Antiglycative effect of fruit and vegetable seed extracts: inhibition of AGE formation and carbonyl-trapping abilities

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ABSTRACT

BACKGROUND: Advanced glycation end-products (AGEs) are the final products derived from the nonenzymatic glycation process. AGEs are involved in the development of several health complications associated with diabetes and aging. Searching for anti-AGE extracts is necessary to mitigate the effects of age-related pathologies.

RESULTS: The antioxidant and antiglycative activities of eight aqueous extracts of fruit and vegetable seeds were evaluated. All seed extracts (3.6 mg mL⁻¹) exhibited anti-AGE activity in protein–glucose assay, ranging from 20 to 92% inhibition compared with aminoguanidine (4.87 mmol L⁻¹). Green pepper extract exerted the highest anti-AGE activity. However, peach and pomegranate extracts exhibited the highest anti-AGE activity in protein–methylglyoxal assay, ranging from 0 to 79% inhibition. Hazelnut, almond and sesame extracts were not effective when methylglyoxal was the promoter. Apricot and peach extracts appeared to inhibit the formation of AGEs through their capacity for direct trapping of 1,2-dicarbonyls (IC₅₀=0.14 mg mL⁻¹). No relationship between antioxidant and phenolic compound content and antiglycative activity was found. Therefore other hydrophilic constituents in addition to phenolic acids must be involved in the antiglycative activity of the extracts.

CONCLUSION: Aqueous extracts of fruits and vegetables can be considered in the prevention of glycation-associated complications of age-related pathologies.

KEYWORDS: advanced glycation end-products (AGEs); seed extract; glycation; glycation inhibitors; phenolic compounds

INTRODUCTION

The Maillard reaction or non-enzymatic glycation process is produced by the interaction between reducing sugars and free amino groups of proteins, lipids and nucleic acids. In the human body the final products that derive from this reaction are called advanced glycation end-products (AGEs). AGEs are a group of complex and heterogeneous products that can be classified according to their structures and characteristics as fluorescent crosslinking AGEs such as pentosidine, non-fluorescent crosslinking AGEs such as methylglyoxal–lysine dimer (MOLD) and non-crosslinking AGEs such as carboxymethyllysine (CML) and pyrraline.¹

It is known that AGEs are involved in the development of several health disorders such as diabetes and its complications,² atherosclerosis,³ Alzheimer's disease and normal aging.⁴ For this reason, the search for AGE formation inhibitors has recently received much attention.^{5–8} Various AGE inhibitors have been developed in the last few years, which can be divided into three groups: (1) inhibitors that prevent glycoxidation through metal ion chelation; (2) 1,2-dicarbonyl-trapping agents; (3) crosslink breakers.⁹ Several AGE inhibitors have been described, a few of them exerting their effects at the early stage of glycation but most of them preventing the formation of AGEs at the late stage of glycation. Inhibition can occur through interference with the initial attachment between reducing sugars and amino groups, through trapping of carbonyls and radicals formed during glycation or through blocking the formation of intermediate Amadori products.¹⁰ Aminoguanidine (AG) and pyridoxamine (PM) are well-known AGE

inhibitors, both being considered as potent carbonyl scavengers. However, although such synthetic compounds have proved to be strong AGE inhibitors, they have also been associated with several adverse effects in *in vivo* assays.^{11,12} Therefore the search for natural products with the ability to inhibit AGE formation is currently being widely pursued.

Many plant extracts have been evaluated for their inhibitory effects on the formation of AGEs, both through preventing glycoxidation and by scavenging reactive 1,2-dicarbonyls such as methylglyoxal (MGO), which are important precursors of AGEs.^{6,13,14} Most studies have been carried out in methanolic, ethanolic or other organic solvents,^{7,13,15} since the inhibitory effects are mainly attributed to polyphenols owing to their potent antioxidant activities.¹⁶ It is known that phenolic compounds possess strong antioxidant abilities as a result of their redox properties; moreover, it has been reported that such antioxidant effects might contribute to the inhibition of protein modifications in the glycation process.¹⁷ Among these studies, only a few have evaluated aqueous extracts of samples.^{18,19}

In this regard, the aim of the present study was to investigate the inhibitory effects on AGE formation of aqueous extracts from eight different fruit and vegetable seeds considered as secondary by-products in the industry. For this purpose, different *in vitro* models of AGEs induced by glucose and MGO were evaluated. Samples were extracted in water, since, despite the fact that the antiglycative effect of aqueous extracts might be lower than that of organic solvent extracts, the extraction procedure is both more economical and environmentally friendly and therefore would be of greater interest for industrial applications. The extracts were also tested for their capacity in direct trapping of MGO. Moreover, antioxidant activity and phenolic compound content were examined in order to study their possible relationship with AGE-inhibitory activity.

MATERIALS AND METHODS

Commercially available fruit and vegetable seeds (green pepper, apricot, hazelnut, peach, sour cherry, sesame, almond and pomegranate) were provided by TIKTA (Ankara, Turkey). Detailed information on the different samples is listed in Table I. D(+)-Glucose, bovine serum albumin (BSA), 400 g L⁻¹ methylglyoxal solution (MGO), sodium azide, aminoguanidine (AG), 5-methylquinoxaline (5-MQ), o-phenyldiamine (OPD), gallic acid, Trolox, fluorescein and phenolic acid standards were purchased from Sigma (St Louis, MO, USA). Sodium dihydrogen phosphate monohydrate, glacial acetic acid, formic acid and high-performance liquid chromatography (HPLC)-grade methanol were acquired from Merck (Darmstadt, Germany). Folin–Ciocalteu reagent and sodium carbonate were obtained from Panreac Quimica (Barcelona, Spain). 2,2'-Azobis(methylpropionamidine)dihydro (AAPH) and pyridoxamine (PM) were purchased from Fluka Chemical (Madrid, Spain). A Pierce bicinchoninic acid (BCA) protein assay kit was obtained from Thermo Scientific (Rockford, IL, USA). The Milli-Q water used was produced using an Elix3 water purification system coupled to an Advantage10 Milli-Q module (Millipore, Molsheim, France). All other chemicals and reagents were of analytical grade.

Preparation of seed extracts

Powder (500 mg) of each seed sample was extracted in water (25 mL × 2) at 50 °C by agitation for 10 min each time. Pellets of the extracts were removed by centrifugation (1400 × g) and supernatants were collected, lyophilised and weighed. Soluble extracts were coded as described in Table I.

Measurement of pH

Each lyophilised extract (250 mg) was mixed with 10 mL of water and vortexed for 3 min. The mixture was held at room temperature for 1 h to separate solid and liquid phases. After carefully removing the supernatant layer, the pH was measured using a CG-837 pH meter (Schott, Mainz, Germany).

Determination of soluble protein

Soluble protein measurements were performed using a modified BCA protein assay.²⁰ According to Thermo Scientific, BCA protein assay reagent A contains sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.1 mL L⁻¹ sodium hydroxide, while reagent B contains 40 g L⁻¹ cupric sulfate. BCA working reagent was prepared by mixing 50 parts of reagent A and one part of reagent B. For sample analysis, 10 mg of lyophilised extract was dissolved in 1 mL of phosphate buffer (0.1 mol L⁻¹, pH 7.4) and vortexed for 10 min. The mixture was held at room temperature for 1 h and centrifuged at 8000 × g for 10 min. A 50 µL aliquot of the supernatant was mixed with 500 µL of BCA working reagent. After incubation for 90 min at 37 °C, the absorbance at 562 nm was recorded using a Synergy² HT-multimode microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA) and quantified using BSA as a standard. BioTek Gen5² data analysis software was used. The limit of quantification was set at 5.15 mg g⁻¹ sample.

Determination of reducing sugars

Reducing sugars were determined according to the method described by Miller.²¹ A calibration curve was constructed using standard glucose solutions in the concentration range 0.25–2 mg mL⁻¹. Each lyophilised extract (50 mg) was suspended in 5 mL of distilled water at 50 °C, vortexed for 20 min and centrifuged at 1400 × g for 15 min at 4 °C. The supernatant was collected. The extraction was repeated twice and the supernatants were pooled. Following treatment with Carrez-I and Carrez-II solutions, the supernatant was used to determine reducing sugars after blank correction. Results were expressed as mg glucose equivalent g⁻¹ sample. The limit of quantification was set at 25.2 mg glucose equivalent g⁻¹ sample.

Determination of total phenolic content

Total phenolic content (TPC) in the extracts was determined by the Folin–Ciocalteu method as described by Singleton et al.²² and adapted to a plate reader. Each lyophilised extract was dissolved in water to obtain a 10 mg mL⁻¹ solution. In a 1.5 mL Eppendorf microtube, 100 µL of sample (appropriately diluted if necessary) and 250 µL of Folin–Ciocalteu reagent (diluted 1:1 (v/v) in methanol) were mixed and vortexed. After exactly 3 min, 500 µL of 75 g L⁻¹ sodium carbonate solution and 4 mL of methanol/water (50:50 v/v) were added, then the mixture was vortexed for a further 10 min and allowed to stand at room temperature in darkness for 60 min. The absorbance at 750 nm was recorded using a BioTek microplate spectrophotometer as described above and quantified using gallic acid as a standard. Results were expressed as mg gallic acid equivalent (GAE) g⁻¹ sample. The limit of quantification was set at 4.5 mg GAE g⁻¹ sample.

Determination of total extracted phenolic acids

Total extracted phenolic acids were determined according to the method described by Kim et al.²³ and Ross et al.²⁴ First, 1 mL of 2 mol L⁻¹ sodium hydroxide containing 13.4 mmol L⁻¹ ethylenediaminetetraacetic acid (EDTA) and 20 mL L⁻¹ ascorbic acid was added to 30 mg of each lyophilised extract. The mixture was flushed with nitrogen and allowed to hydrolyse under agitation for 16 h at room temperature. After hydrolysis, the sample was centrifuged at 2370 × g for 10 min at 4 °C and the supernatant was acidified by adding 0.3 mL of acetic acid. The liberated phenolic acids were extracted with ethyl acetate (2 × 2 mL). The organic layer containing the phenolic acids liberated by base hydrolysis was collected by pipetting off the upper organic (supernatant) layer from the bottom aqueous residue layer. The two organic layers were combined and evaporated to dryness in a speed-vac for 1 h at 45 °C. The residue was dissolved in 1 mL of methanol/water (75:25 v/v) and filtered through a 0.45 µm filter, then the sample was analysed by HPLC. Quantification was conducted with a Shimadzu HPLC system (Kyoto, Japan) equipped with an LC-20AD pump, an SIL-10ADvp autosampler, a CTO-10ASVP oven and an SPD-M20A diode array detector. Chromatographic separation was carried out on a Kinetex C-18 100 Å column (100 mm × 4.6 mm, 2.6 µm; Phenomenex, Torrance, CA, USA). The flow

rate was 0.6 mL min⁻¹ and the injection volume was 5 µL. The mobile phase consisted of 1 mL L-1 formic acid in water (solvent A) and methanol (solvent B) and the gradient program was as follows: 0 min, 25% B; 0–5 min, 25–30% B; 5–10 min, 30–60% B; 10–12 min, 60% B; 12–13 min, 60–80% B; 13–14 min, 80% B; 14–15 min, 80–25% B; 15–18 min, 25% B. The total run time was 18 min and chromatograms were analysed at 254, 280 and 325 nm. The following phenolic acids were identified: p-hydroxybenzoic acid (PHB), syringic acid (SYN), vanillic acid (VA), p-coumaric acid (pCU), caffeic acid (CA), ferulic acid (FA), protocatechuic acid (PCA), gallic acid (GA), gentisic acid (GE), sinapinic acid (SIN) and ellagic acid (EA). The limit of quantification was set at 2 µg g⁻¹ sample.

Determination of benzoic acids

The presence of benzoic acids was determined using the HPLC method described by Lamuela-Raventos and Waterhouse.²⁵ Procedures for sample preparation, quantification and chromatographic separation were the same as those described for total extracted phenolic acid determination. The total run time was 18 min and quantification was made at 280 nm (expressed as gallic acid). The limit of quantification was set at 2.5 µg GAE g⁻¹ sample.

Determination of flavonoids

Flavonoid content was determined using the aluminium chloride method described by Abdel-Hameed.²⁶ Each lyophilised extract was dissolved in water at 25 mg mL⁻¹. Then 80 µL of sample was mixed with 80 µL of aluminium trichloride in ethanol and 100 µL of sodium acetate. The mixture was incubated in darkness for 90 min and centrifuged at 14 926 × g for 3 min. Flavonoids were determined based on the formation of a flavonoid–aluminium complex with absorptivity maximum at 440 nm. Absorbance readings were taken using a BioTek microplate spectrophotometer as described above. Quercetin was used as a reference standard. Results were expressed as µg quercetin equivalent per 100 g sample. The limit of quantification was set at 0.02 µg quercetin equivalent per 100 g sample.

ORAC assay

Alkylperoxyl free radical (ROO•)-scavenging activity was measured by monitoring the fluorescence decay due to ROO-induced oxidation of fluorescein, known as the oxygen radical absorbance capacity (ORAC) assay. The water-soluble azo initiator AAPH was applied as a clean and controllable source of thermally produced ROO• in aqueous media. ROO• were generated by AAPH in a microplate reader at 37 °C. The antiradical activity against AAPH was estimated according to the procedure reported by D'avalos et al.²⁷ A BioTek Synergy² HT-multimode microplate reader with automatic reagent dispense and temperature control was used. All reaction mixtures were prepared in duplicate and four independent assays were performed for each sample. Raw data were processed by the microplate reader, and the area under the curve (AUC) was calculated. ORAC was expressed as Trolox equivalent antioxidant capacity (TEAC) using a standard curve constructed for each assay. Results were expressed as µmol TEAC g⁻¹ sample. The limit of quantification was set at 18.1 µmol TEAC g⁻¹ sample.

ABTS assay

Antioxidant activity was estimated in terms of the radical scavenging activity of samples in aqueous media following the procedure described by Delgado-Andrade and Morales²⁸ with slight modification. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid radical cations (ABTS•+) were produced by reacting 7 mmol L⁻¹ ABTS stock solution with 2.45 mmol L⁻¹ potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The ABTS•+ solution (stable for 2 days) was diluted with distilled water to an absorbance of 0.70 ± 0.02 at 734 nm. Each lyophilised extract was dissolved in water at 10 mg mL⁻¹. Following the addition of 40 µL of sample (appropriately diluted if necessary) and Trolox standard to 200 µL of water and 40 µL of diluted ABTS•+ solution, an absorbance reading was taken after 10 min using a Bio-Tek Synergy² HT-multimode microplate reader.

as described above. Aqueous solutions of Trolox at concentrations of 0.016–0.5 mmol L⁻¹ were used for calibration. The limit of quantification was set at 1.1 µmol TEAC g⁻¹ sample.

In vitro glycation assay with BSA–glucose

The BSA–glucose (Glc) assay was based on Peng et al.⁶ and was used as an in vitro model for comparison of the antiglycation activities of the different seed extracts. First, BSA (35 mg mL⁻¹) and Glc (175 mg mL⁻¹) were dissolved separately in phosphate buffer (0.1 mol L⁻¹, pH 7.4). Then 200 µL of BSA solution containing 0.1 g mL⁻¹ sodium azide (to ensure aseptic conditions) was incubated with 400 µL of Glc solution at 37 °C for 21 days in the absence or presence of seed extract solutions (100 µL, 25 mg mL⁻¹). Blanks containing BSA–Glc but no test sample were kept at -80 °C until measurement. A 4 mg mL⁻¹ solution of AG (32.49 mmol L⁻¹) was used as positive control, corresponding to 4.87 mmol L⁻¹ in the reaction media. In parallel, seed extracts dissolved in phosphate buffer (0.1 mol L⁻¹, pH 7.4) were incubated at 37 °C for 21 days in order to measure their intrinsic fluorescence. The final concentration of each reactant in the reaction medium was 10 mg mL⁻¹ for BSA, 100 mg mL⁻¹ for Glc, 0.6 mg mL⁻¹ for AG and 3.6 mg mL⁻¹ for seed extracts.

In vitro glycation assay with BSA–MGO

The BSA–MGO assay was performed according to the method described by Lunceford and Gugliucci²⁹ with slight modification and was used to evaluate the inhibitory effects of the different seed extracts on protein glycation induced by MGO. First, BSA (35 mg mL⁻¹) and MGO (0.4 mg mL⁻¹) were dissolved separately in phosphate buffer (0.1 mol L⁻¹, pH 7.4). Then 200 µL of BSA solution containing 0.1 g mL⁻¹ sodium azide (to ensure aseptic conditions) was incubated with 400 µL of MGO solution at 37 °C for 14 days in the absence or presence of seed extracts solutions (100 µL, 25 mg mL⁻¹). Blanks containing BSA–MGO but no test sample were kept at -80 °C until measurement. A 4 mg mL⁻¹ solution of AG (32.49 mmol L⁻¹) was used as positive control, corresponding to 4.87 mmol L⁻¹ in the reaction media. In parallel, seed extracts dissolved in phosphate buffer (0.1 mol L⁻¹, pH 7.4) were incubated at 37 °C for 14 days in order to measure their intrinsic fluorescence. The final concentration of each reactant in the reaction medium was 10 mg mL⁻¹ for BSA, 0.23 mg mL⁻¹ for MGO, 0.6 mg mL⁻¹ for AG and 3.6 mg mL⁻¹ for seed extracts.

AGE fluorescence measurement

Measurements of the fluorescent intensity of total AGEs and the intrinsic fluorescence of the different seed extracts after incubation were performed using a BioTek microplate spectrophotometer as described above. The presence of total AGEs was characterized by typical fluorescence with respective excitation and emission maxima at 360 and 420 nm for the BSA–Glc assay and 340 and 420 nm for the BSA–MGO assay. The percentage inhibition of AGE formation by each extract was calculated using the following equation: inhibition (%) = $\{1 - [(fluorescence\ of\ solution\ with\ inhibitor - intrinsic\ fluorescence\ of\ sample) / fluorescence\ of\ solution\ without\ inhibitor]\} \times 100$.

Evaluation of direct MGO-trapping capacity

Direct MGO-trapping capacity was tested using the method described by Peng et al.¹⁵ with slight modification. MGO (0.4 mg mL⁻¹) was dissolved in phosphate buffer (0.1 mol L⁻¹, pH 7.4), OPD (derivatisation agent, 10.8 mg mL⁻¹) was dissolved in methanol and 5-MQ (internal standard, 1 mg mL⁻¹) was dissolved in 500 mL L⁻¹ methanol. PM solution (1 mg mL⁻¹ in 0.1 mol L⁻¹ phosphate buffer, pH 7.4) was used as positive control. A 100 µL aliquot of MGO solution was mixed with 750 µL of phosphate buffered saline (PBS), 50 µL of 5-MQ and 100 µL of either PBS (blank), seed extract solutions (0.005–10 mg mL⁻¹) or PM solution. Therefore the final concentration of each reactant in the reaction medium was 0.04 mg mL⁻¹ for MGO, 0.1 mg mL⁻¹ for PM and 0.0005–1 mg mL⁻¹ for seed extracts. After mixing, samples were incubated at 37 °C for 168 h. Then controls and samples were taken out, 200 µL of OPD was added and each mixture was shaken by vortex for 5 s. After 30 min

(when the derivatisation reaction was complete) the residual MGO was quantified on the basis of the amount of the derivatised product 2-methylquinoxaline (2- MQ) formed in each sample. Quantification was conducted using a Shimadzu HPLC system equipped with an LC-20AD pump, an SIL-10ADvp autosampler, a CTO-10ASVP oven and an SPD-M20A diode array detector. Chromatographic separation was carried out on a Mediterranea-Sea-ODS2 column (150 mm × 3 mm, 5 µm; Tecknokroma, Barcelona, Spain). The flow rate was 0.5 mL min⁻¹ and the injection volume was 10 µL. Isocratic elution was applied using a mobile phase of 5mL L-1 acetic acid/methanol (50:50 v/v). The total run time was 7 min and chromatograms were recorded at 315 nm. The amount of unreacted MGO in each sample could be determined on the basis of the ratio of peak areas of 2-MQ and 5-MQ. The percentage decrease in MGO was calculated using the following equation: MGO decrease (%)=[(amount of MGO in control – amount of MGO in sample with tested seed extract solution or PM solution)/amount of MGO in control] × 100%. IC50 values of samples were evaluated from the dose–response curves of each experiment using Microsoft Excel.

Statistical analysis

Statistical analyses were performed using Statgraphics Centurion XV (Herndon, VA, USA). Data were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) and the least significant difference (LSD) test were applied to determine differences between means. Differences were considered to be significant at $P < 0.05$. Relationships between the different parameters analysed were evaluated by computing Pearson linear correlation coefficients at the $P < 0.05$ confidence level.

RESULTS AND DISCUSSION

As a first step, the solubility of each extract was evaluated. The extracts showed a wide range of solubility varying from 129.8 to 478.1 mg g⁻¹, with pomegranate and green pepper seeds being least soluble and peach, apricot and almond seeds being most soluble (Table 1). Similarly, a wide variability in the pH of the extracts was observed when the lyophilised samples were reconstituted in water at a concentration of 25 mg mL⁻¹, ranging from pH 4.0 for green pepper extract to pH 8.5 for pomegranate extract (Table 2).

Since sugars and proteins are reactants of the Maillard reaction and may be involved in the glycation process, reducing sugars and soluble proteins were analysed in the aqueous extracts. The highest reducing sugar content was observed in sour cherry extract (201.1 mg g⁻¹), followed by peach (136.1 mg g⁻¹) and sesame (124.0 mg g⁻¹) extracts, while almond and hazelnut extracts exhibited the lowest values (41.5 and 34.4 mg g⁻¹ respectively). Soluble protein content varied from 139.7 mg g⁻¹ in sesame extract to 383.3 mg g⁻¹ in hazelnut extract (Table 2).

The antioxidant activities of the aqueous extracts were assessed by two methods: the ORAC assay and the ABTS assay (free radical-scavenging capacity). Table 2 shows the analytical results. The capacity to scavenge O₂⁻ radicals ranged from 107.4 µmol TEAC g⁻¹ extract for peach to 6378 µmol TEAC g⁻¹ extract for pomegranate, a huge difference. Similarly, the ABTS results varied markedly, ranging from 4.6 to 232.7 µmol TEAC g⁻¹ extract. Pomegranate extract had the highest free radical-scavenging activity, followed by green pepper extract, while sour cherry and apricot extracts showed the lowest scavenging activity. No significant correlation was found between ORAC and ABTS, but the capacity of certain extracts such as those of pomegranate and green pepper to scavenge O₂⁻ radicals was related to their capacity to scavenge ABTS radicals.

Since phenolic compounds have been proposed as major contributors to antiglycative activity,⁶ they were also determined (Table 2). TPC varied from 4.8 mg GAE g⁻¹ in apricot extract to 22.2 mg GAE g⁻¹ in pomegranate extract. The proportion of individual phenolic acids in the total extracted phenolic acids was also evaluated (Table 3). Among these phenolic acids, one can note the significant

contributions of caffeic acid in apricot extract (41.3%), ferulic acid in sesame extract (39.9%) and gallic acid in pomegranate extract (39.8%).

With the aim of finding other compounds present in the extracts that may be involved in antiglycative activity, benzoic acids and flavonoids were also determined. Benzoic acid content ranged from 3.5 to 7.9 mg GAE g⁻¹ extract, with almond and pomegranate extracts having the lowest and highest content respectively (data not shown). Flavonoid content was lower than the limit of quantification (0.02 µg quercetin equivalent per 100 g sample) for all extracts except that of pomegranate, whose content was 0.29 µg quercetin equivalent per 100 g sample (data not shown).

In order to evaluate the inhibitory effect of seed extracts against AGE formation *in vitro*, the fluorescence intensity was measured using AG as an AGE inhibitor. Figures 1A and 1B display the inhibitory effects at 25 mg mL⁻¹ on AGE formation in BSA-Glc and BSA-MGO assays respectively (final concentration in reaction medium 3.6 mg mL⁻¹). The results indicated significant differences in AGE-inhibitory activity among most samples ($P < 0.05$). In BSA-Glc assay the AGE-inhibitory rate ranged from 20.7 to 91.9%. Green pepper exhibited the highest inhibitory capacity with a value close to the effect of AG solution (average inhibitory rate 92.7%), followed by sesame and pomegranate with 66.1 and 61.7% inhibition respectively. The lowest inhibitory activity was observed for peach and apricot with 20.7 and 23.2% inhibition respectively. In contrast, both peach and apricot, together with pomegranate, resulted in more than 60% reduction in the formation of fluorescent AGEs in BSA-MGO assay, whereas sesame, hazelnut and almond had no inhibitory activity. In this assay the AGE-inhibitory rate of seed extracts ranged from 0 to 78.6%, with the highest values being lower than the effect of AG solution (average inhibitory rate 99.2%).

Several inhibitors can suppress AGE formation by scavenging certain precursors such as 1,2-dicarbonyls. An evaluation of direct MGO-trapping capacity was carried out in order to observe whether our tested seed extracts could directly scavenge these compounds. Figure 2 shows the different trapping abilities of the samples. All aqueous extracts trapped MGO in a dose-dependent manner and, with the exception of hazelnut and sesame, their activity at a concentration of 10 mg mL⁻¹ was comparable to or higher than the effect of 1 mg mL⁻¹ PM solution (99.6%). The values for IC₅₀ (mg mL⁻¹) are presented in Table 4. As can be observed, apricot and peach had the lowest IC₅₀ (0.14 mg mL⁻¹), followed by sour cherry (0.48 mg mL⁻¹), associated with their high MGO-trapping capacity. In contrast, sesame and hazelnut had the highest IC₅₀, corresponding to their low MGO trapping capacity (Fig. 2). As expected, IC₅₀ from MGO-trapping assay was negatively correlated with antiglycative activity from BSA-MGO assay ($r = -0.890$, $P = 0.003$). In this sense, peach and apricot exhibited the lowest IC₅₀ by MGO-trapping assay and, at the same time, the highest antiglycative activity by BSA-MGO assay together with pomegranate. In contrast, no relation was found for pomegranate between IC₅₀ from MGO-trapping assay and antiglycative activity from BSA-MGO assay. Regarding BSA-Glc assay, no relationship was found between IC₅₀ and anti-AGE capacity according to this assay ($P > 0.05$).

In the present study it has been demonstrated that aqueous extracts of fruit and vegetable seeds possess antiglycative activity. In addition, the samples displayed concentration-dependent MGO-trapping ability. However, as mentioned above, some of the extracts were found to be inhibitors of AGE formation in BSA-Glc assay, whereas little or no effect was observed when these samples were subjected to BSA-MGO assay (Fig. 1). Since inhibitors of glycation can act in multiple steps, it is important to apply different scenarios such as BSA-Glc and BSA-MGO assays to reach a conclusion on their anti-AGE ability. Consequently, both positive and negative inhibitory effects should not be discounted.

In previous studies, several authors have found correlations between TPC and the inhibitory effect on AGE formation of different extracts.^{6,7} The possible association of AGE inhibition and antioxidant

activity was analysed in our assays. No correlation between ORAC or ABTS and antiglycative activity was found in either BSA–Glc or BSA–MGO assay. Regarding the phenolic compound content, it was not correlated with anti-AGE activity in either absolute or relative amounts. Similar results have been observed by Povichit et al.,¹³ who reported that extracts of certain medicinal plants exhibited high antiglycative activity although they had low phenolic content. In accordance with Sun et al.,⁷ such absence of correlation suggests that phenolic compounds are not the sole antiglycative agents of the selected seed extracts and therefore other compounds present in the samples must be involved in their anti-AGE ability. It should be taken into account

that most authors have investigated the preventive effect on AGE formation through extraction of samples with various organic solvents, which are more efficient than water for extracting phenolic compounds.³⁰ As mentioned above, we carried out our assays using aqueous extracts, which might justify the lack of relationship between phenolic composition and antiglycative activity of the extracts.

Another factor to consider is the individual phenolic acid composition in the different aqueous extracts. In this sense, Wu et al.³¹ evaluated the ability of dietary phenolic acids to inhibit glucose-mediated protein glycation. The authors concluded that at a concentration of 1 mmol L⁻¹ most phenolic acids inhibited the glycation process, with vanillic, gallic and ferulic acids being the most significant inhibitors. According to these results, the high inhibitory activity of sesame and pomegranate in BSA–Glc assay could be explained, as their major phenolic acids were ferulic acid and gallic acid respectively (Table 3, Fig. 1A). Recently, Muthenna et al.³² reported ellagic acid to be a potent antiglycating agent in a protein–sugar system, which, together with gallic acid, could explain the high antiglycative activity of pomegranate extract. Regarding green pepper, its anti-AGE ability in this system could be associated mainly with its ellagic acid content, but in addition with its ferulic acid and phydroxybenzoic acid content, which, although at lower intensity, has also been found to be an antiglycative agent.³¹ The anti-AGE effect of the compounds, however, depends on the tested concentrations; thus low, high or no antiglycative activity has been described for ferulic acid at different incubation conditions in protein–sugar model systems.^{19,33,34} In the BSA–MGO system, Gugliucci et al.³⁵ reported high AGE inhibition by caffeic acid. This may justify apricot extract showing one of the highest antiglycative activities in the present study (Fig. 1B), but it does not justify pomegranate or peach extract activity. It should be highlighted that, in accordance with Chompoo et al.,⁵ the presence of more than one inhibitor in an extract may have a synergistic effect. In this way, Rice-Evans et al.³⁶ reported that phenolic compounds may have antagonistic or synergistic effects with themselves or with other constituents of a sample, which may justify the diversity found among the results of the present study. To the best of our knowledge, antiglycative effects of sesame, green pepper, apricot and peach seeds have not been reported before. Regarding pomegranate seed, anti-glycative ability associated with its free radical-scavenging property has been reported by Rout and Banerjee.³⁷

CONCLUSIONS

Eight aqueous extracts of fruit and vegetable seeds were evaluated for their antiglycative activity using different *in vitro* models. All extracts exhibited positive inhibitory effects on the formation of AGEs in BSA–Glc assay, although to different extents. However, BSA–MGO assay and direct MGO-trapping assay showed that some extracts exerted their activity through a different mechanism of action. Aqueous extracts of peach, pomegranate and apricot seeds appeared to inhibit the formation of AGEs through their capacity for trapping 1,2-dicarbonyls. These findings are relevant for focusing on potential extracts to combat the main promoters of aging in humans. On the other hand, no relationship between antioxidant and phenolic compound content and antiglycative activity of the extracts was found. This finding leads us to conclude that other hydrophilic constituents in addition to phenolic acids must be involved in the antiglycative activity of the aqueous seed extracts. Isolation and characterisation of different target compounds are under current investigation by advanced structural identification methodologies.

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LITERATURE CITED

- 1 Rahbar S and Figarola JL, Inhibitors and breakers of advanced glycation endproducts (AGEs): a review. *Curr Med Chem Immunol Endocr Metab Agents* 2:135–161 (2002).
- 2 Vlassara H and Palace MR, Diabetes and advanced glycation endproducts. *J Intern Med* 251:87–101 (2002).
- 3 Vlassara H, Advanced glycation end-products and atherosclerosis. *Ann Med* 28:419–426 (1996).
- 4 Münch G, Thome J, Foley P, Schinzel R and Riederer P, Advanced glycation endproducts in ageing and Alzheimer's disease. *Brain Res Rev* 23:134–143 (1997).
- 5 Chompoo J, Upadhyay A, Kishimoto W, Makise T and Tawata S, Advanced glycation end products inhibitors from *Alpinia zerumbet* rhizomes. *Food Chem* 129:709–715 (2011).
- 6 Peng X, Cheng KW, Ma J, Chen B, Ho CT, Lo C, et al, Cinnamon bark proanthocyanidins as reactive carbonyl scavengers to prevent the formation of advanced glycation endproducts. *J Agric Food Chem* 56:1907–1911 (2008).
- 7 Sun Z, Peng X, Liu J, Fan KW, Wang M and Chen F, Inhibitory effects of microalgal extracts on the formation of advanced glycation endproducts (AGEs). *Food Chem* 120:261–267 (2010).
- 8 Wang X, Zhang LS and Dong LL, Inhibitory effect of polysaccharides from pumpkin on advanced glycation end-products formation and aldose reductase activity. *Food Chem* 130:821–825 (2012).
- 9 Reddy VP and Beyaz A, Inhibitors of the Maillard reaction and AGE breakers as therapeutics for multiple diseases. *Drug Discov Today* 11:646–654 (2006).
- 10 Peng X, Ma J, Chen F and Wang M, Naturally occurring inhibitors against the formation of advanced glycation endproducts. *Food Funct* 2:289–301 (2011).
- 11 Thornalley PJ, Use of aminoguanidine (Pimagidine) to prevent the formation of advanced glycation end products. *Arch Biochem Biophys* 419:31–40 (2003).
- 12 Williams M, Clinical studies of advanced glycation end product inhibitors and diabetic kidney disease. *Curr Diabetes Rep* 4:441–446 (2004).
- 13 Povichit N, Phrutivorapongkul A, Suttajit M, Chaayasut CC and Leelapornpisid P, Phenolic content and in vitro inhibitory effects on oxidation and protein glycation of some Thai medicinal plants. *Pak J Pharmaceut Sci* 23:403–408 (2010).
- 14 Wang J, Sun B, Cao Y and Tian Y, Protein glycation inhibitory activity of wheat bran feruloyl oligosaccharides. *Food Chem* 112:350–353 (2009).

- 15 Peng X, Zheng Z, Cheng KW, Shan F, Ren GX, Chen F, et al, Inhibitory effect of mung bean extract and its constituents vitexin and isovitexin on the formation of advanced glycation endproducts. *Food Chem* 106:475–481 (2008).
- 16 Ahmad MS and Ahmed N, Antiglycation properties of aged garlic extract: possible role in prevention of diabetic complications. *J Nutr* 136:796S–799S (2006).
- 17 Wu CH and Ten GC, Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts. *J Agric Food Chem* 53:3167–3173 (2005).
- 18 Nakagawa T, Yokozawa T, Teresawa K, Shu S and Juneja LR, Protective activity of green tea against free radical- and glucose-mediated protein damage. *J Agric Food Chem* 50:2418–2422 (2002).
- 19 Wu JW, Hsieh CL, Wang HY and Chen HY, Inhibitory effects of guava (*Psidium guajava* L.) leaf extracts and its active compounds on the glycation process of protein. *Food Chem* 113:78–84 (2009).
- 20 Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al, Measurement of protein using bicinchoninic acid. *Anal Biochem* 150:76–85 (1985).
- 21 Miller GL, Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal Chem* 31:426–428 (1959).
- 22 Singleton VL, Orthofer R and Lamuela-Raventos RM, Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. *Meth Enzymol* 299:152–178 (1999).
- 23 Kim KH, Tsao R, Yang R and Cui SW, Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. *Food Chem* 95:466–473 (2006).
- 24 Ross KA, Beta T and Arntfield SD, A comparative study on the phenolic acids identified and quantified in dry beans using HPLC as affected by different extraction and hydrolysis methods. *Food Chem* 113:336–344 (2009).
- 25 Lamuela-Raventos RM and Waterhouse AL, A direct HPLC separation of wine phenolics. *Am J Enol Vitic* 45:1–5 (1994).
- 26 Abdel-Hameed ESS, Total phenolic contents and free radical scavenging activity of certain Egyptian *Ficus* species leaf samples. *Food Chem* 114:1271–1277 (2009).
- 27 Dávalos A, Gómez-Cordovés C and Bartolomé B, Extending applicability of the oxygen radical absorbance capacity (ORACfluorescein) assay. *J Agric Food Chem* 52:48–54 (2004).
- 28 Delgado-Andrade C and Morales FJ, Unraveling the contribution of melanoidins to the antioxidant activity of coffee brews. *J Agric Food Chem* 53:1403–1407 (2005).
- 29 Lunceford N and Gugliucci A, *Ilex paraguariensis* extracts inhibit AGE formation more efficiently than green tea. *Fitoterapia* 76:419–427 (2005).
- 30 Lapornik B, Prošek M and Wondra AG, Comparison of extracts prepared from plant by-products using different solvents and extraction time. *J Food Eng* 71:214–222 (2005).

- 31 Wu CH, Yeh CT, Shih PH and Yen GC, Dietary phenolic acids attenuate multiple stages of protein glycation and high-glucose-stimulated proinflammatory IL-1 β activation by interfering with chromatin remodeling and transcription in monocytes. *Mol Nutr Food Res* 54:127S–140S (2010).
- 32 Muthenna P, Akileshwari C and Reddy B, Ellagic acid, a new antiglycating agent: its inhibition of N ϵ -(carboxymethyl)lysine. *Biochem J* 442:221–230 (2012).
- 33 Bouřsova I, Martin J, Jahoda řL, Duřsek J, Palička V and Drřsata J, Evaluation of in vitro effects of natural substances of plant origin using a model of protein glycooxidation. *J Pharmaceut Biomed Anal* 37:957–962 (2005).
- 34 Silvan JM, Assar SH, Srey C, del Castillo MD and Ames JM, Control of the Maillard reaction by ferulic acid. *Food Chem* 128:208–213 (2011).
- 35 Gugliucci A, Bastos DHM, Schulze J and Souza MFF, Caffeic and chlorogenic acids in *Ilex paraguariensis* extracts are the main inhibitors of AGE generation by methylglyoxal in model proteins. *Fitoterapia* 80:339–344 (2009).
- 36 Rice-Evans CA, Miller NJ and Paganga G, Structure–antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med* 20:933–956 (1996).
- 37 Rout S and Banerjee R, Free radical scavenging, anti-glycation and tyrosinase inhibition properties of a polysaccharide fraction isolated from the rind from *Punica granatum*. *Bioresour Technol* 98:3159–3163 (2007).

FIGURES AND TABLES

Table 1

Description of the tested seed samples and their solubility in water. ^a

Sample extract ID	Common name	Scientific name	Family	Soluble extract (mg g ⁻¹)
Gp	Green pepper	<i>Capsicum annuum</i>	Solanaceae	194.9 ± 0.64b
Ap	Apricot	<i>Prunus armeniaca</i>	Rosaceae	445.8 ± 22.27de
Ha	Hazelnut	<i>Corylus avellana</i>	Betulaceae	436.2 ± 17.04d
Pe	Peach	<i>Prunus persica</i>	Rosaceae	478.1 ± 16.05e
Sc	Sour cherry	<i>Prunus cerasus</i>	Rosaceae	257.1 ± 29.27c
Se	Sesame	<i>Sesamum indicum</i>	Pedaliaceae	224.6 ± 8.06bc
Al	Almond	<i>Prunus dulcis</i>	Rosaceae	442.8 ± 15.77de
Po	Pomegranate	<i>Punica granatum</i>	Punicaceae	129.8 ± 4.31a

^a Results are expressed as mean ± SD for n = 3. Different letters mean significant differences (p < 0.05).

Table 2Characterization of the aqueous extracts from fruit and vegetable seeds. ^a

Sample extract	pH	Soluble protein (mg g ⁻¹)	Reducing sugars (mg g ⁻¹)	ORAC (μmol TEAC g ⁻¹)	ABTS (μmol TEAC g ⁻¹)	TPC (mg GAE g ⁻¹)
Gp	4.0	219.0 ± 10.3b	93.3 ± 2.75c	359.6 ± 5.16c	91.6 ± 35.4c	10.7 ± 0.3d
Ap	4.5	282.0 ± 19.1cd	71.5 ± 0.67b	145.6 ± 6.36ab	8.9 ± 2.1a	4.8 ± 0.2a
Ha	5.5	383.3 ± 42.3e	34.4 ± 0.30a	144.7 ± 2.83ab	17.3 ± 2.1ab	10.2 ± 0.8d
Pe	5.5	209.0 ± 2.5b	136.1 ± 3.42e	107.4 ± 14.28a	10.4 ± 2.3a	8.8 ± 0.2c
Sc	5.5	330.3 ± 28.5de	201.1 ± 5.29f	300.4 ± 0.35c	4.6 ± 1.6a	7.3 ± 0.7b
Se	6.5	139.7 ± 8.3a	124.0 ± 0.01d	272.9 ± 2.55bc	42.7 ± 2.6b	7.8 ± 0.3bc
Al	6.5	227.6 ± 0.9bc	41.5 ± 0.29a	124.7 ± 1.56a	24.3 ± 5.7ab	10.9 ± 0.1d
Po	8.5	368.4 ± 39.8e	119.6 ± 0.79d	6378 ± 160.9d	232.7 ± 16.1d	22.2 ± 1.1e

^a Gp: green pepper. Ap: apricot. Ha: hazelnut. Pe: peach. Sc: sour cherry. Se: sesame. Al: almond. Po: pomegranate. Results are expressed as mean ± SD for n = 4. Different letters mean significant differences (p < 0.05).

TPC: Total phenolic content.

Table 3Proportion of phenolic acids in each seed extract expressed as percentage. ^a

Sample extract	PHB	SYN	VA	pCU	CA	FA	PCA	GA	GE	SIN	EA
Gp	24.0	2.1	5.2	5.2	5.6	18.7	9.6	nd	nd	5.1	24.4
Ap	3.0	1.8	4.8	13.5	41.3	26.7	5.7	0.7	nd	2.5	nd
Ha	5.6	10.4	32.0	4.7	1.2	3.2	22.2	15.4	nd	5.3	nd
Pe	21.5	7.7	1.9	17.5	2.4	17.2	28.0	1.7	nd	2.0	nd
Sc	20.1	3.5	1.1	16.3	20.8	3.3	14.7	4.1	12.2	4.0	nd
Se	7.7	2.6	0.4	30.1	11.3	39.9	4.6	0.1	nd	3.3	nd
Al	9.8	38.4	2.7	10.3	12.3	7.1	12.5	2.7	nd	4.3	nd
Po	11.8	8.8	0.1	8.3	1.6	4.5	4.2	39.8	nd	1.3	19.6

^a Gp: green pepper. Ap: apricot. Ha: hazelnut. Pe: peach. Sc: sour cherry. Se: sesame. Al: almond. Po: pomegranate. PHB: p-hydroxybenzoic. SYN: syringic acid. VA: vanillic acid. pCU: p-coumaric. CA: caffeic acid. FA: ferulic acid. PCA: protocatechuic acid. GA: gallic acid. GE: gentisic acid. SIN: sinapinic acid. EA: ellagic acid. nd < 2 µg g⁻¹ sample

Table 4MGO trapping capacity of the different seed extracts. ^a

Sample extract	IC ₅₀ values (mg mL ⁻¹)
Gp	1.70
Ap	0.14
Ha	3.30
Pe	0.14
Sc	0.48
Se	2.65
Al	2.25
Po	1.60
PM	0.06

^a Gp: green pepper. Ap: apricot. Ha: hazelnut. Pe: peach. Sc: sour cherry. Se: sesame. Al: almond. Po: pomegranate. PM: piridoxamine.

Figure captions

Figure 1. (A): Antiglycative activity of the different seed extracts on the formation of AGEs in BSA-glucose (Glc) assay. (B): Antiglycative activity of the different seed extracts on the formation of AGEs in BSA-methylglyoxal (MGO) assay. The concentration of each extract was 25 mg mL⁻¹ (final concentration in the reaction medium 3.6 mg mL⁻¹). Results are expressed as mean $\pm$ SD for n = 4. Different letters mean significant differences (p < 0.05). AG solution (final concentration 0.6 mg mL⁻¹) presented an antiglycative activity of 92.7% and 99.2% for BSA-Glc and BSA-MGO assays, respectively. Green pepper (Gp), apricot (Ap), hazelnut (Ha), peach (Pe), sour cherry (Sc), sesame (Se), almond (Al) and pomegranate (Po).

Figure 2. Dose-dependent results for MGO trapping capacity experiment at 168 h of the different seed extracts. (A) MGO scavenging abilities of green pepper (Gp), apricot (Ap), sour cherry (Sc) and hazelnut (Ha). (B) MGO scavenging abilities of peach (Pe), sesame (Se), almond (Al) and pomegranate (Po). Results are expressed as mean $\pm$ SD for n = 4. PM solution (final concentration 0.1 mg mL⁻¹) presented a MGO trapping capacity of 99.6%.

Figure 1

Figure 2