JPP JOURNAL OF PROTEINS AND PROTEOMICS
8(1), 2017, pp. 1-12

Research Article

ANTIGLYCATING POTENTIAL OF ELLAGIC ACID AGAINST GLUCOSE AND METHYLGLYOXAL-INDUCED GLYCATION OF SUPEROXIDE DISMUTASE

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Abstract: Ellagic acid (EA) has been reported to have various beneficial pharmacological properties, including strong antidiabetic activity. Glycation plays an important role in diabetes-associated complications. In this study, we have investigated the antiglycating potential of EA by using the important antioxidant enzyme, superoxide dismutase (SOD). The glycation of SOD by glucose or methyglyoxal (MG) and its protection by EA has been studied by activity, SDS-PAGE, ELISA, absorbance and fluorescence measurements. Glycation of SOD resulted in a decrease in enzyme activity, fragmentation/cross-linking, reduced cross-reactivity with anti-SOD antibodies, structural changes, and formation of advanced glycation end products (AGEs) and fibrils. EA provided protection against glucose or MG induced glycation of SOD. The antiglycating potential of EA appears to be comparable with that of quercetin which is reported to be a potent natural inhibitor of glycation. Therefore, EA is an effective anti-diabetic compound, which can be used as a supplement in treating diabetes and its complications.

Keywords: Superoxide dismutase; glycation; glucose; methylglyoxal; ellagic acid.

Introduction

Ellagic acid (EA) is a naturally occurring thermostable polyphenolic molecule (Fig. 1) and is found in a wide variety of fruits and nuts that include raspberries, strawberries, walnuts, grapes and black currants (Ancos *et al.*, 2000; Zafrilla *et al.*, 2001; Vanella *et al.*, 2013; Kang *et al.*, 2016), either in its free form, as glycosides, or as in bound form such as ellagitannins (Amakura *et al.*, 2000; Goswami *et al.*, 2014). EA has many beneficial pharmacological activities including antidiabetic, antimutagenic, antimicrobial, antiviral, anticancer and chemoprotective activity (Ahn *et al.*, 1996; Feldman *et al.*, 1999; Akiyama *et al.*, 2001; Vattem and Shetty, 2002; Ruibal *et al.*, 2003; Losso *et al.*, 2004; Mertens-talcott *et al.*, 2006; Edderkaoui *et al.*, 2008; Malini *et al.*, 2011; Vanella *et al.*, 2013; Amin and Arbid, 2016; Kang *et al.*, 2016). Furthermore, it possesses a strong antioxidant potential which is due to its phenolic groups (Solon *et al.*, 2000; Festa *et al.*, 2001).

Increased oxidative stress has a critical connection with the aetiology of diabetes (Baynes, 1991; Ceriello, 2000; Narkhede *et al.*, 2012; Vinayagam *et al.*, 2016). During diabetes, persistent hyperglycaemia stimulates the production of reactive oxygen species (ROS) (Niedowicz and Daleke, 2005; Yan, 2014). The enhanced production and/or decreased removal of ROS by nonenzymatic/enzymatic antioxidant defences, increases the level of ROS which damages many tissues, resulting in diabetic complications (Lipinski, 2001). Superoxide dismutases (SODs) are important components of the antioxidant defence system in the body (Yasui and Baba, 2006; Kangralkar *et al.*, 2010; Anwar *et al.*, 2014). Protein glycation is a complex network of reactions between the carbonyl groups of reducing sugars with the free amino groups of a protein yielding schiff base which form

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Received: November 21, 2016
Accepted: February 5, 2017
Published: February 10, 2017

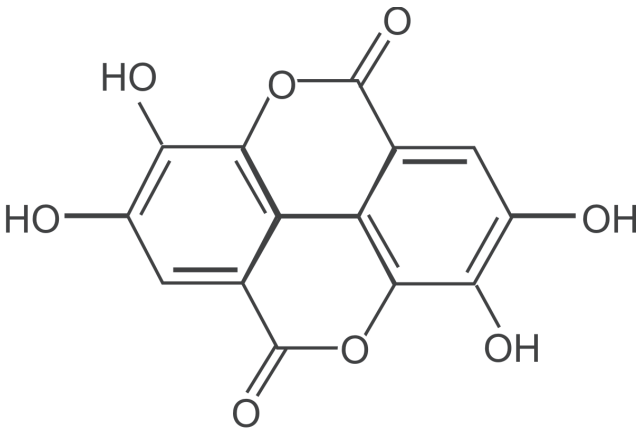

Figure 1: **Chemical structure of ellagic acid.**

amadori products and eventually advanced glycation end products (AGEs) (Neglia *et al*., 1983; Baynes *et al*., 1989; Anwar *et al*., 2014). AGEs formation is accompanied by the formation, among others of a number of ROS, á-oxoaldehydes including methylglyoxal (MG), that further react and damage proteins and other biomolecules (Thornalley *et al*., 1999). The rate of formation of AGEs enhances under diabetic conditions (Younus and Anwar, 2016). The accumulation of AGEs in body tissues is strongly correlated with progression of renal disease (Nishizawa *et al*., 2012), osteoarthritis (Ahmed *et al*., 2014), atherosclerosis (Kizer *et al*., 2014), diabetes (Wang *et al*., 2014; Kerkeni *et al*., 2014) and diabetic complications (Asif, 2015; Hussain *et al*., 2015). Glycation plays a significant role in diabetes, atherosclerosis, chronic renal failure, vascular disease, neurodegenerative diseases and aging (Brownlee, 1995; Anwar *et al*., 2014). Glycation of SOD by glucose and MG has been reported to induce its fragmentation and cross-linking, respectively, leading to reduction in its activity (Kang, 2003; Jabeen *et al*., 2006).

Several antiglycating agents with different mechanisms of inhibiting AGEs formation have been described (Younus and Anwar, 2016). Synthetic compounds which are currently used in the treatment of diabetes are strong antiglycating agents, however, they exert severe adverse effects (Thornalley, 2003; Peng *et al*., 2011). Therefore, recently much interest has been developed in the search of natural phytochemicals from plants that effectively inhibit glycation and have fewer side effects (Jariyapamornkoon *et al*., 2013). For centuries, many plants and their products have been used for their medicinal and remedial values (Abbas *et al*., 2016). On the basis of the link between glycation, oxidative stress and neurodegenerative diseases, it can be suggested that plant derived agents with antioxidant, antiglycating and enzyme inhibitory potential can be a better remedy for diabetes and its complications (Lim *et al*., 2001; Hussain *et al*., 2015). The inhibition of glycation by flavonoids has been reported to be associated with their antioxidant potential (Wu and Yen, 2005). Although EA has been shown to exhibit antidiabetic activity (Ueda *et al*., 2004; Seeram *et al*., 2005; Malini *et al*., 2011; Sepúlveda *et al*., 2011), however, its antiglycating potential has not been explored till date. Therefore, the present study was designed to investigate the antiglycating potential of EA by using the important antioxidant enzyme, SOD.

Materials and Methods

Materials - Bovine erythrocyte Cu,Zn-SOD (EC 1.15.1.1), MG (40% aqueous solution), EA, nitro blue tetrazolium (NBT), NADH, phenazinemethosulfate (PMS), thioflavin T (ThT), *N*, *N*, *N′*, *N′*-tetramethylenediamine (TEMED), bovine serum albumin (BSA), bicinchoninic acid (BCA) and o-phenylenediamine (OPD)/H_2O_2 were purchased from Sigma, St. Louis, MO, USA. Glucose and SDS were the products of Qualigens, Mumbai, India. Acylamide/bisacrylamide, ammonium persulphate and DEAE-cellulose were obtained from SRL, Mumbai, India. Standard molecular weight protein markers (Broad range), Freund's adjuvant and horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG were from Genei, Bangalore, India. All other chemicals used were of analytical grade.

Measurement of SOD concentration and activity - The concentration of SOD was routinely checked by the BCA method employing BSA as the standard (Smith *et al*., 1985). SOD stock (1 mg/ml) was made in 20 mM sodium phosphate buffer, pH 7.4 and stored at -20^{o}C for future use. The activity of SOD was measured spectrophotometrically by using PMS–NADH–NBT system (Nishikimi *et al*., 1972). Briefly, the reaction mixture contained 20 mM sodium phosphate buffer (pH 8.2), PMS (1.9 µM), NBT (184 µM) and NADH (205 µM). For assaying, SOD enzyme was pipetted into a cuvette at room temperature (25^{o}C) containing freshly prepared NBT and NADH. The reaction was started by adding freshly prepared PMS and the absorbance was continuously measured against blank samples using a single beam Shimadzu spectrophotometer. Reagent control lacking the enzyme was taken.

Decrease in absorbance of the reaction mixtures indicates increased activity.

SDS-PAGE - SDS-PAGE analysis was done under reducing conditions according to the method of Laemmli (1970) using 15% separating and 5% stacking gels, followed by overnight staining with 0.1% (w/v) Coomassie Brilliant Blue R250. The gels were destained using 40% (v/v) methanol/10% (v/v) acetic acid.

ELISA - Rabbits were immunized with SOD using Freund's adjuvant, and IgG from the serum of immunized rabbits was purified to homogeneity on DEAE-cellulose matrix after ammonium sulphate precipitation according to the procedure followed in our laboratory (Rehan and Younus, 2006). The cross-reactivity of the antibodies with SOD was determined by ELISA according to the published procedure (Younus *et al.*, 2001; Younus *et al.*, 2006).

Effect of EA on the glycation of SOD - SOD (0.2 mg/ml) was incubated with glucose (0.5 M), MG (10 mM) or a combination of 0.5 M glucose and 10 mM MG in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl for 10 days at 37°C in the presence of 0, 10, 20 and 50 µM EA. EA was dissolved in DMSO and the final DMSO concentration in the incubation mixture was 1%. Incubations were performed in autoclaved tubes and were carried out in a shaking water bath at 37°C. The control, SOD (0.2 mg/ml) dissolved in 20 mM sodium phosphate buffer, pH 7.4 with 0.15 NaCl, was kept at 4°C, until measurements. The effect of EA on the glycation of SOD with glucose or MG or a combination of both was assessed by activity, SDS-PAGE, ELISA, absorbance, intrinsic fluorescence, AGEs specific fluorescence and ThT fluorescence spectroscopic studies.

Absorption measurements - The absorption spectra of SOD (0.2 mg/ml) incubated alone, with glucose or with MG or with a combination of glucose and MG in absence/presence of EA were recorded over the wavelength range of 240-500 nm using a double beam Perkin Elmer spectrophotometer (Lambda 25).

Intrinsic fluorescence measurements - For recording the intrinsic fluorescence spectra of SOD (0.2 mg/ml) incubated alone, with glucose or with MG or with a combination of glucose and MG in the absence/presence of EA, the excitation and emission wavelengths were set at 280 nm and 290-400 nm range, respectively (Du *et al.*, 2013). Both the excitation and emission slit widths were 5 nm. All fluorescence spectra were corrected with respect to their respective blanks. All fluorescence measurements were performed using a Shimadzu spectrofluorometer (model RF-5301PC).

AGE specific fluorescence measurements - AGEs specific fluorescence was monitored by exciting at 350 nm and recording the emission between 400-480 nm. The slit widths were 3 nm for both excitation and emission.

ThT fluorescence measurements - The fibrillar state of SOD was determined via ThT fluorescence measurements. ThT is a benzothiazole dye that gives enhanced fluorescence upon binding to amyloid fibrils and therefore is usually employed to detect amyloid fibrils (Khurana *et al.*, 2005). The fluorescence was measured after adding 6 µM ThT dye in the samples at excitation wavelength of 440 nm and the emission was recorded in the wavelength range of 450-600 nm. The slit widths were 10 nm for both excitation and emission.

Results

Effect of EA on the activity of SOD

Incubation of SOD with 0.5 M glucose or 10 mM MG or a combination of 0.5 M glucose and 10 mM MG for 10 days at 37°C resulted in a decrease in activity of the enzyme as compared to the control (Fig. 2 Panel 2, 3 and 4, respectively). SOD incubated alone in the absence of any glycating agent showed insignificant change in the activity (Fig. 2 Panel 1). SOD incubated alone for 10 days with EA showed a very slight increase in activity with increasing EA concentration (Fig. 2, Panel 1). The activity increased by 2.8% when the enzyme was incubated with 50 µM EA as compared to the control (the sample that had no EA). However, SOD incubated with glucose, MG or a combination of both, and increasing EA showed an increase in activity as compared to the control (Fig. 2, Panel 2, 3 and 4, respectively). The activity increased by 15.9, 13.7 and 10.0% as compared to the control when the enzyme was incubated with glucose, MG or a combination of both glucose and MG, respectively, and 50 µM EA. We than compared the effect of EA on the activity of SOD with that of quercetin which is known to be a potent natural inhibitor of glycation (Sero *et al.*, 2013). The activity increased by 17.3, 18.2 and 11.0% as compared to the control when the enzyme was

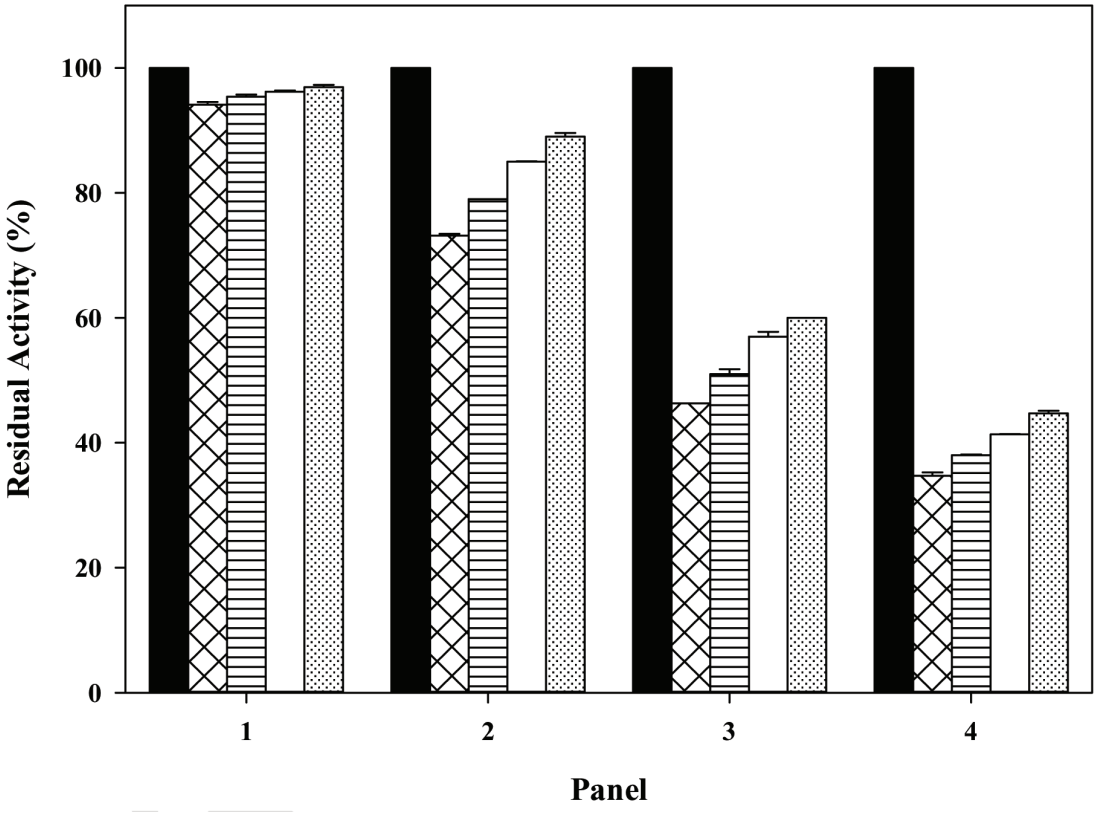

***Figure 2*: Effect of EA on the activity of SOD incubated with glucose, MG or both.** SOD (0.2 mg/ml) was incubated alone (Panel 1), with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (⨻), 10 (≡), 20 (▭) and 50 (▩) µM EA. Each Panel also shows native SOD (0.2 mg/ml) alone in buffer that was not incubated at 37°C for any time period (■). The enzyme activity was then determined under standard assay conditions. Each value represents the average for three independent experiments performed in duplicates.

incubated with glucose, MG or a combination of both glucose and MG, respectively, and 50 µM quercetin (data not shown).

Effect of EA on the fragmentation/aggregation of SOD

SDS-PAGE gel of SOD incubated alone at 37°C for 10 days showed almost no change in the intensity of the band corresponding to the enzyme (Fig. 3A). However, SOD incubated with glucose for 10 days showed a decrease in the intensity of the band corresponding to the enzyme (Fig. 3B). While incubation of SOD with MG or a combination of both MG and glucose for 10 days led to the formation of high molecular weight cross-linked aggregates of the enzyme (Fig. 3C and D). SDS-PAGE of SOD incubated for 10 days in the absence of glucose or MG and increasing concentration of EA showed almost no change in the intensity of the band corresponding to the enzyme (Fig. 3A). However, for the enzyme incubated for 10 days with glucose, a progressive increase in the staining intensity of the SOD band with increasing EA concentration was observed (Fig. 3B). While for SOD incubated for 10 days with MG (Fig. 3C) or a combination of both glucose and MG (Fig. 3D), a progressive decrease in the bands corresponding to the cross-linked aggregates with increasing EA concentration was observed. It is noteworthy that in the case of SOD incubated with MG alone and EA, a gradual increase in the band corresponding to the native enzyme was also observed with progressive increase in EA concentration (Fig. 3C).

Effect of EA on the cross-reactivity of anti-SOD antibodies with SOD

Incubation of SOD alone or with glucose, MG and both glucose and MG results in a decrease in absorbance at 490 nm in ELISA (Fig. 4). When SOD is incubated for 10 days at 37°C alone or with glucose, MG or both glucose and MG and increasing concentration of EA, a progressive increase in

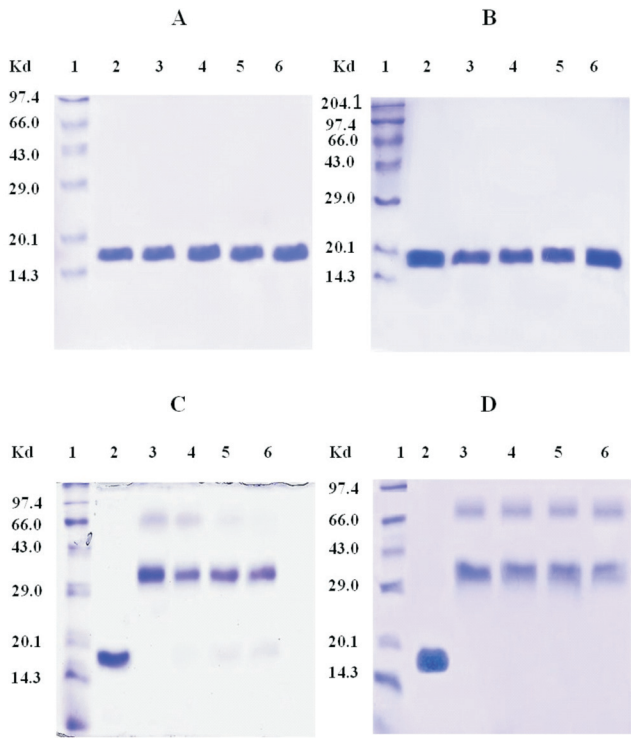

Figure 3: **SDS-PAGE of SOD incubated alone (A), with 0.5 M glucose (B), with 10 mM MG (C) or a combination of 0.5 M glucose and 10 mM MG (D) and with varying concentration of EA for 10 days at 37°C.** Lane 1 shows molecular weight markers (Genei); Lane 2 shows SOD (10 µg) that has not been incubated with glucose, MG or EA. Lanes 3, 4, 5 and 6 show SOD (10 ìg) incubated for 10 days alone or with glucose, MG or a combination of glucose and MG and with 0, 10, 20 and 50 µM EA, respectively.

absorbance at 490 nm (cross-reactivity with anti-SOD antibodies) with increasing EA concentration was observed in all the four cases (Fig. 4).

Effect of EA on the conformation of SOD

The absorption spectra of SOD incubated with glucose, MG and both glucose and MG showed a marked increase in absorbance at 280 nm (hyperchromicity) (Fig. 5 Panel 2, 3 and 4, respectively). Conversely, presence of increasing concentration of EA results in a progressive decrease in absorbance at 280 nm for SOD incubated at 37°C with glucose, MG or both glucose and MG for 10 days. However, in the case of SOD incubated alone for 10 days with increasing EA concentration (control), a very slight decrease in absorbance at 280 nm was observed (Fig. 5 Panel 1).

Incubation of SOD by glucose, MG and both glucose and MG leads to intrinsic fluorescence quenching. However, it was observed that SOD incubated for 10 days at 37°C with glucose, without EA but with 1% DMSO exhibited fluorescence enhancement at 310 nm and not quenching (Fig. 6 Panel 2). The fluorescence intensity of SOD incubated for 10 days at 37°C with glucose, MG or both glucose and MG and increasing concentration of EA (Fig. 6 Panel 2, 3 and 4, respectively), was observed to progressively increase at 310 nm with increasing EA concentration. And fluorescence intensity of the control increased very slightly at 310 nm with increasing EA concentration (Fig. 6 Panel 1).

Effect of EA on the formation of fluorescent AGEs

SOD incubated with glucose, MG or a combination of both glucose and MG showed AGEs specific fluorescence in the wavelength range 400-480 nm. The spectra of fluorescence intensity versus wavelength (400-480 nm) were found to be broad (data not shown). When SOD was incubated for 10 days at 37°C with glucose, MG or both glucose and MG and increasing concentration of EA (Fig. 7 Panel 2, 3 and 4, respectively), AGEs specific fluorescence was observed to progressively decrease at 450 nm in a concentration dependent manner in all the three cases. And insignificant decrease in the fluorescence was observed in the case of the control sample (Fig. 7 Panel 1).

Effect of EA on the formation of fibrils

ThT fluorescence intensity at 480 nm for SOD incubated with glucose, MG and both glucose and MG was observed to be significantly increased as compared to the control. The fluorescence intensity for SOD incubated for 10 days at 37°C with glucose, MG or both glucose and MG and increasing concentration of EA (Fig. 8 Panel 2, 3 and 4, respectively), was found to progressively decrease with increasing EA concentration in all the three cases. And the control was observed to exhibit insignificant decrease in ThT fluorescence at 480 nm (Fig. 8 Panel 1).

Discussion

Diabetes is one of the most common serious metabolic disease worldwide, and all kinds of diabetes are characterized by hyperglycaemia, lipidaemia, oxidative stress and long-term complications affecting the eyes, nerves, blood vessels, skin, and kidneys (Soumya and Srilatha, 2011). A large number of plants and natural

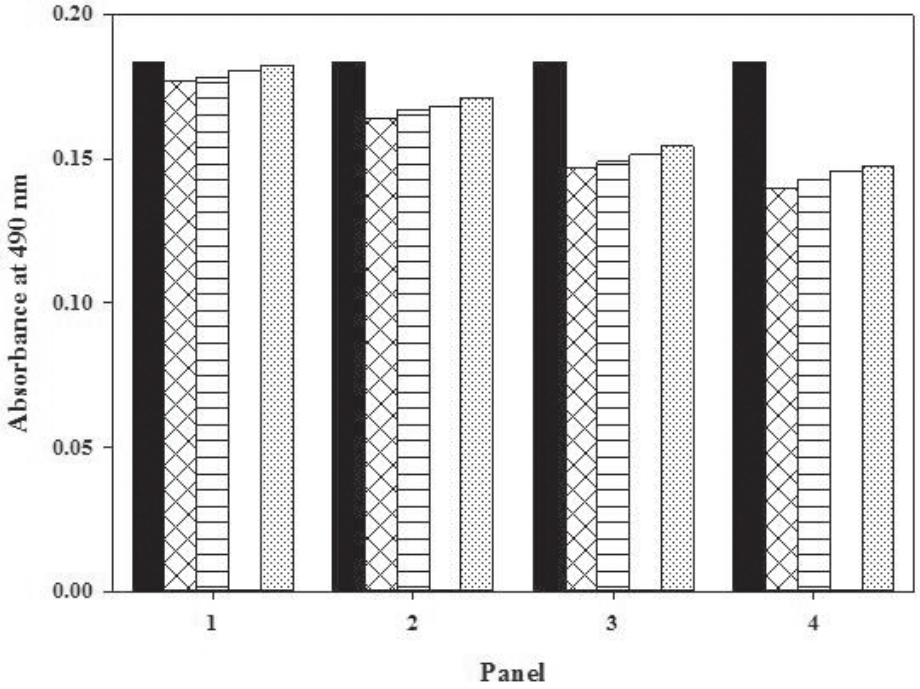

Figure 4: **Effect of EA on the cross-reactivity (ELISA) of anti-SOD antibodies with SOD incubated with glucose, MG or both.** SOD (0.2 mg/ml) was incubated alone (Panel 1), with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (⨷), 10 (≡), 20 (▭) and 50 (⁘) µM EA. Each Panel also shows native SOD (0.2 mg/ml) alone in buffer that was not incubated at 37°C for any time period (■). Each value represents the average for two independent experiments performed in triplicates.

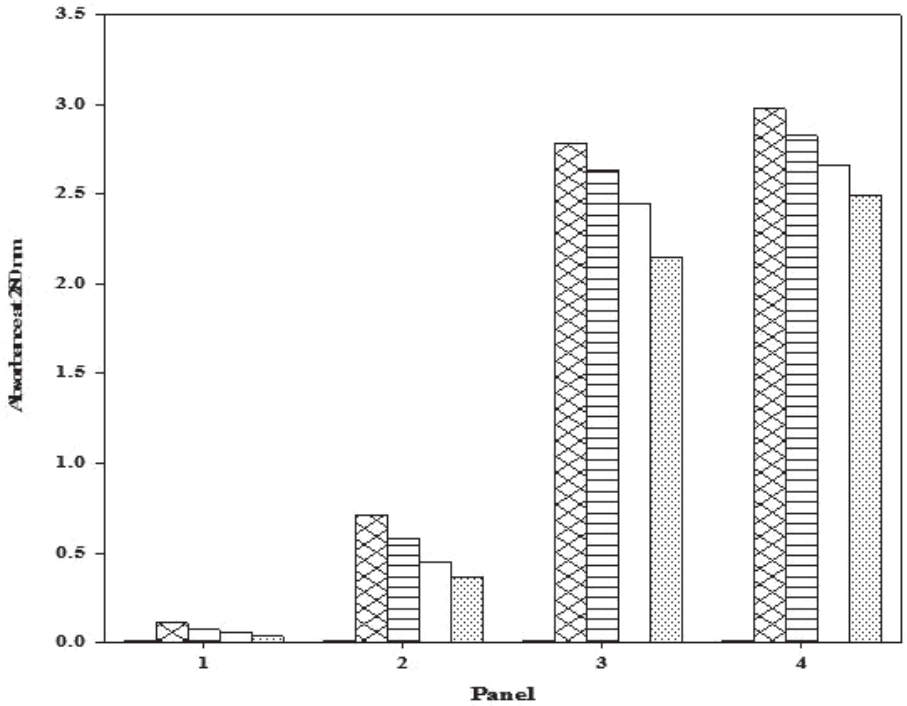

Figure 5: **Effect of EA on the absorption changes induced in SOD due to glycation.** Absorbance at 280 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (⨷), 10 (≡), 20 (▭) and 50 (⁘) µM EA. Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■). Each value represents the average of the experiment performed in duplicates.

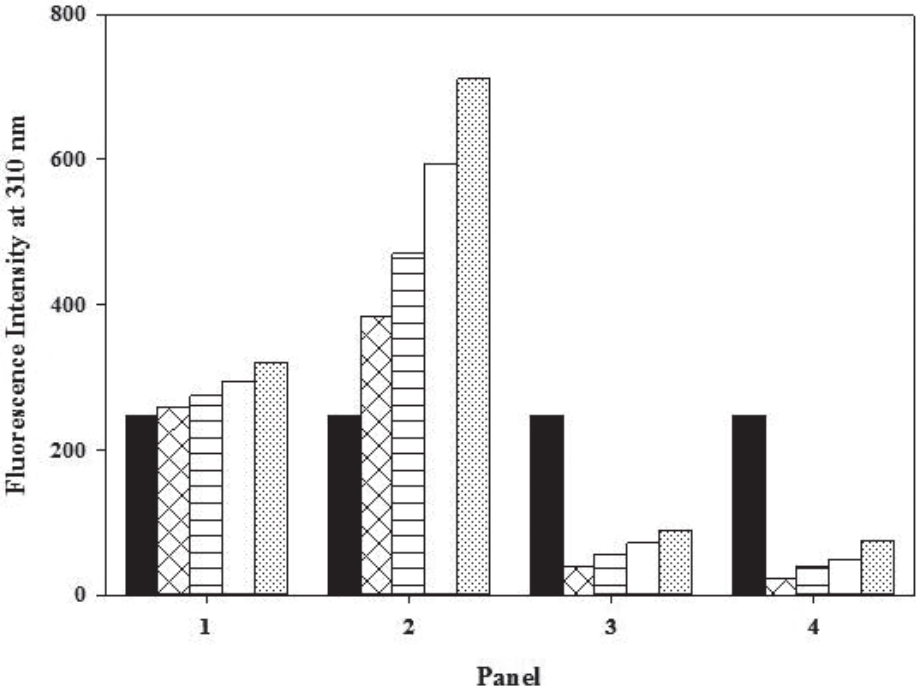

Figure 6: **Effect of EA on the intrinsic fluorescence changes induced in SOD due to glycation.** Fluorescence intensity at the excitation/emission wavelengths of 280/310 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (⨷), 10 (≡), 20 (▭) and 50 (▒) µM EA. Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■). Each value represents the average of the experiment performed in duplicates.

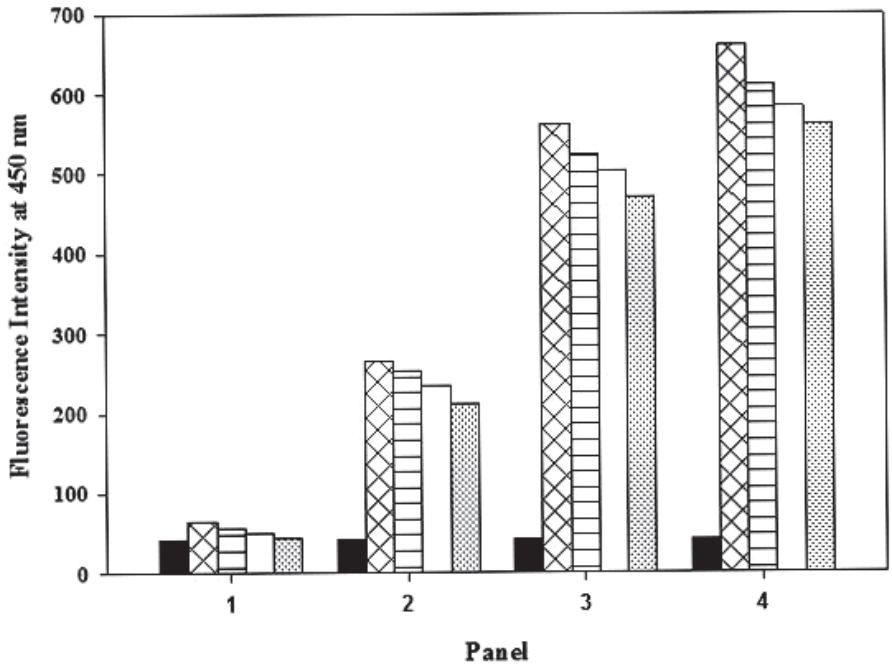

Figure 7: **Effect of EA on the fluorescent AGEs formed of SOD due to glycation.** AGEs specific fluorescence intensity at the excitation/emission wavelengths of 350/450 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (⨷), 10 (≡), 20 (▭) and 50 (▒) µM EA. Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■). Each value represents the average of the experiment performed in duplicates.

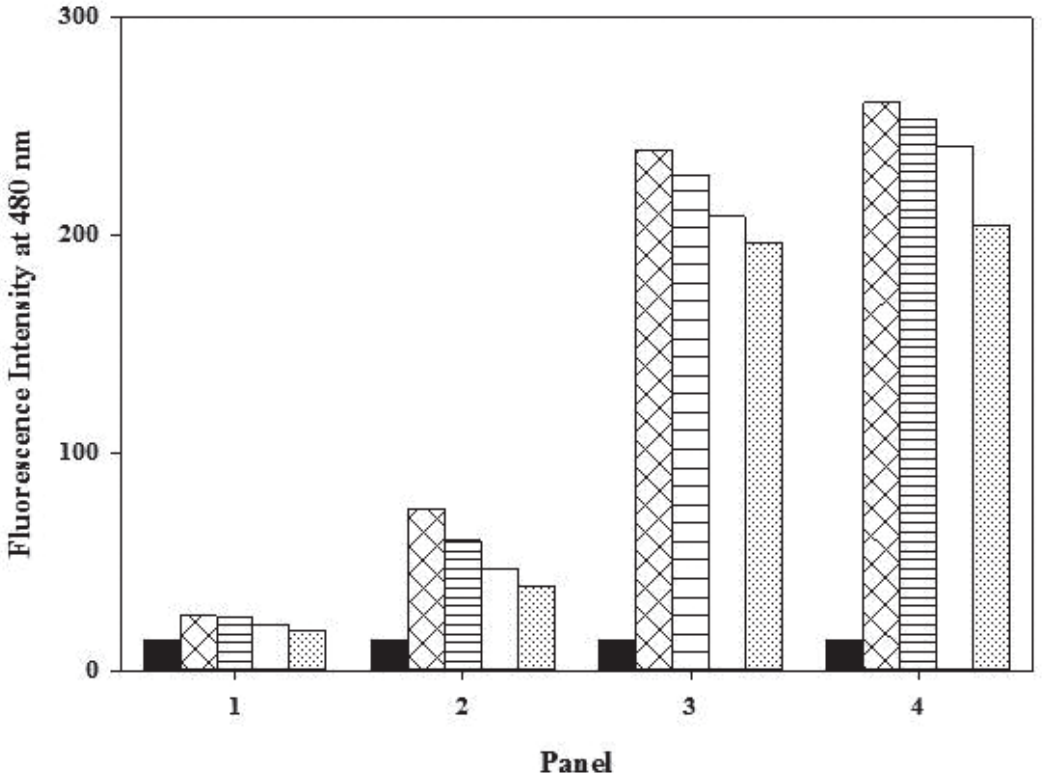

Figure 8: **Effect of EA on the fibrils formed in SOD due to glycation.** ThT fluorescence intensity at the excitation/emission wavelengths of 440/480 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (⨻), 10 (≡), 20 (▭) and 50 (⁙) µM EA. Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■). Each value represents the average of the experiment performed in duplicates.

biomolecules have been discussed in literature for their antidiabetic effects (Coman *et al.*, 2012). However, the mechanism is most often not completely understood. Minimizing glycation can reduce diabetic complications. Prevention of glycation by natural products/compounds is of great importance as they are efficacious agents which are relatively nontoxic, inexpensive and are available in an ingestable form (Younus and Anwar, 2016). The important antioxidant enzyme SOD has been reported to undergo glycation by reducing sugars and MG resulting in its inactivation (Jabeen *et al.*, 2006; Jabeen and saleemuddin, 2006; Khan *et al.*, 2014). We have earlier reported that glycation of SOD by glucose results in the breakdown of the enzyme into small peptides, while glycation by MG leads to the formation of high molecular weight cross-linked aggregates of the enzyme (Khan *et al.*, 2014). MG is a highly reactive á-oxoaldehyde that plays a very significant role in the glycation reactions, formation of AGEs and other complications associated with hyperglycemia and related disorders (Jabeen and saleemuddin, 2006). The objective of the present study was to explore the possible protective effect of EA on the glycation of SOD. The glycation of SOD was carried out with high non-physiological concentration of glucose (0.5 M) or MG (10 mM) which may serve as an appropriate model for the long-term effects of glucose or MG on the enzyme (Jabeen *et al.*, 2006; Khan *et al.*, 2014).

Our results demonstrate that EA effectively protected SOD against glycation. There was a slight increase in activity of SOD incubated with EA in the absence of any glycating agent, which is believed to be due to antioxidant property of EA. However, SOD incubated with glucose, MG or a combination of both, and EA showed a greater increase in the activity as compared to the enzyme that was not incubated with EA. This observed further increase in activity is believed to be due to the antiglycating activity of EA, which inhibited the glycation of SOD in a concentration dependent manner. EA was more protective in the case of

glucose than for MG or MG plus glucose induced glycation, therefore, it appears to be a more effective antiglycating agent for sugars/compounds that are milder glycating agents. The antiglycating potential of EA proved to be comparable with that of the known potent natural inhibitor of glycation, quercetin. It was clearly evident from the SDS-PAGE analysis that EA protected SOD against fragmentation/cross-linking induced by glycation.

Incubation of SOD alone or with glucose, MG and both glucose and MG resulted in reduced cross-reactivity with anti-SOD antibodies in ELISA, which we believe is due to the structural/chemical modification of the epitopes of enzyme due to incubation at 37°C and by glycation. EA protected the enzyme to some extent against the structural/chemical changes induced by incubation at 37°C and glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of EA.

Cu,Zn-SOD from the bovine erythrocyte is a homodimer (Hough *et al.*, 2000) and contains only one tyrosine residue per subunit, with no tryptophan residue in it. Glycation of SOD resulted in an increase in absorbance at 280 nm (hyperchromicity) and hence structural changes. EA led to reduced hyperchromicity and hence protected the enzyme to some extent against the structural changes induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of EA. However, the enzyme was still far from the structure of the native enzyme even at 50 µM concentration of EA in all the three cases. To confirm this further, total intrinsic fluorescence of native and glycated SOD was measured. Glycation of SOD by glucose, MG and both glucose and MG results in intrinsic fluorescence quenching, again indicating structural changes. And EA was again observed to protect against these structural changes to some extent as evident by the increase in fluorescence with increasing EA concentration in all the three cases. However, it was observed that enhancement in the fluorescence intensity and not quenching occurred in the case of SOD incubated with glucose, without EA but 1% DMSO. Therefore, it appears that in the samples of SOD glycated by glucose in the presence of DMSO, the environment around the aromatic residues of the protein is somewhat perturbed which affects their fluorescence (Rabbani *et al.*, 2011; Anwar *et al.*, 2014).

AGEs are a heterogenous group of high molecular weight aggregates and have specific characteristic fluorescence spectra. Thus, we used autofluorescence to detect the formation of AGEs in the samples. SOD incubated with glucose, MG or a combination of both glucose and MG showed AGEs specific fluorescence in the wavelength range of 400-480 nm, therefore AGEs were formed. The spectra of fluorescence intensity versus wavelength were broad (data not shown), and this probably reflects the presence of a number of different fluorescent compounds/AGEs being formed during glycation. EA protected the enzyme to some extent against formation of AGEs induced by glycation as evident by the progressive decrease in AGEs specific fluorescence at 450 nm with increasing EA concentration. ThT dye interacts with the amyloid fibril structures of proteins and then gives strong fluorescence (Khurana *et al.*, 2005). Therefore, we employed this dye to detect whether amyloid fibril structures were formed in SOD upon glycation. Glycation was found to induce the formation of amyloid fibrils in SOD as evident from the increase in ThT fluorescence upon glycation with glucose, MG or both glucose and MG. ThT fluorescence progressively decreased in all the three cases in the presence of increasing concentration of EA. Therefore, EA protected the enzyme to some extent against formation of fibrils induced by glycation.

Conclusion

In summary, in this study we have used different techniques to demonstrate that EA effectively inhibits the glycation of SOD, and its antiglycating potential appears to be comparable to that of quercetin which is currently considered to be one of the most potent natural inhibitor of glycation. EA has also been reported to have other anti-diabetic effects (Malini *et al.*, 2011). Therefore, these findings endorse the idea that treatment with EA supplement can have a positive impact against diabetes by reducing diabetic complications.

Acknowledgement

Research Facilities provided by Aligarh Muslim University are gratefully acknowledged. Fellowship received by S.A from the University Grants Commission-Basic Science Research (UGC-BSR), India is also acknowledged.

Abbreviations

AGEs, advanced glycation end products; BCA, bicinchoninic acid; BSA, bovine serum albumin; EA, Ellagic acid; ELISA,

enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; MG, methylglyoxal; NBT, nitro blue tetrazolium; OPD, o-phenylenediamine; PMS, phenazine methosulfate; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SOD, superoxide dismutase; ThT, thioflavin T.

Conflict of Interest

The authors do not have any conflict of interest with the contents of this manuscript.

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