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Preventive effect of phenolic acids on *in vitro* glycation

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Abstract

The non enzymatic reaction between the carbonyl group of sugars and amino group of proteins, lipids and nucleic acids is termed as glycation. It has been linked to various diseases such as diabetes, cataract, Alzheimer's, dialysis related amyloidosis, atherosclerosis, Parkinson's as well as physiological ageing. In the present study, the progress of glycation was assessed, using methods for the measurement of browning, periodate, fructosamine and carbonyl content. It was found that phenolic acids (gallic acid, cinnamic acid and ferulic acid) caused a decrease in the production of amadori products and carbonyl content and had no significant effect on browning of glucose-lysine mixture, standard glycation reaction. When the effect of gallic acid was checked on the glycated DNA sample, it caused the DNA damage alone and did not enhance the damage of glycated DNA sample. This study is significant as far as understanding the mechanism of phenolic acids on *in vitro* glycation is concerned.

Key Words: Advanced glycation end products (AGEs), carbonyl content, DNA damage, glycation, Maillard reaction, phenolic

1. Introduction

Glycation is a spontaneous process between the sugars and proteins, lipids and nucleic acids. It is a non-enzymatic reaction wherein an interaction takes place between an amino group and a carbonyl group (Ahmed, 2005). It is also one of the post translational modification processes between the sugars and the free amino groups of proteins (Monnier and Cerami, 1981). The research on glycation began with the discovery by Maillard (1912) who observed that heating of amino acids with a reducing sugar results in yellowish brown colour products (Kikuchi *et al*., 2003).

Glycation reaction occurs in three stages: (i) the early, (ii) the intermediate and (iii) the late stage (Ahmed, 2005; Singh *et al*., 2014). In the early and reversible stage, amadori products (or Haynes products) are formed, rearrange themselves and get converted to more stable and irreversible advanced glycation end products (AGEs). In the first stage, the carbonyl group of the sugars attaches to the free amino group to form a Schiff's base non-enzymatically. In the second stage, the Schiff's base undergoes rearrangement to form the Amadori products, also known as the early glycation products. These products are more stable but the reaction is reversible. In the third and irreversible stage, the Amadori products rearrange themselves through complicated chemical rearrangements like oxidation, reductions and hydration and form cross linked proteins. The final brownish product formed is called as the advanced glycation end products (AGEs). They are very stable and accumulate inside and outside the cells and interfere with protein function (Brownlee *et al*., 1984).

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The process of glycation occurs continuously in the body even at the normal blood glucose levels and the damage caused by the glycation products, keeps on accumulating slowly overtime (Gkogkolou and Böhm, 2012). When the blood glucose level increases above the normal level in conditions like hyperglycemia, the deleterious effects of these products are more significantly observed. The process of glycation has also been linked to various diseases such as diabetes, cataract, Alzheimer's, dialysis related amyloidosis (DRA), atherosclerosis, Parkinson's as well as physiological aging (Thorpe and Baynes, 1996). However, it is still not clear that which AGE(s) is/are involved in these complications and what is the mechanism. The complex process of glycation alters the biological activities of proteins, their metabolic processes and inhibits the specific functions of proteins through cross linking, aggregation, precipitation and produce reactive oxygen species (ROS) (Suji and Sivakami, 2004). A large amount of reactive oxygen species (ROS) is produced as a result of subsequent modifications of Amadori products and AGEs and this includes superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), alkoxyl (RO), peroxyl (ROO), hydroxyl radicals (OH) and hypochlorous acid (HOCl) (Ali *et al*., 2014).

The non-enzymatic glycosylation of DNA *in vitro* affects both physical and biological properties of the DNA. AGE product N^{ε}-(carboxymethyl) lysine (CML) accumulates in nuclear proteins like histone, causing extensive DNA strand cleavage (Roberts *et al*., 2003). Nucleic acid can be damaged during glycation in 2 different ways (Pischetsrieder *et al*., 1999). Maillard reaction product can cause DNA strand breakage and act as mutagen (Hiramoto *et al*., 1997). Hydroxyl radical scavengers, spin trap agents, and metal chelators can suppress this damage, indicating that it is mediated by ROS generated by Maillard products. Glycation of DNA can have multiple effects such as strand breaks, unwinding of the double helix, mutations and formation of DNA-protein and nucleotide-nucleotide cross-links (Li Pun and Murphy, 2012).

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Plants are rich in phytochemicals which have been characterized and used in various pharmaceutical industries. Among these phytochemicals, phenolic acids are important non-vitamin antioxidants, naturally present in almost all vegetables and fruits. The biological activity of these phenolic acids is due to their lipophilicity and is affected by the presence of ring substituent hydroxyl groups. The length of the ester moiety is also very important for polyhydroxylated phenolic esters (Tsao, 2010). In our study, we have used cinnamic acid, ferulic acid and gallic acid to check their effect on glycation *in vitro*. A glycation system consisting of glucose and lysine was used to check the parameters like browning, formation of early and middle stage glycation products in the presence and absence of phenolic acids. Results indicate the significant role of these acids in the inhibition of carbonyl content. Although they had no significant effect on browning, the amount of Amadori products generated was significantly decreased in the presence of phenolic acids. In a similar study, the effect of gallic acid was also checked on the glycated DNA sample. It was found that it caused the DNA damage alone and did not enhance the damage of glycated DNA sample. These results indicate the differential roles of phenolic acids in the process of *in vitro* glycation.

2. Materials and Methods

2.1 Materials

Agarose, lysine, methyl glyoxal (MG) and sodium periodate were purchased from Sigma Aldrich. Glucose and trichloroacetic acid (TCA) were procured from Merck India Ltd. The phenolic acids were supplied by SRL, India. pBR 322 was purchased from Thermo Fisher Scientific. All the other chemicals used were of high analytical grade.

2.2 Incubation of phenolic acids with *in vitro* glycation system

Equimolar mixtures of glucose (0.5 M) and lysine (0.5 M) were incubated at 37°C for 14 days and used as standard glycated sample. This glycated sample was incubated with and without phenolic acids (cinnamic acid, ferulic acid and gallic acid - 5 µg/µl)) in 100 mM phosphate buffer pH 7.4 and 0.03% sodium azide at 37°C for 14 days. The extent of browning was measured at 420 nm, using Shimadzu UV 1800 spectrophotometer and relative absorbance was used to plot the graph.

2.3 Periodate oxidation assay

Glycation (Amadori product) was measured by the periodate oxidation method (Ali and Devrukhkar, 2016). 1ml glycated samples were incubated with 0.1 ml of HCl (0.1 M) and 0.1 ml of $NaIO_4$ (0.05 M) for 30 min. at 37°C. To terminate the oxidation, the samples were cooled on ice for 10 min. and mixed with 0.1 ml pre-cooled $ZnSO_4$ (15%) and NaOH (0.7 M). The samples were vortexed throughout the addition and were centrifuged for 10 min. at 9000 X g to remove precipitated zinc periodate. An aliquot of 0.5 ml of the supernatant was transferred to fresh microcentrifuge tubes. Formaldehyde detection reagent was freshly prepared by mixing 46 µl of acetylacetone in 10 ml of 3.3 M ammonium acetate. 1 ml of this reagent was added to each tube and diacetyldihydrolutidine (DDL) was allowed to develop by incubating at 37°C for 1 h. Absorbance was measured at 405 nm, using Shimadzu UV 1800 spectrophotometer and relative absorbance was used to plot the graph.

2.4 Fructosamine assay

Glycated sample (10 ul) was incubated with 0.5 mM NBT (100 ul) in 100 mM carbonate buffer (pH 10.4) at 37°C for 15 min. Absorbance was measured at 530 nm, using Shimadzu UV 1800 spectrophotometer and relative absorbance was used to plot the graph.

2.5 Carbonyl measurement

Glycated sample (0.1 ml) was incubated with 0.4 ml of 10 mM DNPH in 2.5 M HCl in dark. 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 0.5ml of ethanol: ethyl acetate (1:1) for three times and then resuspended in 0.25 ml of 6 M guanidine hydrochloride. Absorbance was taken at 370 nm, using Shimadzu UV 1800 spectrophotometer and relative absorbance was used to plot the graph.

2.6 Incubation of glycated DNA with Gallic acid

0.5 µg of pBR322 plasmid in 100 mM phosphate buffer at pH 7.4 was incubated for 3h at 37°C with lysine (200 mM), MG (200 mM) and $FeCl_3$ (100 µM) in the presence or absence of gallic acid (0.1 µg/µl, 1 µg/µl, and 2.5 µg/µl).

2.7 Analysis by agarose gel electrophoresis

10 µl of samples were mixed with 2 µl 6X gel loading dye and 12 µl of the solution was loaded on to 1% agarose gel. Agarose gel electrophoresis was performed at 80 V till the dye band ran 3/4th of the gel length. Subsequently, the gel was stained with ethidium bromide (1.5 ml of 1 mg/ml EtBr in 300 ml tank buffer) for 20 min. The gel was visualized under UV transilluminator.

3. Results

3.1 Effect of phenolic acids on glycation of lysine

Lysine was glycated with glucose at 37°C for 14 days in a water bath. Phenolic acids were also incubated at the same temperature either alone or along with glycated system. When the absorbance was measured at 420 nm (ëmax for browning), it was found that all the compounds alone had no significant change in the absorbance. However, there was marginal increase in the browning of glucose-lysine mixture in the presence of phenolic acids (Figure 1).

3.2 Effect of phenolic acids on the production of early glycation products

Figure 2 shows the amount of early glycation product, formed by lysine-glucose-phenolic system in comparison to the positive control containing glucose and lysine. The amount of early glycation product was significantly high in the presence of phenolic acids as compared to glucose-lysine mixture.

3.3 Effect of phenolic acids on the production of fructosamines

The reaction between carbohydrate and protein forms unstable Schiff bases that are converted to stable ketoamine products (fructosamine). The amount of fructosamine decreased significantly in the presence of cinnamic acid and gallic acid while there was only a slight decrease in the presence of ferulic acid (Figure 3).

3.4 Effect of phenolic acids on the carbonyl content

It can be seen from Figure 4 that all the three phenolic acids caused a significant decrease in the formation of carbonyls in the glycated sample of glucose and lysine. The most significant decrease was observed in the presence of gallic acid.

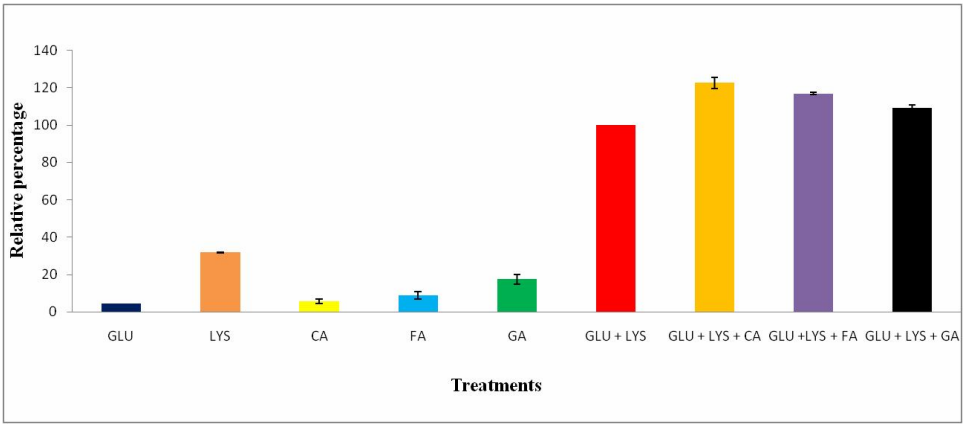

Figure 1: Measurement of browning in the presence of phenolic acids
Glucose and Lysine were incubated with phenolic acids at 37°C for 14 days. The absorbance was taken at 420 nm and tube containing glucose-lysine was used as standard (100%). GLU-Glucose, CA-Cinnamic acid, FA-Ferulic acid, GA-Gallic acid.

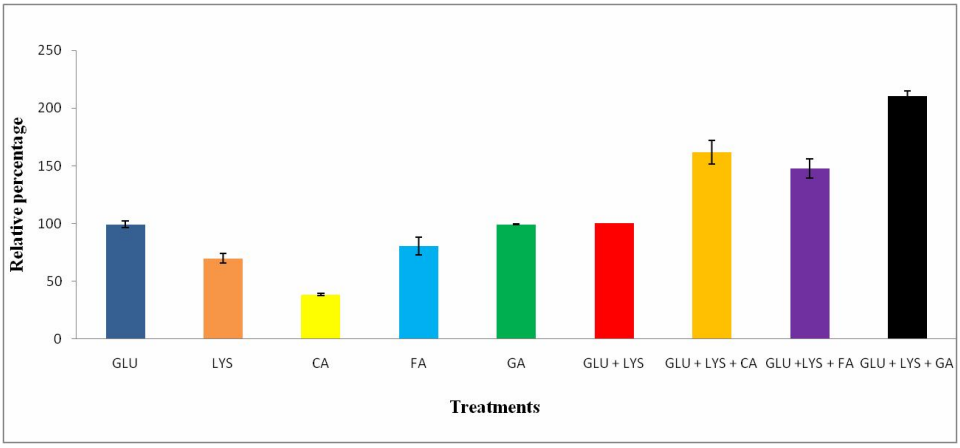

Figure 2: Measurement of early glycation product by periodate method
The amount of periodate was measured in the glycated sample in the presence or absence of phenolic acids. Absorbance was taken at 405 nm and relative absorbance was plotted on the graph using glucose-lysine tube as the standard (100%). GLU-Glucose, CA-Cinnamic acid, FA-Ferulic acid, GA-Gallic acid.

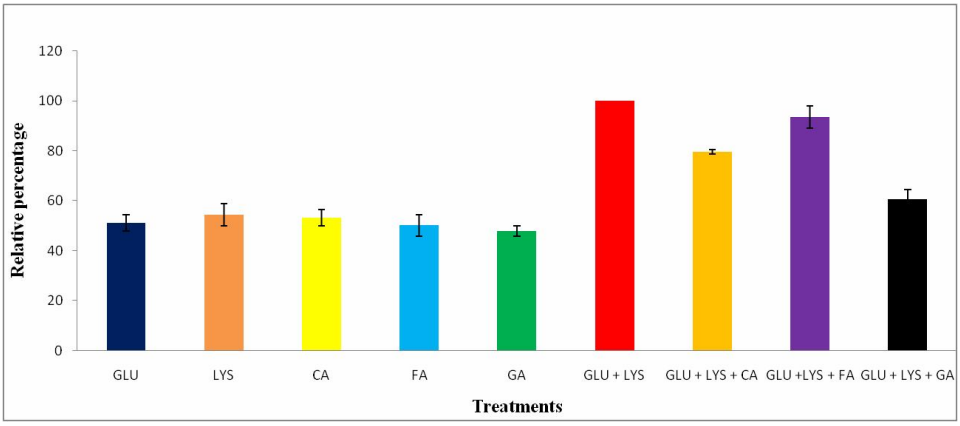

Figure 3: Measurement of fructosamines
The amount of fructosamines was measured in the glycated sample in the presence or absence of phenolic acids. Absorbance was taken at 530 nm and relative absorbance was plotted on the graph using glucose-lysine tube as the standard (100%). GLU-Glucose, CA-Cinnamic acid, FA-Ferulic acid, GA-Gallic acid.

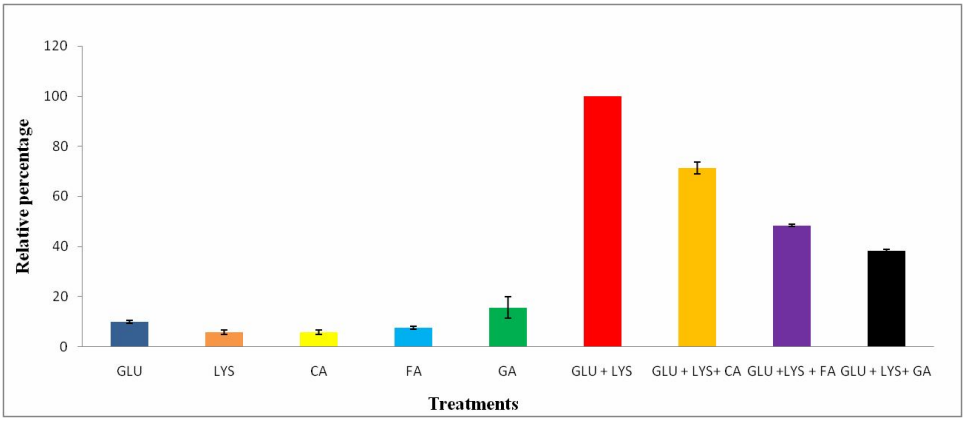

Figure 4: Measurement of carbonyl content

The amount of carbonyl content was measured in the glycated sample in the presence or absence of phenolic acids. Absorbance was taken at 370 nm and relative absorbance was plotted on the graph using glucose-lysine tube as the standard (100%). GLU-Glucose, CA-Cinnamic acid, FA-Ferulic acid, GA-Gallic acid.

3.5 Effect of Gallic acid on the glycated DNA (pBR322)

The incubation of DNA (pBR322) with MG and lysine caused strand breaks and resulted in an increase in open circular form (Figure 5; Lane 5) as compared to unglycated DNA (Figure 5; Lane 1). The incubation of DNA with gallic acid also caused a damage to pBR322 but less significant than glycated sample (Figure 5; Lanes 2, 3, 4 and 5). When glycated DNA was incubated with glallic acid there was no enhancement in damage observed due to the process of glycation (Figure 5; Lanes 5, 6, 7 and 8).

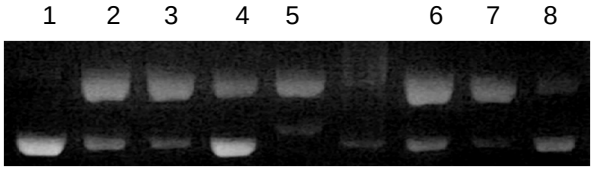

Figure 5: Analysis of glycated pBR322 in the presence of gallic acid

pBR322 (0.5 µg) was incubated with lysine (20 mM), MG (20 mM) and $FeCl_3$ (100 µM) in the presence or absence of different concentrations of gallic acid for 3 hrs at 37 ºC. Incubated samples were loaded onto 1% Agarose gel. Gel was stained with Ethidium bromide and visualized on a UV transilluminator.

Lane Description
Lane 1: pBR322 alone
Lane 2: pBR322 + Gallic acid (0.1 µg/µL)
Lane 3: pBR322 + Gallic acid (1 µg/µL)
Lane 4: pBR322 + Gallic acid (2.5 µg/µL)
Lane 5: pBR322 + MG +Lys+ $FeCl_3$
Lane 6: Lane 5 + Gallic acid (0.1 µg/µL)
Lane 7: Lane 5 + Gallic acid (1 µg/µL)
Lane 8: Lane 5 + Gallic acid (2.5/µg/µL)

4. Discussion

The formation and accumulation of AGEs are major factors in the development of diabetic complications, atherosclerosis, Alzheimer's disease and the normal ageing process. Age-related diseases exhibit increased levels of glycation and its end products, further supporting the idea that sugars and their metabolites may act as damaging molecules, especially when they accumulate in the cells and tissues. Major mechanisms by which AGEs cause damage to the biomolecules include aggregation, precipitation or through ROS formation. Qualitative and quantitative methods have been developed in last few decades to detect the formation of glycation products (Friendman, 1996). These methods include an observation in the change of the color of the glycated sample (browning) and taking absorbance at 420 nm. Some other biochemical methods include the periodate oxidation, fructosamine assay and measurement of carbonyl content of the samples. Recently advanced techniques like spectroflourimetry, HPLC, and LC-MS have found application in the quantification of AGEs (Poulsen *et al*., 2013). There are reports which suggest the availability of artificial and natural compounds that inhibit the AGE formation (Rahbar and Figarola, 2003). These inhibitors can interfere with formation of AGEs at several stages: early, mid and late. Antioxidants are also being used to prevent the formation of AGE-mediated free radicals.

Initially, the formation of maillard product (browning) was detected by incubating a mixture of phenolic acids with lysine and glucose. Glycation system, glucose+lysine showed an increase in intensity of browning with increasing incubation time. In the presence of phenolic acids, there was no significant change in the intensity of browning. It has been reported earlier also that aminoguanidine inhibited the process of glycation without influencing the browning of the glycated system (Requena *et al*., 1993). The effect of phenolic acids was checked on the generation of early glycation products in the previously mentioned system of glucose+lysine. As reported in the results, phenolic acids caused an increase in the early glycation products. This method has advantages as well as disadvantages over other methods. One of the major disadvantage of periodate oxidation method is the nonspecific reaction with endogenous oxidizable moieties, for example, aldehydes or ketones (Roth *et al*., 2012). These oxidizable groups are also present in phenolic acids.

Amadori products are formed as a result of stabilization of first intermediates, Schiff's bases, of glycation process. Fructosamine assay is more sensitive method to measure these early glycation products. Among the three phenolic acids gallic acid, caused most significant decrease in the formation of Amadori products. Cinnamic

acid and ferulic acid also caused decrease in the formation of these products. Adisakwattana *et al* (2014) have also reported the decrease in the formation of fructosamines by more than 50% by the *Mesona chinensis* Benth extract containing phenolic acids.

Carbonyls are generated as a result of modification of Amadori products and the carbonyl content increases with increase in the glycation process. Gallic acid caused decrease in the carbonyl content of glucose+lysine system by more than 2.5-fold. Similarly, the presence of cinnamic and ferulic acids also caused a significant decrease in the carbonyl content but less as compared to gallic acid. A recent report also suggests that have reported that polyphenols present in pomegranate caused more decrease in the carbonyl content than the well known antiglycating agent, aminoguanidine (Liu *et al*., 2014).

All the results presented here indicate the role of phenolic acids in the reduction of glycation products. Gallic acid seems to be most potent as antiglycating agent. There are many reports which suggest a similar role for phenolic acids in the prevention of AGEs (Chih-Yu *et al*., 2011; Ramkissoon *et al*., 2013; Chen *et al*., 2016). However, in most of the earlier reports plant extracts have been used where equivalents of gallic acid were used to check the effect of these compounds. This is a preliminary study wherein pure phenolic acids have been used and their effect on the glycation of DNA and protein were assessed.

Gallic acid, the most potent phenolic acid in this study, was also used to evaluate the role of phenolic acids in the glycation of DNA. For this purpose, plasmid DNA, pBR322, was used as it is a sensitive indicator of single strand breaks, arising due to damage by glycation and free radicals (Levi and Werman, 2001). Gallic acid caused damage to the plasmid alone, *i.e*., even in the absence of glycating system, MG and lysine. Also, it did not enhance the damage of metal-induced glycated DNA. Recently, it has been shown that gallic acid induced DNA damage in human oral cancer SCC-4 cells (Weng *et al*., 2015). The results presented in previous studies suggest that DNA strand breakage is induced only by co-incubation of lysine and MG (Ali *et al*., 2014; Ali and Sharma, 2015) indicating that free radicals formed during the glycation reaction damage DNA.

5. Conclusion

AGEs are continuously generated in the body during normal metabolic processes. The effect of these molecules is observed when there is an increase in the production during hyperglycemia. Several mechanisms have been suggested to prevent the formation of AGEs and their resultant damaging potential inside the body. Phenolic acids have been used as natural products for many pharmaceutical and therapeutic purposes since long. We report here the role of these compounds in the prevention of AGEs. Gallic acid was found to be the most potent AGE inhibitor among the three phenolic acids used. This is a preliminary work wherein attempt has been made to characterize the significance of these compounds in the glycation process. Further work needs to be carried out to understand the mechanism of prevention of AGE formation by the phenolic acids.

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Conflict of interest

We declare that we have no conflict of interest.

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