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Aqueous extract of some indigenous medicinal plants inhibits glycation at multiple stages and protects erythrocytes from oxidative damage–an in vitro study

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Abstract *Azadirachta indica*, *Emblica officinalis*, *Syzygium cumini* and *Terminalia bellirica* are common in Indian system of traditional medicine for the prevention of diabetes and its complications. The aim of the present study was to comprehensively and comparatively investigate the antiglycation potential of these plant extracts at multiple stages and their possible protective effect against glycated albumin mediated toxicity to erythrocytes. Antiglycation activities of these plant extracts was measured by co-incubation of plant extract with bovine serum albumin-fructose glycation model. The multi-stage glycation markers- fructosamines (early stage), protein carbonyls (intermediate stage) and AGEs (late stage) are investigated along with measurement of thiols and β aggregation of albumin using amyloid-specific dyes–Congo red and Th T. Protection of erythrocytes from glycated albumin induced toxicity by these plant extracts was assessed by measuring erythrocytes hemolysis, lipid peroxidation, reduced glutathione and intracellular antioxidant capacity. Total phenolics, reducing power and antioxidant activities of the plant extracts were also measured. In vitro glycation assays showed that plant extracts exerted site specific inhibitory effects at multiple stages, with *T. bellirica* showing maximum attenuation. In erythrocytes, along with the retardation of glycated albumin induced hemolysis and lipid-peroxidation, *T. bellirica* considerably maintained cellular antioxidant potential. Significant positive correlations were observed between erythrocyte protection parameters with total phenolics. These plant extracts especially *T. bellirica* prevents glycation induced albumin modifications and subsequent toxicity to erythrocytes which might offer additional protection against diabetic vascular complications.

Abbreviations

ABTS	2, 2′azino-bis (3-ethylbenzothizoline-6-sulphonic acid)
AGEs	Advanced glycation end products
BHT	Butylated hydroxyl toluene
BSA	Bovine serum albumin
DNPH	di-nitro phenyl hydrazine
DPPH	2,2′-diphenyl-1-picrylhydrazyl
DTNB	5, 5-Dithiobis- 2-nitrobenzoic acid
FC	Folin–Ciocalteu
GAE	Gallic acid equivalents
LPO	Lipid peroxidation
MDA	Malondialdehyde
OS	Oxidative stress
RP	Reducing power
Th. T	Thioflavin T

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Introduction

Hyperglycemia is widely accepted as a factor which contributes to the development, progression and complications of diabetes (Brownlee 2001). Glycation of proteins leads to partial loss of protein activity and is one serious consequences of chronic hyperglycemia (Yamagishi and Matsui 2010). The glycation reaction is subdivided into three main stages: early, intermediate, and late (Baker et al. 1985). In the early stage, various reducing sugars (fructose/glucose/pentose, etc.) react

with the free amino groups of proteins to form Schiff bases, fructosamines and Amadori products. In the intermediate stage, Amadori products degrade into a variety of carbonyl compounds (glyoxal, methylglyoxal, and deoxyglucosones). In the late stage, yellow-brown, often fluorescent, insoluble, irreversible advanced glycation end-products (AGEs) are formed. Furthermore, there is a growing body of evidence to indicate that glycated protein, AGEs and their signal-transducing receptor interactions induce the formation of reactive oxygen species, leading to concomitant oxidative stress (OS) and vascular inflammation, thereby playing a central role in the pathogenesis of various vascular complications in diabetes (Goh and Cooper 2008). The levels of OS are much higher in diabetic patients than healthy individuals, and OS is often associated with decreased serum levels of vitamin C and, antioxidant enzymes such as catalase, superoxide-dismutase and glutathione peroxidase, and increased levels of malondialdehyde, conjugated diene, AGEs and protein carbonyls (Goh and Cooper 2008; Martín-Gallán et al. 2003). Thus identification of compounds that specifically inhibit each step of glycation and the formation of the intermediate products of this important pathway may provide an effective approach for generating novel therapeutic interventions to delay and prevent diabetic vascular complications (Rahbar and Figarola 2003).

Collectively, data from various biochemical, animal and epidemiological studies strongly suggests that glycation of circulating proteins plays a critical and causative role in the pathogenesis of diabetes (Cohen 2003). Albumin is one of the main circulating proteins which undergoes glycation due to its abundance in serum (35 g/l), long half-life (21 days) and presence of multiple glycation sites (Barnaby et al. 2011). Glycation induced modifications have a detrimental impact on albumin structure as its–1) conformation changes, (Rondeau and Bourdon 2011) 2) free thiols gets oxidized to disulfide linkages or thiol radicals (S-S or S, respectively) (Bourdon et al. 1999), 3) initially globular albumin refolds into amyloid fibrils comprising cross- β structure (the β aggregates specifically bind to amyloid specific dyes–Congo red and thioflavin T (Th T) (Bouma et al. 2003). These structural changes affect the ability of albumin to bind drugs and small solutes which have important clinical implications (Vetter and Indurthi 2011). Further increased level of glycated albumin transduces various signaling pathways which contribute to the malfunction of various cells from kidney, eye, heart, blood, etc. (Rondeau and Bourdon 2011) and eventually leads to cellular death. Since circulating erythrocytes are continuously interacting with glycated albumin during hyperglycemic conditions in diabetes. During circulation, the interaction of glycated albumin with erythrocytes causes cell aggregation (Candiloros et al. 1996) and subsequently induces OS in vessel wall resulting in the development of diabetic complications (Wautier et al. 1996). Thus,

erythrocytes are important in orchestrating the toxic effects of glycated albumin.

Azadirachta indica (Meliaceae), *Emblica officinalis* (Euphorbiaceae), *Syzygium cumini* (Myrtaceae) and *Terminalia bellirica* (Combretaceae) are commonly used in Indian system of traditional medicine for the prevention of diabetes and complications. In experimental studies using diabetic human and animal models, these plants have been shown to reduce diabetes symptoms effectively (Mukherjee et al. 2006; Modak et al. 2007). The summary of these plants along with information regarding the active constituents, reported antiglycation activities is presented in Table 1. It was evident that *S. cumini* has not studied so far for its effect in glycation reaction and others (*A. indica*, *E. officinalis* and *T. bellirica*) have been scarcely documented for their antiglycatory effects. Also, there are no systematic studies evaluating the effect of these plants extracts on glycation induced various modifications of albumin and cellular consequences of glycated albumin. The aim of present study was to comprehensively and comparatively investigate the antiglycation potential of these plant extracts and their possible protective effect against glycated albumin mediated toxicity to erythrocytes. The multistage glycation markers-fructosamines (early stage), protein carbonyls (intermediate stage) and AGEs (late stage) are investigated along with measurement of thiols and β aggregation of albumin using amyloid-specific dyes–Congo red and Th T. Moreover, correlation of the protective effects with the extracts total phenolics, reducing power and antioxidant potential is also presented.

Materials and methods

Chemicals

Bovine serum albumin (BSA) [fraction V, initial fractionation by heat shock, purity- 98 % (electrophoresis), remainder mostly globulins and fatty acids depleted], Di-nitro phenyl hydrazine (DNPH), 2,2′-diphenyl-1-picrylhydrazyl (DPPH), 2, 2′ azino-bis (3-ethylbenzothizoline-6-sulphonic acid (ABTS), 5, 5-Dithiobis- 2-nitrobenzoic acid (DTNB), Th. T, Congo-red, thiobarbituric acid and m-aminophenyl boronic acid were obtained from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals were of analytical grade.

Plant materials and preparation of plant extracts

A. indica (leaf)*, E. officinalis* (fruit) and *S. cumini* (seed) and *T. bellirica* (fruit) were procured from an herbal store (Ambadas Vanaushadhalaya, Pune) in the powder form. Aqueous plant extracts (1 mg/ml) were prepared in double distilled water as per method of Saraswat et al. (2009) with some modification. Extracts were then kept on shaker

Table 1 Summary of plants used for present study with their active constituents and reported antiglycation activity

Sr. no	Scientific names	Common names	Parts used	Active constituents	Reported antiglycation activity
1.	*Azadirachta indica*	Neem, Nimba	Leaf	Nimbocinone, nimolinone, kulactone, nimocinolides, isonimocinolide, nimbin, salanin, azadirachtin, flavonoids, myricetin, meldenindiol, vilasinin, margosinolide, isomargosinolide, desacetyldihydronimbic acid.	Inhibited albumin AGEs formation (Gutierrez et al. 2011)
2.	*Emblica officinalis*	Amla, Indian gooseberry	Fruit	High concentration of polyphenol with low and high molecular weight gallotannins.	Inhibited albumin AGEs formation (Nampoothiri et al. 2011)
3.	*Syzygium cumini*	Jambolan, black plum, Jamun, Jambul	Seed	Phenolic compounds, such as Kaempferol 7-O-methylether and sterols.	No report available.
4.	*Terminalia bellirica*	Belliric myrobalan, bahera	Fruit	Termilignan, thannilignan, 7-hydroxy-3′,4′ -(methylenedioxy) flavone, anolignan B, gallic acid, ellagic acid, β-sitosterol, arjungenin, belleric acid, bellericoside and cannogenol 3-O-β-D-galactopyranosyl-(1→4)-O-α-L-rhamnopyranoside.	Inhibited albumin (Nampoothiri et al. 2011) and insulin glycation (Kasabri et al. 2010)

(150 rpm) for 3 h, followed by centrifugation (3,000 rpm, 10 min) (SuperspinR- V/FM, Plasto craft, India). The clear supernatant was used for further studies. The extracts were stored at 4 °C in plastic vials, till further use. All the analyses were performed in triplicates.

Measurement of antiglycation potential of plant extracts

In vitro glycation of BSA

Albumin glycation was performed according to the method of McPherson et al. (1988) with some modifications. Glycated samples were prepared by incubating 1 ml of BSA (10 mg/ml), 1 ml of fructose (250 mM) in 1 ml of potassium phosphate buffer (200 mM, pH 7.4 containing 0.02 % sodium azide) along with aqueous plant extracts (1 ml) and incubated at 37 °C for 4 days. Positive control (BSA + fructose–plant extract) was maintained under similar conditions and all the incubations were performed in triplicates. Before incubation, all the solutions were filtered through 0·22 μm membrane filters in sterile plastic-capped vials to maintain sterility and strict asepsis was maintained during the entire process.

After the incubation period, it was ensured that all the samples were free of microbiological contamination. The unbound fructose from the samples and positive control was removed by dialysis against the phosphate buffer (200 mM, pH 7.4) and dialysates were subsequently stored at 4° C for further analysis.

The antiglycation potential of plant extracts was determined by estimation of seven parameters from dialysates, i.e.–i) fructosamines, ii) protein carbonyls, iii) AGEs,

iv) protein thiols, v) Congo Red absorbance, vi) Th. T fluorescence, and vii) amount of glycated albumin by affinity chromatography.

Estimation of fructosamines

Nitroblue tetrazolium assay was used to determine the level of fructosamines (Baker et al. 1985). For this purpose, 40 μL of glycated samples and positive control were added to the 0.8 mL of nitroblue tetrazolium (0.75 mM in sodium carbonate buffer 100 mM, pH 10.35) and incubated at 37 °C for 30 min. After incubation absorbance was measured at 530 nm (UV-10 Spectrophotometer, Thermo Scientific, USA) and the % inhibition of fructosamine by plant extracts were calculated using the following equation:- Inhibitory activity$(\%)=[(A_0-A_1)/A_0]\times 100$, Where A_0 is the absorbance value of the positive control at 530 nm and A_1 is absorbance of the glycated albumin samples coincubated with plant extracts at 530 nm.

Protein carbonyl estimation

Protein carbonyl groups were estimated by the method of Uchida et al. (1998). Briefly, 0·5 mL glycated samples and positive control were mixed with an equal volume of DNPH (10 mM in 2.5 M-HCl) and incubated at room temperature for 1 h. Proteins were precipitated by adding 0·5 mL of tricholoroacetic acid (20 % w/v), precipitate washed three times with 1 mL of ethanol: ethyl acetate (1:1, v/v) mixture and then solubilized in 1 mL of 6 M urea. The absorbance was read at 365 nm and protein carbonyl concentration was calculated by using the molar extinction coefficient ($\varepsilon 365$ nm=

21 mM per cm). The results were expressed as % inhibition as calculated by the following formula- % Inhibition=$[(A_0-A_1)/A_0]\times100$, where A_0 is the absorbance of protein carbonyls of the positive control and A_1 is the absorbance of glycated albumin samples coincubated with plant extracts.

AGEs fluorescence measurement

The formation of total AGEs were assessed by monitoring the production of these fluorescent products of glycated albumin samples and positive control at excitation and emission wavelengths of 370 and 440 nm (slit = 10 nm) respectively on Perkin Elmer luminescence spectrometer LS50B (USA) (Sattarahmady et al. 2007). The results were expressed as percent inhibition as calculated by the formula: %Inhibition=$[(F_0-F_1)/F_0]\times100$, where F_0 is the fluorescence of the positive control and F_1 is the fluorescence of the glycated albumin samples co- incubated with plant extracts.

Protein thiol estimation

Thiol groups of glycated albumin samples and positive control were measured according to Ellman's assay using DTNB (Ellman 1959). Briefly, 250 µL samples and control were incubated with 750 µL of DTNB (0·5 mM) for 15 min. and the absorbance was measured at 410 nm. The free thiol concentration was calculated using standard curve performed with standard BSA concentrations (0·8 to 4 mg/ml, corresponding to 19–96 nmol total thiols). The results were expressed as % protection to protein thiols by comparing with positive control.

Binding of Congo red

Congo red binding to amyloid cross-β structure was estimated by measuring absorbance at 530 nm (Klunk et al. 1999). For this purpose, 500 µL of glycated samples and positive control were incubated with 100 µL of Congo red (100 µM in phosphate buffer saline (PBS) with ethanol (10 % v/v)) for 20 min. at room temperature. Absorbance was recorded for the Congo red incubated samples as well as for Congo red background. The results were expressed as % inhibition calculated by the formula- Inhibition %=$[(A_0-A_1)/A_0]\times100$, where A_o is the absorbance at 530 nm of positive control and A_1 is the absorbance at 530 nm of the glycated albumin samples co-incubated with plant extracts.

Th. T fluorescence

For determination of β aggregation, Th. T, another marker for amyloid cross β structure was used. Glycated samples and positive control (100 µL) were incubated with 32 µM Th. T for 1 h. at room temperature (LeVine 1999). Fluorescence was measured at excitation and emission wavelengths of 435 and 485 nm (slit, 10 nm) respectively with correction for background signals without Th. T. The results were expressed as % inhibition, calculated by the formula- Inhibition %=$[(F_0-F_1)/F_0]\times100$, where F_0 is the florescence of the positive control and F_1 is the florescence of the glycated albumin samples co-incubated with plant extracts.

Phenyl boronate affinity chromatography

The extent of glycation of glycated albumin samples was determined using phenyl boronate affinity chromatography (Dean et al. 1985). For this purpose, 0.2 mL of glycated albumin samples and positive control were passed through a phenyl boronate affinity column (2×1 cm) equilibrated with 50 mM potassium phosphate containing 1 M NaCl (pH 7.8). The unbound fractions containing non-glycated protein were washed with the 50 mM taurine/NaOH buffer (pH 8.7) containing 20 mM $MgCl_2$. While bound glycated proteins were eluted using same buffer with 50 mM Tris/HCl. The absorbance of fractions was measured at 280 nm.

Estimation of glycated albumin toxicity to erythrocytes

In vitro treatment of erythrocytes with glycated albumin samples

The freshly obtained erythrocytes of sheep blood were washed with PBS (pH 7.0) three times and then suspended in 0.15 M NaCl (pH 7.4) to obtain 4 % cell suspension. 1 mL aliquots of the erythrocytes suspension were incubated with 1 mL glycated albumin samples along with positive control and solvent control (PBS) for 4 h. at 37 °C with intermittent shaking (Konyalioglu and Karamenderes 2005). The effect of glycated albumin samples co-incubated in presence or absence of plant extracts on erythrocytes was evaluated by monitoring various cellular parameters such as–hemolysis, reduced glutathione (GSH), lipid peroxidation (LPO) and intracellular antioxidant power as FRAP value. The levels of above parameters were expressed in percentage by considering the values of positive control sample as 100 %.

Hemolysis of erythrocytes

The toxicity of glycated albumin samples and positive control was evaluated by the erythrocytes lysis assay as adapted by Luize et al. (2005) with some modifications. After the incubation period, 0.5 mL of treated erythrocytes suspension was taken in tubes, centrifuged at 2,000 rpm for 10 min. and the absorbance of supernatant were read at 540 nm. Each experiment was performed in triplicate and percent hemolysis was calculated as compare to positive control sample.

Estimation of LPO

It was assessed by measuring malondialdehyde (MDA), an end product of fatty acid peroxidation and the estimation was done by the method of Placer et al. (1966). In brief, 0.2 mL of treated erythrocytes was mixed with 1 mL of 10 % trichloroacetic acid and tubes were centrifuged at 2,000 rpm for 15 min. 1 mL of supernatant was transferred to another tube and to this 2 mL of thiobarbituric acid (0.67 % in 0.25 M HCl) was added. Tubes were mixed and kept in a boiling water bath for 25 min. Further tubes were placed in ice-cold water for 10 min, followed by centrifugation at 1,000 g for 10 min. The absorbance of supernatant was measured at 532 nm and the concentration of MDA was calculated using a molar extinction coefficient value of 1, 53,000 L M^{-1} cm^{-1}.

Estimation of GSH

This was estimated in the treated erythrocytes by using the method of Ellman (1959). Briefly, 0.25 mL erythrocytes were added to 0.5 mL of precipitating buffer (5 % trichloroacetic acid in 1 mM EDTA). After protein precipitation, the samples were centrifuged at 2,000 g for 10 min. to obtain the supernatant. A 0.1 mL fraction of the supernatant was mixed with 2.5 mL phosphate buffer (0.1 M, pH 8.0); and color was developed by adding 0.1 mL of 0.01 % DTNB. The absorbance of the yellow colored complex was measured within 10 min. at 412 nm against a blank sample without cells. The GSH concentrations of samples were calculated using a molar extinction coefficient value of 14,150 L M^{-1} cm^{-1}.

Ferric reducing antioxidant power (FRAP) assay

The intracellular antioxidant power of the erythrocytes was determined by the FRAP assay, following the method given by Benzie and Strain (1998). 50 μl of treated erythrocytes hemolysate was added to 1.5 ml freshly prepared and prewarmed (37 °C) FRAP reagent (300 mM acetate buffer, pH 3.6: 10 mM 2, 3, 5-triphenyltetrazolium chloride (TPTZ) in 40 mM HCl: 20 mM $FeCl_3.6H_2O$ in a ratio of 10:1:1) and incubated at 37 °C for 10 min. Absorbance of the sample was recorded at 593 nm against a reagent blank (1.5 ml FRAP reagent + 50 μl distilled water).

Measurement of plants extracts antioxidant potential

The antioxidant potential of plant extracts was measured by four in vitro assays through estimation of- i) Total phenolic content, ii) Reducing power (RP) and their capacity in scavenging iii) DPPH and iv) ABTS radicals.

Estimation of total phenolic content

Total phenolic content was determined using the Folin–Ciocalteu reagent (Lim and Quah 2007). Briefly 0.3 mL of methanolic extracts were thoroughly mixed with 1.5 mL of freshly diluted Folin–Ciocalteu reagent (1 N), to which 1.2 mL of sodium carbonate solution (7.5 %) was added and the mixture was incubated for 30 min. in dark. After incubation, the absorbance was measured at 765 nm. Gallic acid (0.002–0.02 mg/mL) was used as a reference standard. The concentration of phenolic content was expressed as mg of gallic acid equivalents (GAE) per gm dry weight.

Estimation of RP

The RP was evaluated according to the method of Oyaizu (1986). The mixture containing 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of $K_3Fe(CN)_6$ (1 % w/v) was added to 1 mL of the methanolic plant extract and incubated at 50 °C for 20 min. After incubation, 2.5 mL of tricholoroacetic acid (10 % w/v) was added and the solutions were centrifuged at 3,000 rpm for 10 min. The upper layer (2.5 mL) was collected and mixed with 2.5 mL distilled water and 0.5 mL of $FeCl_3$ (0.1 %, w/v). The absorbance was measured at 700 nm against a blank.

DPPH radical scavenging ability

The DPPH radical scavenging activity was estimated by measuring the decrease in the absorbance of methanolic solution of DPPH (Brand-Williams et al. 1995). In brief, to 5 mL DPPH solution (3.3 mg of DPPH in 100 mL methanol), 1 mL of methanolic plant extracts were added, incubated for 30 min. in the dark and the absorbance (A_1) was read at 517 nm. The absorbance (A_0) of a reaction control (methanol instead of plant extract) was also recorded at the same wavelength. Scavenging ability (%) was calculated by using the formula: DPPH radical scavenging activity$(\%)=[(A_0-A_1)/A_0]\times 100$, where A_0 was the absorbance of reaction control and A_1 was the absorbance of extracts.

ABTS radical scavenging ability

The ABTS cation radical scavenging activity of the extracts was determined according to the modified method of Re et al. (1999). A stock solution of ABTS was produced by mixing 7 mM aqueous solution of ABTS with potassium persulfate (2.45 mM) in the dark at ambient temperature for 12–16 h before the use. The radical cation solution was further diluted until the initial absorbance value of 0.7±0.005 at 734 nm was reached. For assaying test samples, 980 μL of ABTS solution was mixed with 20 μL of the aqueous plant extracts. The decrease in absorbance was recorded at 0 min. and after 6 min.

Scavenging ability relative to the reaction control (without plant extract as 100 %) was calculated by using the formula: ABTS radical scavenging activity(%)=[(Initial reading− final reading)/Initial reading]×100, where initial reading is absorbance at 0 min. and final reading is absorbance at 6 min.

Statistical analysis

Data were expressed as the mean and standard deviations of triplicate values. The statistical analysis was carried out using the Microsoft Excel software package (Microsoft Corp.). Pearson correlation matrix was applied to the analytical data to find the relationships between the different analytical methods, which were expressed as the correlation coefficient 'R'.

Results

Antiglycation potential of plant extracts

Effect of plant extracts on multiple stages of albumin glycation

The early glycation product i.e. fructosamines were found to be inhibited by plant extracts with the range from 71 to 86 %, where *A. indica* exhibited maximum inhibition (Fig. 1a). While the protein carbonyl compounds, i.e. intermediate stage markers was found to be inhibited by 20–30 % and in presence of *A. indica* extract it was maximally reduced (Fig. 1b). Fluorescence analysis of the late stage glycation moieties-AGEs as depicted in Fig. 1c, suggested that plant extracts can inhibit them in the range from 7 to 40 %. *E. officinalis* illustrated maximum hindrance and in *S. cumini* coincubated albumin samples negligible reduction was noted.

Effect of plant extracts on thiol oxidation during glycation

The influence of plant extracts on thiol groups alteration were examined in present experimental conditions. In positive control samples, the free thiols were significantly decreased and interestingly all plant extracts illustrated significant protection against thiol oxidation. Results indicated thiols protection value ranged from 82 to 90 % with extracts where in presence of *T. bellirica* extract strong shielding from denaturation was evident, as thiol concentrations were almost similar to native BSA (Fig. 1d).

Effect of plant extracts on β aggregation during glycation

Glycation is a key mechanism to induce the conformational changes of protein by increasing the level of amyloid cross β-structure, which plays a fundamental role in the protein aggregation. The ability of the plant extracts to retard aggregation of glycated albumin which leads to amyloidosis was investigated by using two amyloid markers, i.e. Congo Red and Th. T. With these markers mixed response of plant extracts was evident (Fig. 1e). In glycated albumin samples co-incubated with *S. cumini* and *T. bellirica* extracts similar results were exhibited while slight discrepancies was evident in the glycated albumin samples of *A. indica* and *E. officinalis* extracts. The presence of *S. cumini* demonstrated potent inhibition with both the amyloid markers.

Overall effect of plant extracts on albumin glycation

On the basis of chemical structure, AGEs are of two types–a) fluorescent and crosslinking AGEs and, b) nonfluorescent and noncrosslinking AGEs. Since AGEs measurement is nonspecific, to resolve ambiguities, the overall impact of plant extracts on albumin glycation was investigated by another sensitive method, i.e. affinity chromatography. Through boronate affinity chromatography, glycated and non-glycated albumin fractions can be separated. As seen from Fig. 2, all plant extracts reduced the amount of glycated albumin remarkably and in *T. bellirica* extract maximum decline in the glycated protein was noted.

As shown Fig. 1f, cumulatively most promising effect against glycation is observed in the presence of *T. bellirica* extract and in *E. officinalis* extract least antiglycation activities were detected. Interestingly *A. indica* showed potent attenuation at the early stages of glycation. Conversely, in the latter phases its activity was reduced as it displayed intermediate AGEs inhibition with least thiol protection and Th. T fluorescence. On the whole based on these complementary assays, analyzed plant extracts showed variation in attenuation at multiple stages of albumin glycation, it's subsequent cross linking and consequential OS (Fig. 3).

Prevention of glycated albumin toxicity in erythrocytes by plant extracts

To ascertain the effects of plant extracts in glycated albumin induced toxicity to erythrocytes, various glycated albumin samples coincubated with plant extracts and positive control were tested for various cellular oxidative injury parameters.

Invasion of ROS to erythrocyte membrane may lead to cell hemolysis. As hypothesized the positive control glycated albumin sample showed detrimental effect on erythrocytes as it caused significant increase in hemolysis elucidating cellular oxidative damage. The ability of the plant extracts to retard free-radical-induced injury of erythrocytes was investigated and results are illustrated in Fig. 4a. Erythrocytes hemolysis was found to be reduced in presence of plant extracts as the inhibition values ranged from 30 to 62 % and *T. bellirica* showed maximum attenuation with a percentage inhibition of 62.31 %. Remarkable protection was noted in *E. officinalis*
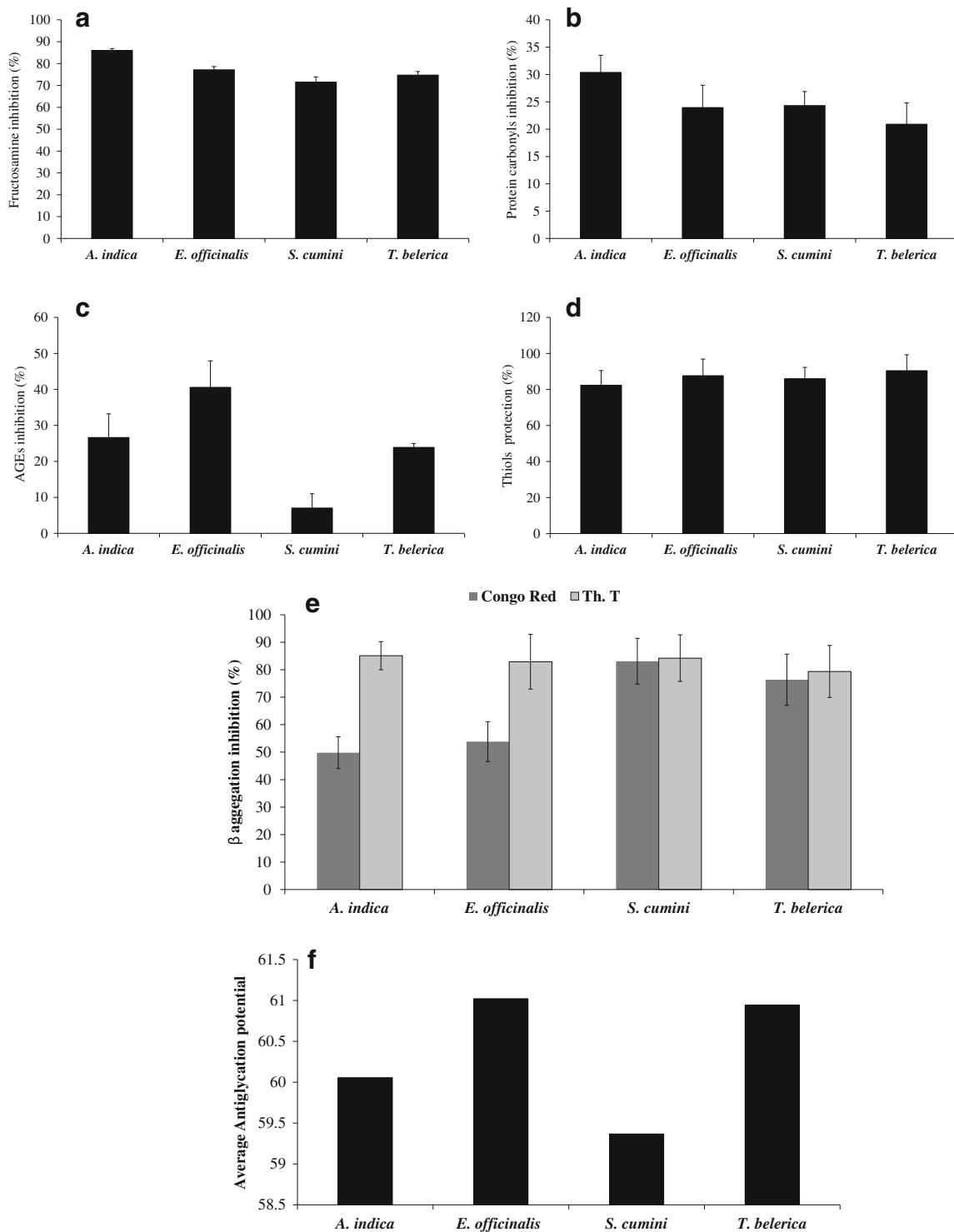
Fig. 1 The effect of plant extracts (1 mg/ml) on albumin glycation modifications in terms of (**a**) Fructosamine inhibition, (**b**) Protein carbonyls inhibition, (**c**) AGEs inhibition, (**d**) Protein thiol protection, (**e**) β aggegation inhibition and (**f**) Average antiglycation activity of plant extracts. Values are means ($n=3$), with standard deviations represented by *vertical bars*. Results of the measured parameter are expressed as % inhibition with respect to positive control

and *T. bellirica* extracts, containing a phenolic concentration higher than 100 (mg GAE/gm sample). The glycated albumin induced LPO was reduced by 30– 52 % by presence of plant extracts in glycated albumin, where *T. bellirica* showed highest decline by 52 % (Fig. 4b). As shown in Fig. 4c, the levels of GSH were

Fig. 2 The effect of plant extracts (1 mg/ml) on the amount of glycated albumin upon in vitro glycation as analysed by phenyl boronate affinity chromatography
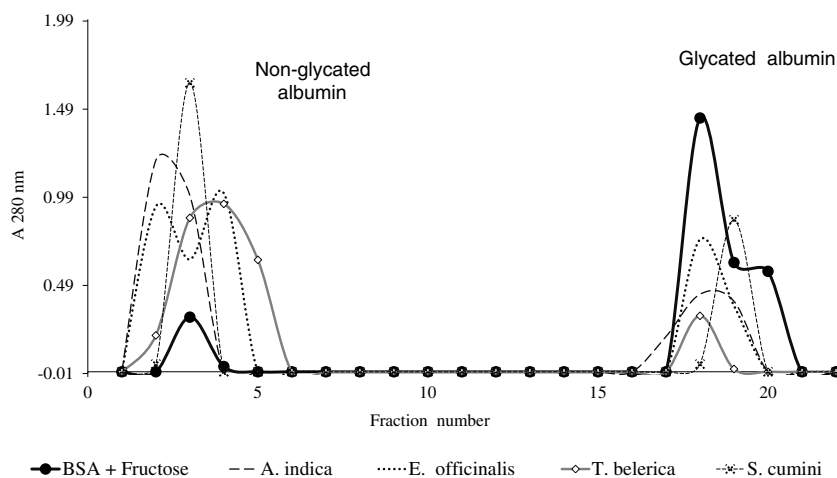
remarkable retained in presence of *T. bellirica* extract (100 %) and with *E. officinalis* extract it was minimally maintained (80 %). Functional consequences of the oxidative damage further leads to decrease in total antioxidant capacity of erythrocytes which was estimated in terms of FRAP values. These levels were showed to be sheltered by presence of plant extracts in glycated albumin sample (Fig. 4d). The FRAP value ranged from 80 to 100 % and the highest cellular antioxidant level was demonstrated in presence of *T. bellirica* and *E. officinalis* extracts (100 %) while *A. indica* and *S. cumini* extracts displayed almost similar values of 80–81 %.

Overall erythrocytes results suggest that oxidative toxicity was rescued greatly by the treatment of *T. bellirica* extract (80 %) coincubated glycated albumin sample, as depicted in Fig. 4e. Whereas, comparatively less protection was exerted to erythrocytes by other three plants.

Antioxidant potential of plant extracts

A possible mechanism by which selected plants inhibit the albumin glycation could be through their antioxidant properties. Therefore, a systemic evaluation of antioxidant potential of plants extracts was carried out using different complementary assays as shown in Fig. 5.

Phenolic content of plant extracts

There was a wide range of phenolic levels in the plant extracts as the values varied from 47 to 147 mg GAE/gm in the following increasing order: *A. indica* < *E. officinalis* < *S. cumini* < *T. bellirica* < *E. officinalis*. The *E. officinalis* extract and *T. bellirica* extract contained three fold total phenolic compared to the *A. indica* extract.

RP of plant extracts

All methanolic plant extracts had shown considerable reducing activity indicating Fe^{3+} transformation to Fe^{2+} in the presence of plant extracts. The plant extracts showed significant variation in the absorbance values as ranged from 1 to 7.9. RP of *T. bellirica* was incredibly high, indicating that it has strong capacity of donating electrons. Moderately high RP value is shown by *E. officinalis* (4.29), while lowest reducing capacity was demonstrated by *A. indica* which also had least phenolic compounds.

DPPH and ABTS radical scavenging activities of plant extracts

The plant extracts DPPH scavenging activities was ranged from 86 to 95 %. *T. bellirica* exhibited the highest activity, closely pursued by *E. officinalis* and *A. indica* whereas lowest scavenging activity was demonstrated by *S. cumini* extract. Elevated ABTS radical scavenging ability was showed by all extracts had ranging from 97 to 100 %. *S. cumini* and *T. bellirica* extracts showed the highest scavenging capacity followed by *E. officinalis* (98.77 %) and *A. indica* (97.63 %).

Association between antiglycation potential and erythrocyte protection with antioxidant activity of plant extracts

Pearson correlation analysis was used to investigate potential relationships between antiglycation activities, erythrocytes parameters with antioxidant properties of individual extracts. It was found that the protein thiol protection ability of plant extracts were correlated positively to their total polyphenols and RP (Total polyphenols vs. thiol protection $R^2 = 0.92$: RP vs. thiol protection $R^2 = 0.944$) rather than scavenging activities. It was found that the antiglycation activity values were slightly
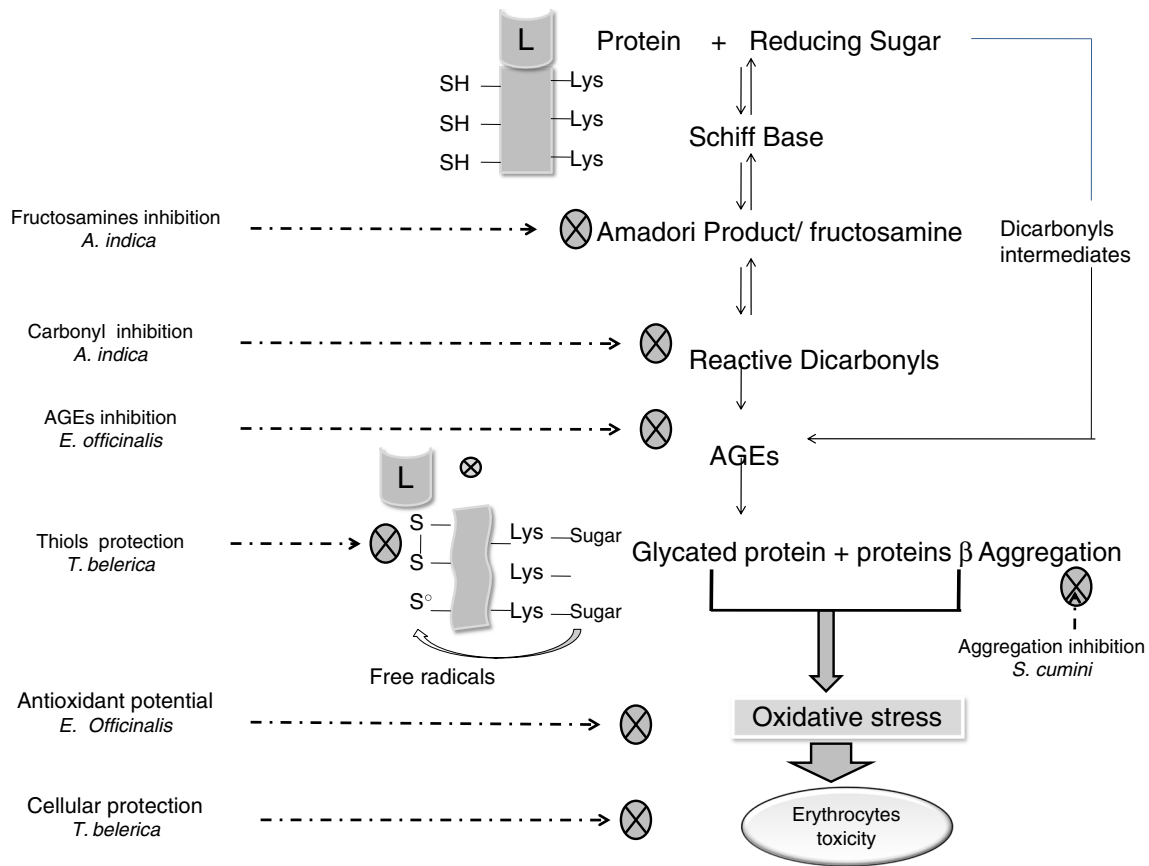
Fig. 3 Proposed schematic model of the glycation induced modifications of the structure and the properties of the albumin molecule. Before glycation, native albumin is in its normal globular conformation with the bound ligand (L), reduced thiol groups (SH) and free Lysine residues (Lys). In hyperglycemia, the reducing sugar reacts with proteins leads to the formation of Schiff base, Amadori products, protein carbonyls, AGEs and glycate the protein. The glycated protein has drastically alerted conformation with no attached ligand (L), oxidized thiols into disulfure linkages or thiyl radicals (S-S or S, respectively) and Lys bound to sugar. Glycation also induces the β aggregation of proteins and collectively leads to OS. To understand the complete modifications and inhibition at various steps by plants the biochemical analysis has been done using different markers. Based on the biochemical analysis, at each potential site the most promising plants with maximum inhibitory potential have been recognized (shown in *boxes*). The latter can prevent glycation ($\otimes$) either at the formation of protein fructosamine, carbonyls, AGEs, β-aggregation, oxidation of thiols and finally resulting OS which results in cellular protection

better correlated to total polyphenols rather than to RP (Total polyphenols vs. AGEs inhibition $R^2=0.352$: RP vs. vs. AGEs inhibition $R^2=0.259$). Secondly, significant positive correlations ($p>0.01$) were also observed between erythrocyte parameters, i.e. hemolysis reduction, LPO inhibition activities and FRAP levels with total phenolic contents as illustrated in Fig. 6a, b and c respectively.

Discussion

Inhibition of protein glycation is a complex process and glycation inhibitors may act by various mechanisms at different steps that can delay or prevent the glycation process, such as: i) at an early stage scavenging hydroxyl radicals and superoxide radicals and reducing the generation of reactive carbonyl or dicarbonyl groups, 2)

during the glycation process, blocking the carbonyl or dicarbonyl groups in reducing sugars, Schiff bases or Amadori products, 3) metal ion chelation to inhibit AGE formation, 4) inhibiting the formation of late-stage Amadori products, 5) breaking the crosslinking structures in the formed AGEs and 6) AGEs receptor (RAGE) blocking (Wu et al. 2011). Based on this background, to completely elucidate the effect of 4 plant extracts at concentrations of physiological relevance on albumin glycation, its structural modifications and cellular effects; a series of experiments were performed in the present study.

The observed results for AGEs inhibition by *T. bellirica*, *E. officinalis* and *A. indica* extracts are supported by other recent studies (Gutierrez et al. 2011; Nampoothiri et al. 2011; Kasabri et al. 2010). However inhibitory effects of the analyzed extracts were not limited to AGEs formation (which account for only a
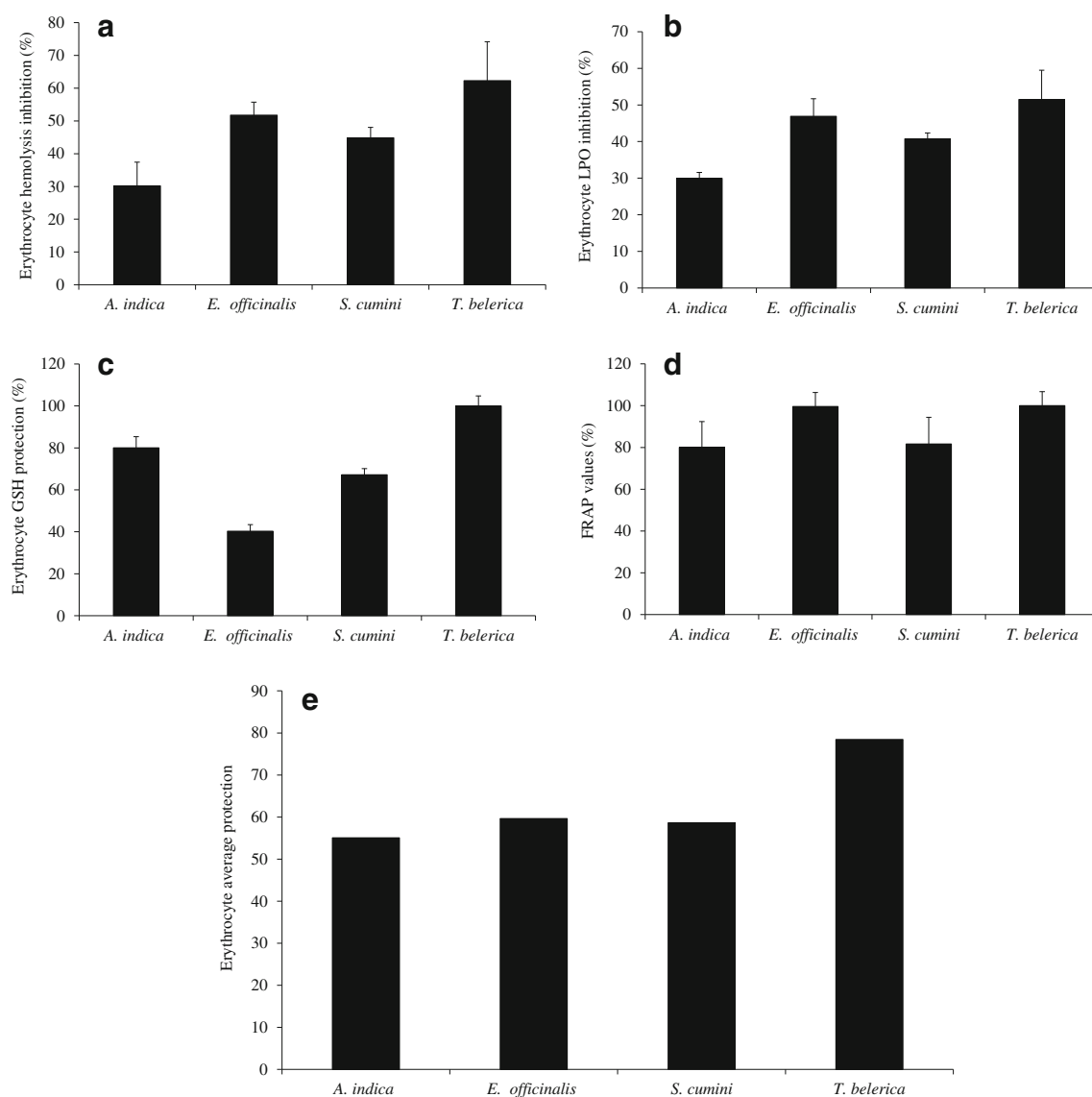
Fig. 4 The effect of plant extracts (1 mg/ml) on glycated albumin toxicity on erythrocytes in terms of (**a**) hemolysis prevention, (**b**) LPO inhibition, (**c**) GSH protection, (**d**) FRAP levels and (**e**) Average erythrocyte protection. Results of the measured parameter are expressed as % inhibition with respect to positive control

small percentage of physiologically occurring cross-linking structures), but extend to other structural and functional alteration of glycated albumin (thiol oxidation, β aggregation, antioxidant properties) implicated in the pathophysiology of diabetic complications (Cohen 2003). The pragmatic different degree of activities against various glycation products by the plant extracts may reflect their selective mechanism of action. Analogous study by Wu et al. (2010) for individual dietary phenolic acids also demonstrated the variation in inhibition at multiple stages of glycation (Amadori products, AGEs-specific fluorescence, late glycation products and protein-AGEs crosslinking). The reason for variation had not been clarified and warrants further studies.

The major source of thiols in plasma is albumin and glycation modifies thiol group(s) to form disulfide bonds and intermolecular aggregates. A major molecular modification of protein structure due to glycation is the loss of protein thiol groups, which is the direct reflection of excess free radical generation (Mossine et al. 1999). Glycation-induced modifications have a determinant impact on albumin functional properties, which is related to alteration in its conformation. In the present study an effort has been made to understand these modifications in presence of antidiabetic plant extracts. It was seen that presence of extracts during glycation protected thiol groups from oxidation, indicating their strong reducing potential. Since the significant correlation was found in thiol protection with phenol content and reducing potential of the plant extracts.

Fig. 5 Antioxidant potential of plants extracts (1 mg/ml) in terms of total phenolic content, RP and scavenging activities of ABTS and DPPH radicals. Values are means ($n=3$), with standard deviations represented by *bars*


☐ Total phenolic content (mg GAE/gm sample)   ☐ RP (A700nm)

☐ DPPH (radical scavenging activity %)   ■ ABTS radical scavenging activity (%)
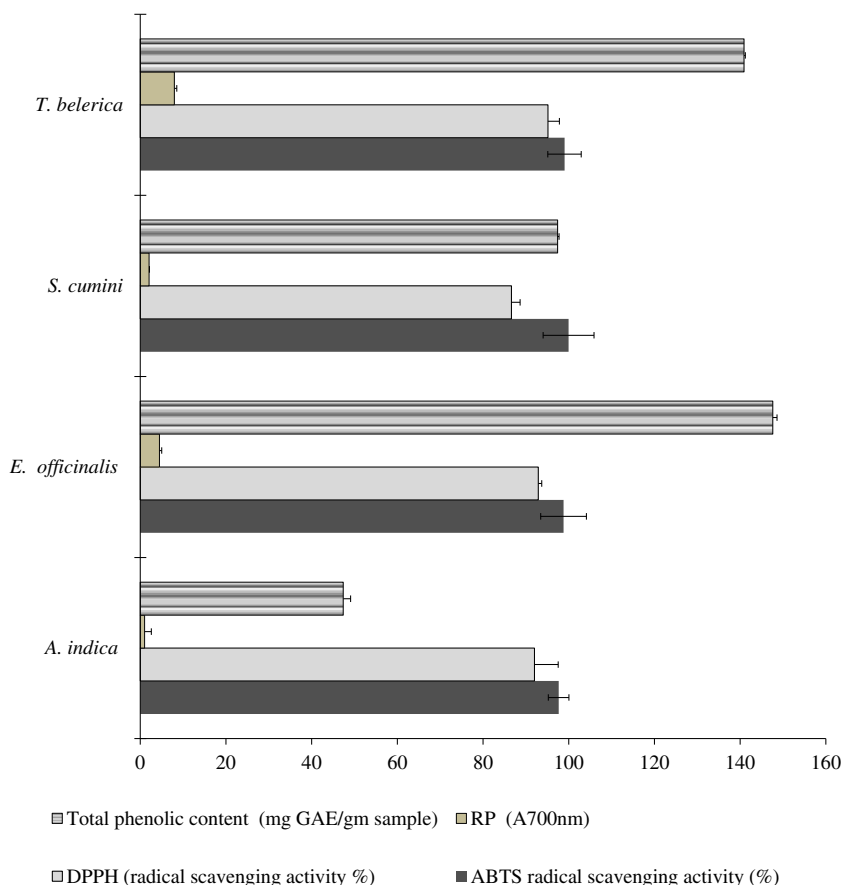
Our data clearly establish that plant extracts can suppress the level of amyloid cross β-structure of albumin. This beneficial effect of *S. cumini* and *T. bellirica* should be additionally evaluated to understand the mode of inhibition. According to Hudson et al. (2009), Th. T fluorescence can be bias as polyphenolics directly interact with Th. T and suggested another method using Congo Red. In the present study, this may justify the observed differences among both these aggregation marker's levels in the glycated albumin samples of *A. indica* and *E. officinalis* extracts.

Importantly, the total phenolic content correlated positively with measures of AGEs and protein thiols protection as phenolic-rich extracts generally exhibited stronger effects. This is supported by several lines of evidence strongly suggest the antiglycation activities of certain plant phenolics (Peng et al. 2008). Nevertheless, a deviation from phenolics-antiglycation property was also observed in the present study, for instance *A. indica* extract which contained relatively least phenolic content, significantly inhibited fructosamines and protein carbonyls, on the contrary *E. officinalis* extract with relatively high phenolic content displayed lowest carbonyl inhibition. Therefore, though phenolic content served as a reasonable indicator of an extract's overall antiglycation and antioxidant potential, action in an individual assays depends on the quantities and properties of specific phenolics in the extracts. Hence, no direct structure–activity relationship has been established to explain the anti-glycation activity of the phenolic acids that was shown in the above assays.

Earlier we have described the glycated albumin toxicity in hepatocytes (Tupe and Agte 2010) and there are reports about its toxicity to various cells like brain endothelial cells (Bouma et al. 2003), platelets and monocytes etc. (Rondeau and Bourdon 2011). Despite the determinant role of erythrocytes in the etiology of diabetic complications, there are no reports on the effect of glycated albumin on various cell parameters of erythrocytes. Erythrocytes are more frequently exposed to albumin than other body tissue and thus, are more susceptible to oxidative damage induced by glycated albumin toxicity. Major pathological consequence of glycation is free radical induced erythrocytes membrane peroxidation which includes increased membrane rigidity, decreased cellular deformability, reduced erythrocyte survival and lipid fluidity (Waczulíkova et al. 2000). Therefore the effect of glycation in presence and absence of plant extracts was evaluated in the present study. The plant extracts erythrocyte hemolysis inhibition capacity was identical to the LPO inhibition ability

Fig. 6 Correlation of erythrocyte parameters with total phenolic content. The plot of phenolic content (mg GAE)/gm sample), versus (**a**) Hemolysis inhibition (%), (**b**) LPO inhibition (%) and (**c**) FRAP value (%)
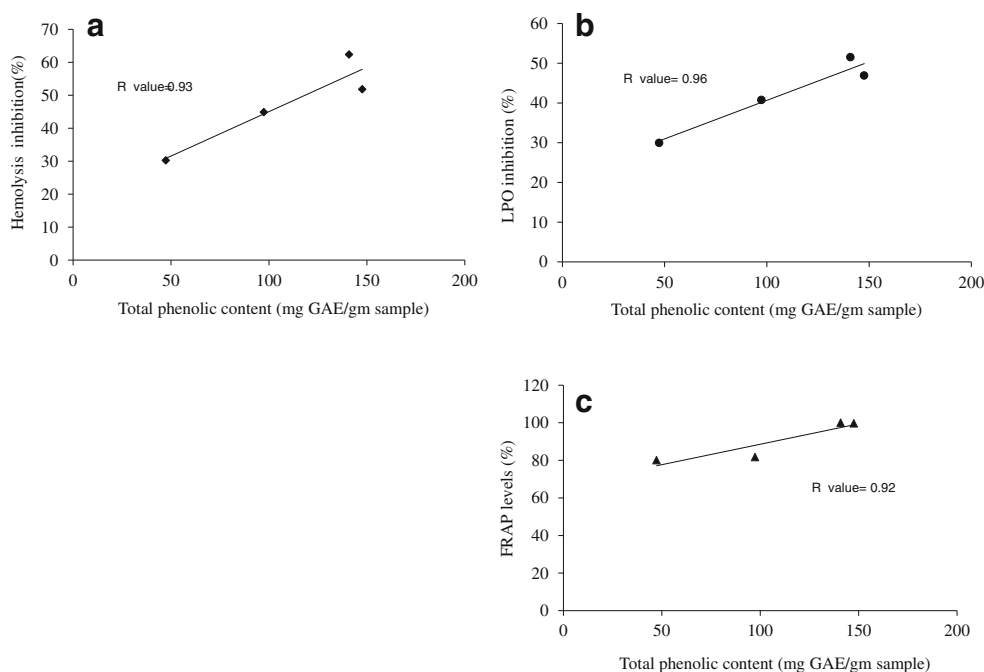
where *T. bellirica* and *E. officinalis* showed similar effects. This result shows that the natural antioxidants of the *T. bellirica* and *E. officinalis* extract strongly decrease the LPO of erythrocytes, thus preventing their hemolysis.

In addition to LPO, oxidants affect vital –SH groups of proteins which are highly active, protects membrane proteins and preserves their stability and may be targeted during OS (Martín-Gallán et al. 2003). In our experimental system, a complete preservation of erythrocytes GSH content was observed in erythrocytes pretreated with *T. bellirica* coincubated glycated albumin followed by *A. indica*. The FRAP values of erythrocytes were retained completely by *T. bellirica* and *E. officinalis extracts*. Thus results clearly demonstrated sufficient protection against oxidative toxicity in erythrocytes treated with *T. bellirica* and *E. officinalis* incubated albumin samples and the phenolic richness of the extract could contribute to this observation. This is assisted by other indirect reports, whereby phenolic-rich plant extracts exerted modulatory effects on erythrocyte oxidative damage (Paiva-Martins et al. 2009). As suggested by Asgary et al. (2005) phenols afford beneficial effects in preventing oxidative damage to erythrocyte membranes by inhibiting peroxidation of lipids, protecting -SH groups and increase cellular resistance to oxidative stress therefore, inhibited hemolysis. Though the bioavailability of phenolics from the specific extracts has yet to be determined, their abundance in certain samples suggests that the cumulative concentration of phenolics with antiglycation and antioxidant activity likely provides some degree of protection against endogenous AGEs formation and OS.

Conclusion

Though for various synthetic AGEs inhibitors encouraging results have been obtained in vitro and in diabetic animals, still most of the clinical trials have been more or less disappointing in part because of side- effects. Thus, demand for natural, specific and safe- nutritional or herbal interventions has increased in patients inclined toward no pharmaceutical or alternative healing practices. In conclusion, our result's support the view that antidiabetic plants are promising sources to inhibit glycation, OS and protect erythrocytes. This also suggests that observed therapeutic potential is not limited to the plants included in the study since similar or additional compounds are present in the several antidiabetic plants. These new findings provide evidence for a novel mechanism by which antidiabetic plants might have additional protective effects and lay the groundwork for future in vivo investigations against diabetic vascular complications.

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