooth muscle cells from different metabolic precursors. *Metabolism* 2008, 57: 1211–1220.
7. Abd El-Mageed, NM: Hepatoprotective effect of feeding celery leaves mixed with chicory leaves and barley grains to hypercholesterolemic rats. *Pharmacogn Mag* 2011, 7: 151–156.
8. Ahmed B, Alam T, Varshney M, Khan SA: Hepatoprotective activity of two plants belonging to the Apiaceae and the Euphorbiaceae family. *J Ethnopharmacol* 2002, 79: 313–316.
9. Momin RA, Nair MG: Antioxidant, cyclooxygenase and topoisomerase inhibitory compounds from *Apium graveolens* Linn. seeds. *Phytomedicine* 2002, 9: 312–318.
10. Zidorn C, Johrer K, Ganzera M, Schubert B, Sigmund EM, Mader J, Greil R, et al.: Stuppner H: Polyacetylenes from the Apiaceae vegetables carrot, celery, fennel, parsley, and parsnip and their cytotoxic activities. *J Agric Food Chem* 2005, 53:2518–2523.
11. Fazal SS, Singla RK: Review on the pharmacognostical and pharmacological characterization of *Apium graveolens* Linn. *Indo Global J Pharm Sci* 2012, 2: 36-42.
12. RM, Aguiniga VJ, Saucedo JV, Sosa IA: In vitro and in vivo antidiabetic and antiglycation properties of *Apium graveolens* in Type 1 and 2 diabetic rats. *Intern J Pharmacol* 2014, DOI: <https://doi.org/10.3923/IJP.2014>.
13. Kooti W, Ghasemiboroon M, Asadi-Samani M, Ahangarpour A, Zamani M, Amirzargar A: The effects of hydro-alcoholic extract of celery on lipid profile of rats fed a high fat diet. *Adv Environ Biol* 2014, 6: 325–331.

14. Tsi D, Tan BK: The mechanism underlying the hypocholesterolaemic activity of aqueous celery extract, its butanol and aqueous fractions in genetically hypercholesterolaemic RICO rats. *Life Sci* 2000, 66: 755–767
15. Rashmi ST, Neena MS, Shamim AS, Devyani VP, Juhi UP, Amrita AK, Nisha GK: Aqueous extract of some indigenous medicinal plants inhibits glycation at multiple stages and protects erythrocytes from oxidative damage—an in vitro study. *J Food Sci Technol* 2015, 52:1911–1923. DOI: <https://doi.org/10.1007/s13197-013-1211-8>.
16. Cheng MC, Lin LY, Yu TH, Peng RY: Hypolipidemic and antioxidant activity of mountain celery (*Cryptotaenia japonica* Hassk) seed essential oils. *J Agric Food Chem* 2008, 56: 3997–4003.
17. Zhang W, Peng Y, Huang YL, Song S, Bi K: Simultaneous determination of seven flavonoids and one coumarin of *Genkwa Flos* by RP-HPLC. *J Shenyang Pharmaceutical University* 2011, 28: 206–210.
18. Zhou L, Li J, Yan C: Simultaneous determination of three flavonoids and one coumarin by LC-MS/MS: application to a comparative pharmacokinetic study in normal and arthritic rats after oral administration of *Daphne genkwa* extract. *Biomed Chromatogr* 2018. DOI: <https://doi.org/10.1002/bmc.4233>.
19. Trinh H, Yoo Y, Won KH, Ngo HTT, Yang JE, Cho JG, Lee SW, et al.: Evaluation of in-vitro antimicrobial activity of *Artemisia apiacea* H. and *Scutellaria baicalensis* G. extracts. *J Med Microbiol* 2018. <https://doi.org/10.1099/jmm.0.000709>.
20. 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. *Pak J Pharm Sci* 2010, 23:403–408.
21. Ardestani A., Yazdanparast R: *Cyperus rotundus* suppresses age formation protein oxidation in a model of fructose-mediated protein glycooxidation. *Inter J Biol Macromol* 2007, 41, 572–578.
22. Levine RL, Garland D, Oliver CN, Amici A, Climent I: Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* 1990, 186: 464–478.
23. Ellman GL: Tissue sulfhydryl groups. *Archives Biochem Biophys* 1959, 82: 70–77.
24. Tupe R, Agte V: Role of zinc along with ascorbic acid and folic acid during long-term in vitro albumin glycation. *Brit J Nutr* 2010, 103: 370–377.
25. Klunk W, Jacob RF, Mason RP: Quantifying amyloid by Congo red spectral shift assay. *Methods Enzymol* 1999, 309: 285–305.
26. Peng X, Cheng KW, Ma J, Chen B, Ho CT, Lo C: Cinnamon bar proanthocyanidins as reactive carbonyl scavengers to prevent the formation of advanced glycation endproducts. *J Agric Food Chem* 2008, 56: 1907–1911.
27. Sang S, Shao X, Bai N, Lo CY, Yang CS, Ho CT: Tea polyphenol (–)- epigallocatechin-3-gallate: a new trapping agent of reactive dicarbonyl species. *Chem Res Toxicol* 2007, 20: 1862–1870.
28. Hsu WH, Lee BH, Lu IJ, Pan TM: Ankaflavin and monascin regulate endothelial adhesion molecules and endothelial NO synthase (eNOS) expression induced by tumor necrosis

- factor- α (TNF- α) in human umbilical vein endothelial cells (HUVECs). *J Agric Food Chem* 2012, 60: 1666–1672.
29. Yao Y, Sang W, Zhou M, Ren G: Phenolic composition and antioxidant activities of 11 celery cultivars. *J Food Sci* 2010, 75: C9-13. <https://doi.org/10.1111/j.1750-3841.2009.01392>.
 30. Lin L, Lu S, Harnly JM: Detection and quantification of glycosylated flavonoid alonates in celery, chinese celery, and celery seed by LCDAD-ESI/MS. *J Agric Food Chem* 2007, 55: 1321–1326. <https://doi.org/10.1021/jf0624796>.
 31. Younus H, Anwar S: Prevention of non-enzymatic glycosylation (glycation) Implication in the treatment of diabetic complication. *Int J Health Sci (Qassim)* 2016, 10: 261-277.
 32. Goh SY, Cooper ME: Clinical review: The role of advanced glycation end products in progression and complications of diabetes. *J Clin Endocrinol MeTable* 2008, 93: 1143–1152.
 33. Prochazkova D, Bousova I, Wilhelmova N: Antioxidant and prooxidant properties of flavonoids. *Fitoterapia* 2011, 82: 513–523.
 34. Rahbar S, Figarola JL: Novel inhibitors of advanced glycation endproducts. *Arch Biochem Biophys* 2003, 419, 63–79.
 35. Nakagawa T, Yokozawa T, Terasawa K, Shu S, Juneja LR: Protective activity of green tea against free radical-and glucose-mediated protein damage. *J Agr Food Chem* 2002, 50: 2418–2422.
 36. Shao X, Chen H, Zhu Y, Sedighi R, Ho CT, Sang S: Essential structural requirements and additive effects for flavonoids to scavenge methylglyoxal. *J Agric Food Chem* 2014, 1: 453-458.
 37. Grzegorzczuk-Karolak I, Goł G, Gburek J, Wysokińska H, Matkowski A: Inhibition of advanced glycation end-product formation and antioxidant activity by extracts and polyphenols from *Scutellaria alpina* L. and *S. altissima* L. *Molecules* 2016, 21: 739-749, DOI: <https://doi.org/10.3390/molecules21060739>.
 38. Li X1, Lu Y, Deng R, Zheng T, Lv L: Chemical components from the haulm of *Artemisia selengensis* and the inhibitory effect on glycation of β -lactoglobulin. *Food Funct* 2015, 6:1841-1846. <https://doi.org/10.1039/c5fo00117j>.
 39. Ardestani A, Yazdanparast R: *Cyperus rotundus* suppresses age formation and protein oxidation in a model of fructose-mediated protein glycooxidation. *Int J Biol Macromol* 2007,41: 572–578.
 40. Bains Y, Gugliucci A: *Ilex paraguariensis* and its main component chlorogenic acid inhibit fructose formation of advanced glycation endproducts with amino acids at conditions compatible with those in the digestive system. *Fitoterapia* 2017, 117: 6-10. DOI: <https://doi.org/10.1016/j.fitote.2016.12.006>.
 41. Sompong W, Cheng H, Adisakwattana S: Ferulic acid prevents methylglyoxal induced protein glycation, DNA damage, and apoptosis in pancreatic β -cells. *J Physiol Biochem* 2017, 73: 121-131. <https://doi.org/10.1007/s13105-016-0531-3>.
 42. Chen H, Virk MS, Chen F: Phenolic acids inhibit the formation of advanced glycation end products in food simulation systems depending on their reducing powers and structures. *Int J Food Sci Nutr* 2016, 67:400-11. <https://doi.org/10.3109/09637486.2016.1166187>.

43. Kooti W, Daraei N: A Review of the Antioxidant Activity of Celery (*Apium graveolens* L). J Evid Based Complementary Altern Med 2017, doi: 10.1177/2156587217717415.
44. Ferrars RM, Czank C, Zhang Q, Botting NP, Kroon PA, Cassidy A, Kay CD: The pharmacokinetics of anthocyanins and their metabolites in humans, British J pharmacol 2014, 171: 3268-3282.
45. Liu Z, Hu M: Natural polyphenol disposition via coupled metabolic pathways, Expert opinion on drug metabolism & toxicology, 2007, 3, 389-406.
46. Kalt W, Liu Y, McDonald JE, Vinqvist-Tymchuk MR, Fillmore SA: Anthocyanin metabolites are abundant and persistent in human urine, Journal of agricultural and food chemistry 2014, 62: 3926-3934.
47. Kalt W, McDonald JE, Liu Y, Fillmore SA: Flavonoid metabolites in Human Urine during Blueberry Anthocyanin Intake, Journal of agricultural and food chemistry 2017, 65: 1582-1591 Singh VP, Bali A, Singh N, Jaggi AS: Advanced glycation end products and diabetic complications. Korean J Physiol Pharmacol 2014, 18: 1-14
48. Hsu TH, Lin FY, Chen HY: Antioxidant and antiglycation properties of different solvent extracts from Chinese olive (*Canarium album* L.) fruit. Asian Pac J Trop Med 2015, 8:1013-1021. <https://doi.org/10.1016/j.apjtm.2015.11.013>.
49. Younus H, Anwar S: Prevention of non-enzymatic glycosylation (glycation): Implication in the treatment of diabetic complication. Int J Health Sci (Qassim) 2016, 10: 261-277.
50. Bouma B, Kroon-Batenburg LM, Wu YP: Glycation induces formation of amyloid cross-beta structure in albumin. J Biol Chem 2003, 278: 41810-41819.
51. Marzban L, Verchere CB: The role of islet amyloid polypeptide in type diabetes. Canadian J Diabetes 2004, 28: 39-47.
52. Mossine VV, Linetsky M, Glinsky GV: Superoxide free radical generation by Amadori compounds: the role of acyclic forms and metal ions. Chem Res Toxicol 1999, 12:230-236.
53. Berlanga J, Cibrian D, Guillén I, Freyre F, Alba JS, Lopez-Saura P: Methylglyoxal administration induces diabetes-like microvascular changes and perturbs the healing process of cutaneous wounds. Clin Sci (Lond) 2005, 109: 83-95.
54. Yoon SR, Shim SM: Inhibitory effect of polyphenols in *Houttuynia cordata* on advanced glycation end-products (AGEs) by trapping methylglyoxal. LWT-Food Sci Technol 2015, 61: 158-163
55. Shao X, Chen H, Zhu Y, Sedighi R, Ho CT, Sang S: Essential structural requirements and additive effects for flavonoids to scavenge methylglyoxal. J Agric Food Chem 2014, 62: 3202-3210.
56. Chesne S1, Rondeau P, Armenta S, Bourdon E: Effects of oxidative modifications induced by the glycation of bovine serum albumin on its structure and on cultured adipose cells. Biochimie 2006, 88:1467-1477.