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Original article

Reversal of glycoxidative damage of DNA and protein by antioxidants

Pamela Jha*, Adiba Riyaz Momin, Dinesh Kumar** and Ahmad Ali****
**Amity Institute of Biotechnology, Amity University, Mumbai-410206, Maharashtra, India*
***Department of Life Sciences, University of Mumbai, Vidyanagari, Santacruz (East), Mumbai-400098, Maharashtra, India*

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Abstract

Diabetes is a metabolic and endocrinological disorder. It is a manifestation of hyperglycemia and glucotoxicity for a long period of time. The high glucose concentration leads to secondary complications of diabetes like cataract, cardiovascular and, neurological disorders. The reactive carbonyl group of glucose reacts with amino groups of biomolecules like DNA and proteins leading to their structural alterations. In the present study, effect of non-enzymatic glycation was checked on proteins and the structure of DNA. It was observed that there was an increase in the generation of early and advanced glycation products when BSA was incubated with methylglyoxal. There was a decrease in the amount of glycation products in the presence of most of the antioxidants except sodium acetate. The glycation system caused strand breakage and led to change in the conformation of pBR322 plasmid, from supercoiled to open circular. This structural damage of DNA increased in the presence of $FeCl_3$, indicating the role of metal ions in the generation of free radicals. Antioxidants like sodium azide, eugenol and melatonin significantly reversed the DNA damage, induced by glycation system, as shown by agarose gel electrophoresis. Eugenol and mannitol were able to show significantly high percentage of DPPH inhibition and ABTS, as compared to sodium acetate and sodium azide. The amount of eugenol and mannitol used were able to show similar antioxidant capacity, as compared to 50 µg of vitamin C, a standard and potent antioxidant. It can be concluded from this study that generation of free radicals is the major route for glycation-induced DNA damage as shown by the reversal of damage in the presence of antioxidants. The study indicates the significance of natural antioxidants in the prevention of glycation related diseases.

Key words: Antioxidants, DNA damage, DPPH assay, free radicals, glycation, TEAC assay

1. Introduction

Glycation is a non-enzymatic reaction between the reducing sugars and biomacromolecules. The reaction takes place between the carbonyl group of sugars, such as glucose and the amino group of proteins, lipids and nucleic acids (Ahmed, 2005). This interaction leads to formation of Schiff's base which further gets converted rapidly to amadori products. The chemical rearrangements of Schiff's bases and Amadori products through various processes like oxidation, reduction, hydration, aggregation and precipitation lead to formation of irreversible and more stable advanced glycation end-products (AGEs). These brownish products are also known as maillard products as they were first reported by maillard in 1912 (Kikuchi *et al*., 2003). AGEs accumulate inside and outside the cells and interfere with metabolic reactions and biomolecules function (Brownlee *et al*., 1984). The rate of accumulation of AGEs depends on the concentration and reactivity of sugars as well as duration of interaction between the proteins and sugars. The amount of glycated products increases manifold during the hyperglycemic conditions.

It has been reported that accumulated AGEs are involved in many pathological conditions such as diabetes, cataract, dialysis related amyloidosis (DRA), atherosclerosis, neurodegenerative disorders as well as physiological ageing (Singh *et al*, 2001). The mechanism by which AGEs cause these disorders include the structural alteration of biomolecules, adduct formation and generation of reactive oxygen species. Long shelf life proteins are more prone to glycation by sugars. Glycated proteins lose their function because of structural alterations like cross-linking, aggregation, precipitation and side-chain group interactions (Suji and Sivakami, 2004). Superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), alkoxyl (RO), peroxyl (ROO), hydroxyl radicals (OH) and hypochlorous acid (HOCl) are the some of reactive oxygen species (ROS) produced during metabolism and glycation process (Ahmad *et al*., 2014). These reactive molecular species cause damage to biomolecules especially nucleic acids. The damage to biomolecules due to glycation-induced ROS is known as glycoxidative damage. Glycation leads to strand breaks, unwinding of double helix, mutations and nucleotide-nucleotide cross-links in DNA (Li Pun and Murphy, 2012). The strategies to prevent the accumulation of glycation products include inhibition of interaction between carbonyl and amino groups, progression of Amadori to AGEs and prevention of generation of ROS. Many natural and artificial compounds are known to prevent glycation and glycation-mediated ROS generation (Ali *et al.*, 2014). In the present study, some of the well known antioxidants like mannitol, melatonin, sodium azide, sodium acetate and eugenol have been used to characterise their role in the process of glycoxidation. The effect of these chemicals was checked on the process of glycation, oxidative damage of DNA and their antioxida nt capacity. The results indicate

Author for correspondence: Dr. Ahmad Ali
Assistant Professor, Department of Life Sciences, University of Mumbai, Vidyanagari, Santacruz (East), Mumbai-400098, Maharashtra, India
E-mail: ahmadali@mu.ac.in
Tel.: +91-9870941656

that some of these compounds have the potential to prevent the glycation as well as glycoxidative damage of biomolecules.

2. Materials and Methods

2.1 Materials

Agarose, lysine, methyl glyoxal (MG) and eugenol were purchased from Sigma Aldrich. BSA was procured from VWR Life Science. pBR322 was purchased from Thermo Fisher Scientific. Melatonin, Mannitol, Sodium acetate were purchased from Himedia. Sodium azide was procured from Loba Chem, Mumbai. All the other chemicals used were of high analytical grade.

2.2 Incubation of antioxidants BSA-MG glycation system

Bovine albumin serum (10 mg/ml) and methylglyoxal (20 mM) were incubated with and without presence of antioxidants (melatonin (2 mM), mannitol (250 mM), sodium azide (250 mM), sodium acetate (200 mM), eugenol (6 µM) at 37°C for 7 days. 100 mM phosphate buffer (pH 7.4) and 3 mM sodium azide were also added in all incubated samples for maintaining pH and antibacterial activity, respectively. These all incubated reaction mixtures were analysed for glycation study.

2.3 Effect of antioxidants on the generation of early glycation products

The amount of early glycation products were measured according to the earlier methods with minor modification (Banan and Ali, 2016). 100 µl of 0.5 mM NBT prepared in 100 mM carbonate buffer (pH 10.4) was mixed with glycated samples (10 µl) and was kept for incubation at 37 °C for 15 min. Then assay mixture volume raised to 1 ml with distilled water to make sufficient volume for absorbance. The intensity was measured at 530 nm using Shimadzu UV-Vis 1800 spectrophotometer.

2.4 Effect of antioxidants on the carbonyl content

Protein oxidation method was used to measure the amount of advanced glycation end products as per the methods mentioned in a previous paper (Banan and Ali, 2016). Glycated sample (0.1 ml) was incubated with 0.4 ml of 10 mM DNPH in 2.5 M HCl in dark for an hour. 0.5 ml of 20% TCA was added and kept in ice for 5 min. and then centrifuged at 10000 X g at 4°C for 10 min. Pellet obtained was washed with 0.5 ml of ethanol: ethyl acetate (1:1) for thrice and then resuspended in 0.1 ml of 6 M guanidine hydrochloride. The volume made upto 1 ml with distilled water and intensity was recorded at 370 nm using Shimadzu UV-Vis 1800 spectrophotometer.

2.5. Incubation of glycated DNA with antioxidants

0.5 µg of pBR322 plasmid was incubated for 3 h at 37°C with lysine (200 mM), MG (20 mM) and $FeCl_3$ (100 µM) in the presence or absence of antioxidants [melatonin (2 mM), mannitol (250 mM), sodium azide (250 mM), sodium acetate (200 mM), and eugenol (6 µM)].

2.6. Effect of maillard reaction on DNA

Maillard products are generated at a rapid rate when the sugars and amino groups are incubated at higher temperature. The effect of these products was checked on the structure of DNA and the reaction was set up as per the methods reported by Ali and Sharma (2015). 0.5 µg of pBR322 plasmid incubated with Mailliard products (Lysine and glucose) with and without presence of $FeCl_3$, and sodium azide for 3 h at 100°C. Mailliard product along with $FeCl_3$ and sodium azide was freshly incubated with pBR322 and no incubation for 3 h at 100°C.

2.7 Analysis by agarose gel electrophoresis

10 µl of glycated samples were mixed with 2 µl 6X gel loading dye and whole mixture was loaded on to 1% agarose gel. Agarose gel electrophoresis was performed at 100 V till the dye band ran 3/4th of the gel length. Subsequently, the gel was stained with ethidium bromide (5 µg/ml) for 20 min. The gel was visualized under UV Transilluminator.

2.8 DPPH Assay

The samples were incubated for 3 h at 37°C with lysine (200 mM), MG (20 mM) and $FeCl_3$ (100 µM) in the presence or absence of antioxidants [melatonin (2 mM), mannitol (250 mM), sodium azide (250 mM), sodium acetate (200 mM), and eugenol (6 µM)].The free radical scavenging activity was estimated using DPPH as described by Schlesier *et al*. (2002) . The assay mixture contains 0.5 ml of 0.3 mM DPPH solution and 0.1 ml respective samples. The mixture was kept in dark at 37°C for 30 min. The absorbance was measured at 517 nm by spectrophotometer. The ability to scavenge DPPH radical was calculated using following formula:

$$\% \text{ Inhibition} = [(A_{control} - A_{sample}) / (A_{control})] \times 100$$

2.9 Trolox equivalent antioxidant capacity (TEAC) assay

The antiradical properties of the samples, mentioned in DPPH assay were also determined using the TEAC assay. The TEAC assay is based on the reduction of the 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation by antioxidants and was adapted with minor modifications from Madhujith *et al*. (2006). ABTS+radical cation was prepared by mixing ABTS stock solution (7 mM in water) with 2.45 mM potassium persulfate ($K_2S_2O_8$). This mixture was kept in dark for 12-24 h until the completion of reaction which was checked by the stability of the absorbance in the range of 0.700 ± 0.030 at 734 nm. Glycated sample (1 ml) was allowed to react with the ABTS solution (1 ml) and the absorbance was taken at 734 nm after 7 min, using a spectrophotometer (Shimadzu UV1800). Appropriate solvent blanks were run in each assay. The scavenging capacity of glycated samples was compared with that of vitamin α-C and percentage inhibition calculated as ABTS radical scavenging activity:

$$\% = [(A_{control} - A_{sample}) / (A_{control})] \times 100$$

where $A_{control}$ is the absorbance of ABTS radical + methanol; A_{sample} is the absorbance of ABTS radical + glycated sample/standard.

3. Results

3.1 Effect of antioxidants on the generation of early glycation products

The formation of unstable Schiff bases through interaction between proteins and reducing sugar was transformed into stable early glycation products. The amount of fructosamine content declined significantly in the presence of sodium azide (14.92%), sodium acetate (6.63%), melatonin (8.45%) and eugenol (10.59%). However, there was no effect observed in decrement of early glycation products formation in the presence of mannitol (Figure 1).

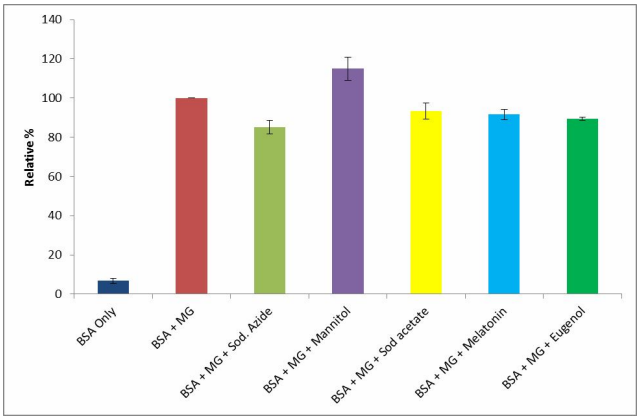

Figure 1: Effect of antioxidants on the generation of early glycation products.

BSA (10 mg/ml) was incubated with MG (20 mM) along with the presence/absence of antioxidants [melatonin (2 mM), mannitol (250 mM), sodium azide (250 mM), sodium acetate (200 mM), eugenol (6 µM)]. Phosphate buffer (100 mM, pH 7.4) and sodium azide (3.0 mmol/l) also added and kept at 37°C for 7 days. Glycated samples are used for measurement of fructosamines content with NBT method. Absorbance was measured at 530 nm post-incubation. BSA along with MG was considered as control (100%) for calculating the relative percentage. Results are expressed as Means ± SEM (n =3), Statistical significance, p<0.05. (BSA-bovine albumin serum, MG-methyl glyoxal).

3.2 Effect of antioxidants on the carbonyl content

The carbonyl assay indicated its reduction in the presence of sodium azide (10.06%), sodium acetate (13.49%), melatonin (22.08%) and eugenol (2.48%). However, there was no decrease in the formation of carbonyl content of the glycated sample in the presence of mannitol (Figure 2).

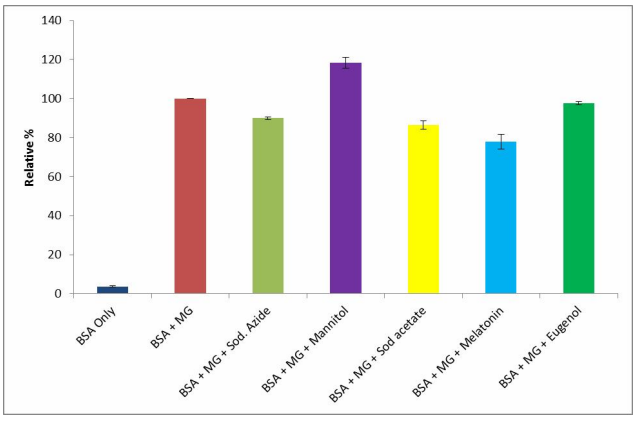

Figure 2: Effect of antioxidants on the carbonyl content.

BSA (10 mg/ml) was incubated with MG (20 mM) along with the presence/absence of antioxidants [melatonin (2 mM), mannitol (250 mM), sodium azide (250 mM), sodium acetate (200 mM), eugenol (6 µM)]. Phosphate buffer (100 mM, pH 7.4) and sodium azide (3.0 mmol/l) also added and kept at 37°C for 7 days. Glycated samples are used for measurement of carbonyl content with DNPH method. Absorbance was measured at 370 nm. BSA along with MG was considered as control (100%) for calculating the relative percentage. Results are expressed as Means ± SEM (n =3), Statistical significance, p<0.05. (BSA- bovine albumin serum, MG-methyl glyoxal).

3.3 Effect of antioxidants on the glycated DNA (pBR322)

The incubation of DNA (pBR322) with methyl glyoxal and lysine caused strand breaks and resulted in an increase in open circular form (Figure 3; Lane 3) as compared to unglycated DNA (Figure 3; Lane 1). The presence of antioxidants like sodium azide (Figure 3; Lane 4), melatonin (Figure 3; Lane 6) and eugenol (Figure 4; Lane 5) reversed the damage to DNA. On the other hand there was no damage reversal of glycated DNA in the presence of melatonin (Figure 3; Lane 5) and sodium acetate (Figure 3; Lane 7).

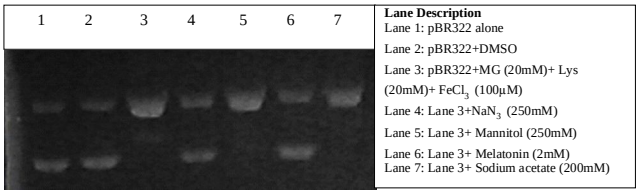

Figure 3: Effect of antioxidants on DNA damage.

pBR322 (0.5 µg) was incubated with MG (20 mM) and $FeCl_3$ (100 µM) in the presence or absence of antioxidants for 3 h at 37°C. Incubated samples were loaded onto 1% agarose gel. Gel was stained with ethidium bromide(5µg/ml) and visualized on a UV transilluminator.

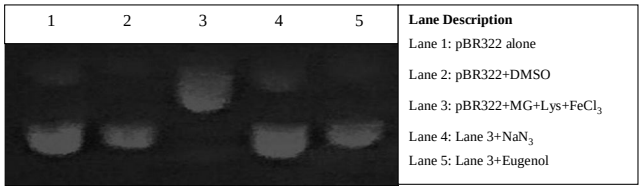

Figure 4: Effect of eugenol on DNA damage.

pBR322 (0.5 µg) was incubated with MG (20 mM) and $FeCl_3$ (100 µM) in the presence or absence of antioxidants for 3 h at 37°C. Incubated samples were loaded onto 1% agarose gel. Gel was stained with ethidium bromide (5µg/ml) and visualized on a UV transilluminator.

3.4 DNA damage by Mailliard products

The Mailliard product formed during the heating process of lysine and glucose was checked for DNA damage. It was observed that Mailliard products caused significant DNA damage as indicated by the conversion of supercoiled DNA to circular DNA (Figure 5, Lane 2). However, $FeCl_3$ did not enhance the damage to DNA (Figure 5, Lane 4). This damage was reversed by sodium azide (Figure 5, Lane 5).

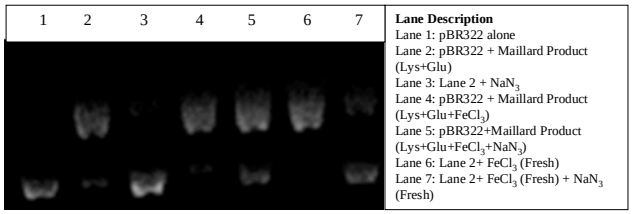

Figure 5: DNA damage by Mailliard reaction.

0.5 µg of pBR322 plasmid incubated with Mailliard products (lysine-20 mM and glucose-100 mg/ml) with and without presence of $FeCl_3$ (100 µm), and sodium azide (250 mM) for 3h at 100°C. Mailliard product along with $FeCl_3$ and sodium azide was freshly incubated with pBR322 and no incubation for 3 h at 100°C. Incubated samples were loaded onto 1% agarose gel. Gel was stained with ethidium bromide (5 µg/ml) and visualized on a UV transilluminator.

3.5 DPPH assay

The inhibition of % DPPH was calculated with vitamin C as positive control. As per the graph shown in Figure 6, the inhibition percentage was found to be maximum in eugenol (27.5 %) which is comparable to vitamin C (31%). The other antioxidants were also found to have significant inhibition of DPPH radical, *viz*., in presence of sodium azide (22%), mannitol (26%) and melatonin (23%). However, there was negligible inhibition in the presence of with sodium acetate (6%).

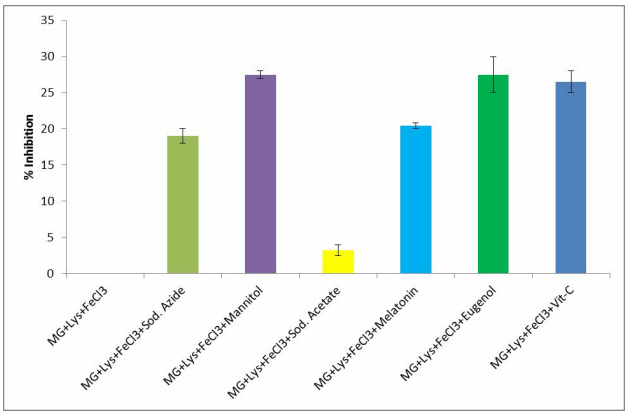

Figure 6: DPPH assay.

Lysine (20 mM, MG (20 mM) and $FeCl_3$ along with antioxidants [melatonin (2 mM), mannitol (250 mM), sodium azide (250 mM), sodium acetate (200 mM), eugenol (6 µM)] at 37°C for 3 h. Glycated samples are used for antioxidant activity in DPPH method. Absorbance was measured at 517 nm and % inhibition was calculated. Results are expressed as Means ± SEM (n = 2), Statistical significance, $p<0.01$.

3.6 Trolox equivalent antioxidant capacity (TEAC) assay

As per the graph shown in Figure 7, the inhibition percentage was found to be maximum in eugenol (39%) which is comparable to vitamin C (25.5%). The other antioxidants were also found to have significant inhibition of DPPH radical, *viz*., in presence of sodium azide (9%), mannitol (21%) and melatonin (19%). However, there was negligible inhibition in the presence of with sodium acetate (4%).

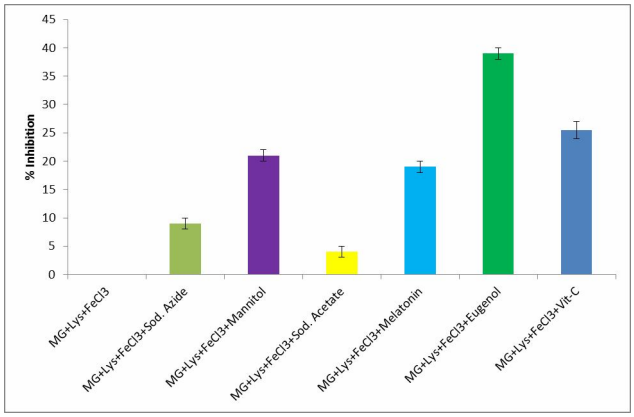

Figure 7: Trolox equivalent antioxidant capacity (TEAC) assay.

Lysine (20 mM, MG (20 mM) and $FeCl_3$ along with antioxidants [melatonin (2 mM), mannitol (250 mM), sodium azide (250 mM), sodium acetate (200 mM), eugenol (6 µM)] at 37°C for 3 h. Glycated samples are used for antioxidant activity in TEAC method. Absorbance was measured at 734 nm and % inhibition was calculated. Results are expressed as Means ± SEM (n = 2), Statistical significance, $p<0.01$.

4. Discussion

Hyperglycemia is the major causative factor of diabetes. The blood glucose level crosses the normal level and becomes toxic to the cells and tissues, a condition known as glucotoxicity. An increased level of glucose leads to increased formation of advanced glycation end products. These AGEs cause structural alteration of most of the biomolecules and lead to their loss of function. As a result of this glucotoxicity and increased AGE level, the individual suffers from secondary complications of diabetes and neurodegenerative disorders (Parbhakar, 2016). The generation of oxidative species is the part of normal metabolism, but it get enhanced in hyperglycaemic condition and other pathological complications (Wang *et al*., 2016). Many group of researchers have made efforts to develop a drug which can prevent the formation of AGEs or reduce their harmful effects. Some of the artificial and natural compounds have been used as antiglycating agent (Rahbar and Figarola, 2003). Antioxidants have been also shown to be effective partially in preventing the glycation mediated damage to biomolecules (Sadowska-Bartosz and Bartosz, 2015).

In the present study, the effect of some commonly used natural compounds (eugenol, melatonin, mannitol, sodium acetate and sodium azide) was checked on the process of glycation of proteins. The amount of glycation products were measured in the presence and absence of these compounds by established methods like NBT assay and protein oxidation methods. Most of the compounds could reduce the amount of both early and advanced glycation end products. However, there was no effect of mannitol on the formation of glycation products. These observed antiglycating effects may be due to their inhibitory role in the formation of AGEs, metal-chelation or antioxidant properties of these compounds (Sadowska-Bartosz and Bartosz, 2015).

In the next step, the role of these compounds in process of glycoxidation was analysed using pBR322 as a model DNA system. The conformation of DNA changed from supercoiled to open circular in the presence of glycation system (lysine and methylglyoxal). It has been shown earlier that the presence of a metal ion increases the damage of the DNA by glycation (Ali *et al*., 2014, Ali and Sharma, 2015). There was complete reversal or prevention of the glycation-induced damage of DNA in the presence of most of the compounds used in the study except mannitol and sodium acetate. Most of these compounds are known to possess antioxidant properties. These results indicate that glycation-mediated DNA damage is due to the generation of reactive oxygen species in the system. However, when the metal ion was used alongwith Maillard products (generated by boiling the mixture of lysine and glucose for three hours), there was no significant increase in the damage of the DNA. The order of addition of metal ion (before and after boiling) did not have any difference on the results. This may be due to enough free radicals generated during the process of boiling as compared to the earlier experiment which was carried out at 37°C.

It can be clearly seen from the results discussed in previous sections that most of these compounds have significant role in prevention of glycation and glycoxdative damage of DNA. Experiments were

designed to quantify the antioxidant potential of these compounds by DPPH and ABTS assays. It can be interpreted from the results of both the assays that most of the compounds have significant antioxidant potential. It was observed that eugenol shows the best antioxidant potential as compared to other antioxidants used in this study. In fact, the concentration of eugenol was lowest among the compounds used in the present study. In another study, eugenol was shown to have significant inhibition of lipid peroxidation even at a very low concentration (Gülçin, 2011). On the other hand, sodium acetate could not inhibit the generation of free radicals. Coincidentally, sodium acetate did not prevent the formation of glycation products. The active role of antioxidants has been established in inhibition of glycation process by acting as radical trapping agents (Khalifah *et al*., 1999).

5. Conclusion

AGEs have the propensity to generate reactive oxygen species and further accelerate the protein glycation under hyperglycemic conditions in diabetic patients Some compounds can specifically inhibit the formation of glycation products at early stage and some others at the later stage. An ideal drug should be able to inhibit the AGE formation as well as scavenge the free radicals generated during the process of glycation. We report here a positive correlation between the antioxidants and their antiglycating potential of most of the compounds used in the study against *in vitro* glycation system. The mechanism of action of these compound can be further characterised for their applications as a drug for the treatment of secondary complications of diabetes.

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Conflict of interest

We declare that we have no conflict of interest.

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