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Protective effect of *Trigonella foenum-graecum* and *Cinnamomum zeylanicum* against diabetes induced oxidative DNA damage in rats

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Diabetes mellitus has become a global epidemic as its prevalence is steadily increasing everywhere at an alarming rate, most markedly in the India. Traditional Indian system of medicine based on herbal therapies is being practiced since ancient times and has been proved very effective against number of diseases. *Trigonella foenum-graecum* (*Tfg*) and *Cinnamomum zeylanicum* (*Cz*), the two spices, possess multiple medicinal properties. Hence present study is aimed to evaluate the protective effects of these plants on the oxidative DNA damage in diabetic rats. The presence of DNA base oxidation was investigated by modified comet assay which uses bacterial repair enzymes, formamidopyrimidine glycosylase and Endonuclease III which can measure oxidatively damaged purines and pyrimidines, respectively. Diabetes induced alteration in total antioxidant capacity (TAC), generation of superoxide anion and the levels of GSH, GSSG, NADH, NAD^+, NADPH and $NADP^+$ were monitored. Diabetes was induced by intravenous injection of alloxan, followed by oral administration of aqueous suspension of *Tfg* seeds and bark of *Cz*. Treatment of *Tfg* and *Cz* significantly restored the altered level of superoxide anion, TAC, ratios of GSH/GSSG, NAD(P)H/$NAD(P)^+$ and NADH/NAD^+ in the diabetic group. Furthermore, both the treatment effectively decreased the level of oxidatively damaged purine and pyrimidine bases.

Keywords: *Cinnamomum zeylanicum*, Hyperglycemia, Oxidative DNA damage, *Trigonella foenum-graecum*

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia resulting from either lack of insulin production or resistance to insulin. Both genetic and environmental factors contribute in the development and progression of diabetes. The pervasiveness of DM has risen to epidemic proportions worldwide. In diabetic condition, oxidative stress induced by the presence of excessive reactive oxygen species (ROS) and reactive nitrogen species (RNS) is closely associated with chronic inflammation, leading to potential tissue damage. All the important biomolecules including proteins, lipids and DNA are susceptible to oxidative damage. Diabetes mellitus induced oxidative stress, has also been reported to damage DNA[1]. Oxidative DNA damages include oxidiation of purines and pyrimidines bases, single-strand (SSBs) and double-strand (DSB) breaks as well as DNA-DNA and DNA-protein cross links[2]. ROS may also damage DNA indirectly, through reaction with lipids, proteins, and other cellular components to produce electrophilic species that can react with DNA[3]. Both, in type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM), induction of oxidative stress due to hyperglycemia have been reported as major cause of complications[1]. It has also been demonstrated that hyperglycemia is associated with an increased production of free radicals in the mitochondria and may contribute to a greater DNA damage[2,3].

The management of diabetes involves both the non pharmacological and pharmacological approaches. Due to the high cost and serious side effects of hypoglycemic drugs, the focus has been shifted to herbal medicines which are not only easily available but also having no toxic effects on other body functions. More than 10000 plants have been described to be experimentally or ethnopharmacologically used in the treatment of diabetes[4] and various plants extracts with medicinal values have been screened by many scientists in the field of diabetic research[4]. Most of the plants contain glycosides, alkaloids, terpenoids, flavanoids, carotenoids *etc*. that are frequently implicated as having antidiabetic effects. Several plants, such as *Azardirachta indica*, *Eugenia jambolana*, *Momordica*

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charantia, Ocimum sanctum, Gymnema sylvestre and *Trigonella foenum-graecum* L. *etc* are being used commonly in the treatment of diabetes. In the present study, two such medicinal plants that are widely used to manage diabetes, *Trigonella foenum-graecum* (*Tfg*) and C*innamomum zeylanicum* (*Cz*), commonly known as fenugreek and cinnamon, respectively, are selected to study their effects on oxidative stress and DNA damage in experimental diabetic rats. Studies on different animal models proved that fenugreek has strong antidiabetic properties[5]. Several studies have demonstrated that fenugreek seed extract, mucilage of seeds, and leaves can decrease blood glucose levels in human and experimental animals[5,6,9]. Cinnamon, a natural product with long history of safety, is rich in polyphenolic compounds that have been shown to improve the antioxidant status. Cinnamon belongs to the family *Lauraceae*, and its main components are cinnamaldehyde, cinnamic acid, tannin and methylhydroxychalcone polymer (MHCP). Cinnamon bark possesses significant anti-diabetic, anti-allergic, anti-ulcerogenic, antipyretic and antioxidant properties[7,8,10]. Both are being used as folk medicine not only in diabetes but also in number of other diseases[9,10]. The antihyperglycemic and antioxidant effect of *Tfg* seed and *Cz* bark have already been reported[5-10].

The present study is undertaken to evaluate the status of total antioxidant capacity (TAC), nonenzymatic antioxidants reduced and oxidized glutathione namely (GSH and GSSG), reduced and oxidized pyridine nucleotides ($NAD(P)^+$ and NAD(P)H), and their ratio $NAD(P)H/NAD(P)^+$ in tissues of diabetic rats, which will give estimates of redox status of the cell resulting in damage to the vital molecule, DNA. Attempts have also been made to evaluate the mechanism of DNA damage by measured oxidatively damaged purines and pyrimidines bases by the modified comet assay. In the modified comet assay, two bacterial enzymes, formamidopyrimidine glycosylase (Fpg) and endonuclease III (Endo) are used in conjunction with comet assay which remove oxidatively damaged purine and pyrimidine bases, respectively, and create a break in the DNA strand by the associated action of endonuclease which are then measured by comet assay and compared with lysis buffer treated slides. Aqueous suspension of powdered *Tfg* seed and bark of *Cz* were given as treatment to diabetic animals to analyse whether they have any protective effects against altered redox balance and DNA damage. The standard antidiabetic drug, glibenclamide, was taken as positive control.

Materials and Method

Chemicals

Chemicals used in the present study were of highest purity/analytical grade. Dimethyl sulfoxide, alloxan monohydrate, endonuclease III (Endo III) and formamidopyrimidine-DNA glycosylase (Fpg), oxidized cytochrome C, phorbol 12-myristate-13-acetate (PMA), reduced and oxidized glutathione, N-ethylmaleimide (NEM), oxidized and reduced pyridine nucleotides $NAD(P)^+$ and NAD(P)H), nicotinamide, N-ethyldibenzopyrazine ethyl sulphate (PES), 3-(4,5-dimethythiazolyl)- 2,5- diphenyl tetrazolium bromide (thiazolyl blue or MTT), alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, glucose-6-phosphate, ethylenediaminotetraacetic acid tetrasodium salt, agarose (normal melting and low melting), Hisep, trypan blue, 4-(2-hydroxyethyl)-pierazine-1-ethanesulphonic acid (HEPES) buffer, potassium hydroxide, metmyoglobin, trolox and ABTS [2, 2′-azinobis-(3-ethylbanzothizoline-6-sulfonic acid) disodium salt] were purchased from Sigma Chemical Co., St. Louis, Mo. Sarcosine, was obtained from Merck, Germany.

Experimental animals

Adult male albino rats of Wistar strain (*Rattus norvegicus*) weighing about 140 ± 10 g were used in the present study. Rats were obtained from the animal facilities of Defence Research and Development Establishment, Gwalior, India, and were maintained in a light (light-dark cycle of 12 h each) and temperature (25° ± 2°C) controlled animal room of our department on standard pellet diet (obtained from Amrut Rat & Mice Feed, New Delhi, India) and tap water *ad libitum*. Rats were acclimatized for one week prior to the start of the experiment. The study was approved by the institutional ethical committee on animal use of Jiwaji University (Ethical Code IAEC/JU/2011/01).

Experimental design

Rats were randomly divided into five groups of six animals each, and were given following treatments:
Group 1: Control (healthy rats with normal blood glucose level); Group 2: Diabetic (i.v. injection of alloxan 55 mg/kg body weight); Group 3: Diabetic + *Tfg* seed powder (1740 mg/kg body weight); Group 4: Diabetic + Cz bark powder (200 mg/kg body weight);

and Group 5: Diabetic + Glibenclamide (0.6 mg/kg body weight)

Induction of experimental diabetes and treatment

Diabetes was induced in the overnight fasted adult rats by single intravenous injection of 55 mg/kg body weight of alloxan monohydrate dissolved in normal saline (0.85% NaCl)[5]. Diabetes was confirmed by blood glucose estimation after 48 h by an electronic glucometer. The rats with fasting glucose level above 300 mg/dL were considered diabetics and selected for the study.

Trigonella foenum-graecum (*Tfg*) seeds and *Cinnamomum zeylanicum* (*Cz*) bark were purchased from the local herbal market, cleaned, dried and finely powdered. The plant materials were identified by the School of Studies in Botany, Jiwaji University, Gwalior. Aqueous suspension of both powdered *Tfg* seeds and *Cz* bark were prepared and doses 1740 mg/kg body weight[5,10] and 200 mg/kg body weight, respectively[11,12], were given orally to the diabetic rats with the help of cannula, daily for two weeks. For positive control a group of rats was given glibenclamide at a dose of 0.6 mg/kg body weight orally for two weeks[13] and a separate group of rats received equivalent volume of water for the same period of time and served as the control.

Collection of blood, isolation of lymphocytes and tissues

The blood was collected 24 h after the last treatment *via* ocular bleeding in tubes containing EDTA from the animals for lymphocyte separation and also in tubes without EDTA for serum separation. Blood samples were diluted with equal volume of phosphate buffer saline (PBS), layered on the top of a Ficoll solution (1.077 g/mL). The interface containing the lymphocytes was collected after sedimentation of erythrocytes at 1000 g for 10 min at room temperature. The collected lymphocytes were washed twice with phosphate buffer saline (PBS), pH 7.4, and cell viability was checked by trypan blue dye exclusion test[14]. The lymphocytes samples with viability >95% were used for various estimations. After blood collection, rats were humanly killed by cervical dislocation; different tissues were excised off, washed with 0.9% NaCl and used for different estimations.

Estimations

Superoxide anion release was measured by superoxide dismutase inhibitable reduction of ferricytochrome c[15]. PMA (polymormphonucleocytes) (3×10^5) were incubated in PBS-EDTA buffer (pH 7.4) with phorbol-12, 13-dibutyrate (PDBu) in a total volume of 1 mL at 37°C for 15 min. The final concentration of ferricytochrome c and PDBu were 50 nM/L and 100 nM/L, respectively. The change in absorbance was measured spectrophotometrically at 550 nm for 10 min with a double beam Shimadzu UV-160A spectrophotometer (Shimadzu Seisakusho Ltd., Kyoto, Japan) at room temperature. The amount of superoxide anion released into the medium was calculated by using molar extinction coefficient of reduced cyochrome c, $2.1X\ 10^4\ M^{-1}\ cm^{-1}$ and the concentration is expressed as n mole $O_2^{\cdot-}$/ 10^6 cells/min.

Total antioxidant capacity (TAC) in serum was measured by the method described by Rice-Evans and Miller[16]. The reagents were mixed as follows: 8.4 µL of sample, 489 µL of buffer (0.1 M PBS, pH 7.4), 36 µL of 70 µM metmyoglobin, and 300 µL of 5 mM ABTS [2, 2′-azinobis-(3-ethylbanzothizoline-6-sulfonic acid) disodium salt], with vortexing. The reaction was started by addition of 167 µL of 450 µM H_2O_2 and the absorbance change per min was recorded at 734 nm for 6 min. The total antioxidant capacity in serum was calculated by calibration curve using trolox (2.5 mm) as standard and values were expressed as mmol trolox equivalent/L.

The levels of reduced and oxidized glutathione (GSH and GSSG) were estimated by the method described by Hssin and Hilf[17]. Approximately 250 mg of tissues were homogenised in solution consisted of 3.75 mL phosphate EDTA buffer (0.1 M sodium phosphate buffer containing 5.0 mM EDTA, pH 8.0) and 1.0 mL of 25% HPO_3, which were used as protein precipitant and GSH or GSSG was assayed in supernatant obtained after centrifugation at 10000 g for 30 min. To 0.5 mL of the 10000 g supernatant, 4.5 mL of phosphate EDTA buffer, pH 8.0, was added for GSH estimation. The final mixture contained 0.1 mL diluted tissue supernatant, 1.8 mL phosphate EDTA buffer and 0.1 mL o-phthaldehyde (1 mg/mL) and fluorescence at 412 nm was determined with excitation at 350 nm. For estimation of GSSG, to 0.5 mL of 10000 g supernatant, 0.2 mL of N-ethylmaleimide (NEM, 0.04 M) was added and incubated at room temperature for 30 min to interact with GSH present in the tissue. To this mixture 4.3 mL of 0.1 N NaOH was added. 0.1 mL of this mixture was used for the assay of GSSG in the manner identical to GSH except that 0.1 N NaOH was

used as diluents in place of phosphate EDTA buffer. Both GSH and GSSG standard curves were prepared in similar manner using the concentration range of 10 to 100 µg. The levels in tissues were expressed as µg GSH or GSSG g^{-1} tissue.

Oxidized and reduced pyridine nucleotides (NAD^+, $NADP^+$, NADH and NADPH) were assayed by the method of Zerez *et al.*[18]. A 10% homogenate was prepared in 1 M Tris-HCl buffer (pH 8.0) and centrifuged at 9500 g for 20 min in a refrigerated centrifuge. To 0.1 mL of resulting supernatant, 1.9 mL extraction buffer (containing 100 mM Na_2CO_3 and 10 mM nicotinamide) was added and kept at 0˚C for 30 min. The frozen mixture was thawed quickly at room temperature water bath and divided into two equal parts, one part was used for estimation of NAD^+ + NADH or $NADP^+$ + NADPH and the other part was incubated at 60ºC for 30 min, quickly chilled and used for the estimation of only reduced form of pyridine nucleotides. For assay of total NAD (NAD^+ + NADH) or NADH, the reaction mixture containing 2.0 µM N-ethyldibenzopyrazine ethyl sulphate (PES), 0.5 µM 3-(4,5-dimethythiazolyl)- 2,5- diphenyl tetrazolium bromide (thiazolyl blue or MTT), 0.25 U of alcohol dehydrogenase, 600 µM ethanol and 0.1 mL extract (for NAD^+ + NADH) or 0.1 mL heat treated extract (for NADH estimation) in the total volume of 1.0 mL. Absorbance change per min was recorded at 570 nm for 5 min. For assay of total NADP ($NADP^+$ + NADPH) or NADPH, the reaction mixture containing 5.0 µM Na_4EDTA, 2.0 µM PES and 0.5 µM MTT, 1.3 U glucose-6-phosphate dehydrogenase, 1.0 µM glucose-6-phosphate and 0.1 mL extract (or 0.1 mL heat treated extract) in the total volume of 1.0 mL. Absorbance change was recorded at 570 nm for 5 min. For standard curve, varying concentrations of NAD^+, NADH, $NADP^+$ and NADPH ranging from 1 to 4 pmole in a total volume 0.5 mL were taken. The rest of the procedure remained similar to the estimation of NAD^+ or $NADP^+$. The results were expressed as pmole NAD(P) or NAD(P)H present gm^{-1} tissue.

Modified bases were estimated by Fpg- Endo enzyme treatment in combination with the comet assay based on Collin's protocols[19] and described earlier[20]. A homogenate (25% *w/v*) of fresh tissue was prepared in chilled homogenizing buffer (0.075 M NaCl containing 0.024 M EDTA, pH 7.2) in a Potter Elvehjem homogenizer with a single stroke. The nuclei were obtained by centrifugation at 700 g for 10 min at 4°C and the pellet was gently resuspended in 3.0 mL of chilled homogenizing buffer. Seventy five µL of normal melting agarose (1% prepared in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.9% NaCl) was quickly layered on end-frosted slide, covered gently with another slide, and allowed to solidify. The upper slide was carefully removed and the precoated slide was coated with 100 µL of a mixture containing equal volumes of sample (nuclei preparation) and low melting agarose (2% in phosphate buffer saline). The slides were placed in cold lysis buffer (2.5 M NaCl, 0.1 M Na_2EDTA, 0.01 Tris-HCl, 1% Triton X-100, 10% DMSO, pH 10) overnight at 4°C (minimum of 12 h). For the enzyme treatment, four slides were prepared from each cell treated sample: "lysis", "buffer", "Fpg" and "Endo III". After lysis, slides "buffer", "Fpg" and "Endo III" were washed 3 times at 4°C, 5 min each time, with enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, adjusted to pH 8 with KOH). Then 50 µL of enzyme buffer, Fpg solution or Endo III (1 µg/mL of Endo III or Fpg) solution are placed onto the gels of the corresponding slides, covered with coverslips and incubated at 37°C for 30 min in a moist box. The slide labelled as "lysis" remained in the lysis solution during the incubation of rest of the slides. At the end of the incubation period, the cover slips were removed and all slides, including the "lysis" slides, were placed in an electrophoresis chamber containing electrophoresis solution (0.3 M NaOH, 1 mM EDTA). Electrophoresis was carried out for 30 min at constant voltage of 0.8 V/cm (measured between the electrodes across the platform carrying the slides) and the current around 300 mA. After electrophoresis slides were neutralized by adding Tris-HCl buffer (0.4 M, pH 7.5) in a drop-wise fashion onto the slides. The whole procedure was performed in dim light to minimize the artefactual DNA damage. Just before visualization, each slide was stained with 50 µL of ethidium bromide (20 µg/mL), rinsed with water and covered with a cover slip. The slides were observed at 10X magnification with a Leica Optiphase microscope equipped with an excitation filter of 515-560 nm and barrier filter of 590 nm. A total of 100 cells were scored per tissue per animal (50 from each replicate slide). The nuclei were divided into five different categories on the basis of percentage of DNA in the tail using TriTek CometScore™ Freeware v1.5 software. The nuclei having 0-10% of tail DNA were

Stages	Criteria of classification	% of DNA in tail	Visual appearance
0	0-10%	0.001224	
I	10-25%	11.921560	
II	25-50%	48.226644	
III	50-75%	70.715544	
IV	>75%	91.362786	

Fig. 1 — Different stages of DNA damage in isolated nuclei

categorized under 0 stage, 10-25% tail DNA under stage I, 25-50% tail DNA under stage II, 50-75% tail DNA under stage III and the nuclei having tail DNA >75% were categorized under stage IV (Fig. 1). The results are expressed as DNA damage index, calculated as #0 + #1 + #2 + #3 + #4/ # of cell scored where # is the total number of nuclei counted.

Statistical analyses

Results are expressed as mean ± S.E. of six sets of observations taken on different days. Statistical analyses were performed using Sigma Stat Statistical software version 2.0. All the statistical analyses were performed using one-way analysis of variance post hoc Bonferroni's multiple comparison test applied across the treatment groups. Significance was based on P value < 0.05.

Results

Effect of Tfg seeds and Cz bark on superoxide anion release and total antioxidant capacity (TAC) in tissues of control and diabetic rats

The alloxan induced diabetes caused significantly increased rate of generation of superoxide anion ($O_2^{\cdot -}$) in the lymphocytes of rats. The increase in the levels of superoxide anion was 160% in diabetic group when compared with the control. When diabetic rats were treated with *Tfg*, *Cz* and glibenclamide at a dose of 1740 mg/kg, 200 mg/kg and 0.6 mg/kg body weight for two weeks, superoxide anion levels were decreased by 48%, 38% and 53%, respectively, in the lymphocytes, when compared with untreated diabetic group (Fig. 2).

The total antioxidant capacity (TAC) was also affected by alloxan induced experimental diabetes. The decrease in TAC and recovery after treatment was not very high as compared to the other parameters, but it was statistically significant. The decrease in TAC in the serum of diabetic rats was 42%, when compared with control (Fig. 2). The

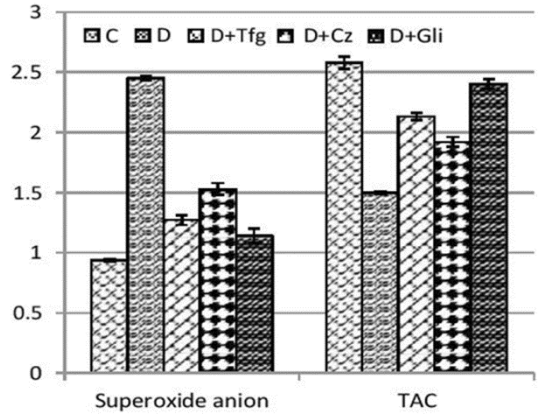

Fig. 2 — Effect of *Tfg* and *Cz* on the level of superoxide anion in lymphocytes and total antioxidant capacity in serum of control and diabetic group. Where, *Tfg- Trigonella foenum-graecum*, *Cz- Cinnomomum zeylanicum*, Gli- Glibenclamide

diabetic group receiving treatment of *Tfg*, *Cz* and glibenclamide orally for two weeks showed a increase of 42%, 27% and 60%, respectively, in the serum of diabetic rats, when compared with untreated diabetic group (Fig. 2).

Effect of Tfg seeds and Cz bark on the levels of GSH, GSSG and their ratio GSH/GSSG in tissues of control and diabetic rats

The present study showed that oral administration of aqueous suspension of *Tfg* seeds and *Cz* bark powder significantly increased in the levels of GSH and corresponding decrease in the levels of GSSG, which leads to increase in GSH/ GSSG ratio, when compared with untreated diabetic group. Administration of alloxan caused 52% decrease in the level of GSH in the liver and 39% decrease in the brain while increase in the levels of GSSG were 72% in the liver and 48% in the brain of diabetic group when compared with control. Decreased levels of GSH and increased levels of GSSG caused decrease in the ratio of GSH/GSSG in the tissues of diabetic rats. The ratio of GSH/GSSG was decreased by 72% in the liver and 59% in the brain, respectively, when compared with control (Table 1).

When the diabetic rats were given treatment of *Tfg*, *Cz* and glibenclamide for two weeks, GSH levels were increased by 51%, 21%, 72% in the liver and 41%, 19%, 54% in the brain, while the levels of GSSG were decreased by 28%, 9%, 33% in the liver and 21%, 7%, 29% in the brain, respectively, when compared with untreated diabetic group (Table 1). Similarily, when the *Tfg*, *Cz* and glibenclamide treated diabetic rats were compared with untreated

diabetic group, increase in the ratios of GSH/GSSG were 110%, 34%, 159%, in the liver and 88%, 28%, 118%, respectively, in the brain.

Effect of Tfg seeds and Cz bark on the levels of NADPH, $NADP^+$, NADH, NAD^+ and their ratio NADPH/$NADP^+$, NADH/NAD^+ in tissues of control and diabetic rats

The present study clearly showed that the alloxan induced diabetes caused decrease in the levels of NADPH and NADH and corresponding increase in the levels of oxidized pyridine nucleotide *i.e.* $NADP^+$ and NAD^+ indicating the redox imbalance and conditions of oxidative stress. There was 23% and 44% decrease in the level of NADPH and corresponding 239% and 189% increase in the levels of $NADP^+$ in the liver and the brain, respectively, in diabetic rats when compared with the control (Table 2). Similarly decrease in the levels of NADH by 64%, 58% and corresponding increase in the levels of NAD^+ by 454%, 87% were observed, in the liver and the brain of diabetic group, respectively, when compared with control group (Table 3).

The increase in the levels of NADPH was 17%, 10%, and 23% in the liver and 38%, 22%, and 56% in the brain on treatment with *Tfg*, *Cz* and glibenclamide at dose of 1740 mg/kg, 200 mg/kg and 0.6 mg/kg body weight, respectively, for two weeks. The decrease observed in $NADP^+$ levels was 45%, 28%, and 60% in the liver and 46%, 37%, and 53% in the brain of rats given similar treatment of *Tfg*, *Cz* and glibenclamide orally for two weeks, respectively, (Table 2).

When the diabetic rats were given *Tfg*, *Cz* and glibenclamide treatment for two weeks, 82%, 51% and 137% increase in NADH levels while 51%, 31% and 67% decrease in the NAD^+ levels in the hepatic tissue likewise 72%, 44% and 118% increase in NADH levels while 31%, 19% and 34% deacrease in NAD^+ levels in the brain, respectively, were observed when compared with untreated diabetic group (Table 3).

The redox status measured by the ratio of NADPH/$NADP^+$ and NADH/NAD^+, an indicator of oxidative stress, was found to be significantly reduced by 78% and 93% in the liver and 24% and 78% in the brain of diabetic rats when compared with control. After oral administration of *Tfg*, *Cz* and glibenclamide, the levels were restored by increase in the ratio of both NADPH/$NADP^+$ and NADH/NAD^+ as 115%, 56%, 210% and 211%, 158%, 605% in the liver and 161%, 97%, 242% and 150%, 80%, 237% in the brain of diabetic rats, respectively, when compared with untreated group (Tables 2 & 3). Treatment of *Tfg* and *Cz* showed protection against diabetes induced disturbances in the redox status in the diabetic rats.

Effect of Tfg seeds and Cz bark on the level of DNA damage in tissues and the lymphocytes of control and diabetic rats

The diabetes induced damage to purine and pyrimidine bases were monitored by modified comet assay. In this assay, two bacterial glycosylases, Fpg and Endo III, which remove the damaged purines and

Table 1 — Effect of oral administration of *Trigonella foenum graecum* seeds and *Cinnamomum zeylanicum* bark powder for two weeks on GSH, GSSG and their ratio GSH/GSSG in the liver and the brain of control and diabetic rats

	Groups	GSH	GSSG	GSH/GSSG
Liver				
	Control	1631.1 ± 9.7	421.4 ± 2.8	3.87
	Diabetic	785.5 ± 2.1***	722.8 ± 2.9**	1.08
	Diabetic + *Tfg* (1740 mg/kg)	1183.5 ± 2.4***	521.3 ±3.9***	2.27
	Diabetic + *Cz* (200 mg/kg)	953.5 ± 13.2**	656.7 ± 2.1**	1.45
	Diabetic + Gli (0.6 mg/kg)	1351.5 ± 2.3***	481.7 ± 2.2***	2.80
Brain				
	Control	425.4 ± 3.5	291.1 ± 23.6	1.46
	Diabetic	261.6 ± 2.4**	431.8 ± 2.7***	0.60
	Diabetic + *Tfg* (1740 mg/kg)	370.1 ± 3.2**	341.7 ± 2.7**	1.13
	Diabetic + *Cz* (200 mg/kg)	310.8 ± 2.9*	400.9 ± 2.4*	0.77
	Diabetic + Gli (0.6 mg/kg)	402.9 ± 4.2***	308.3 ± 4.7**	1.31

GSH and GSSG levels are expressed as µg GSH or GSSG g $tissue^{-1}$.

Results are mean ± S.E. of six set of observation. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$ and $^{\#}$ $P > 0.05$ when compared with control.

Diabetic rats were given aqueous suspension of powdered *Trigonella foenum graecum* seed and *Cinnamomum zeylanicum* bark orally at a dose of 1740 mg/kg body weight (*Tfg*) and 200 mg/kg body weight (*Cz*), with the help of cannula, daily for two week.

Glibenclamide (Gli) is taken as positive control and 0.6 mg/kg body weight was given.

pyrimidines, respectively, and create strand breaks at the abasic sites are used. In present study, diabetic group showed extensive DNA damage and the damage index was increased by 777% in the liver, 1182% in the brain and 795% in the lymphocytes in lysis treated slides in rats, respectively, when compared with control while 207%, 199% and 138% damage were found in the liver, the brain and the lymphocytes of diabetic group, respectively, when compared with buffer treated slides of the same group. The treatment of slides with Fpg and Endo III caused significantly high increase in the damage index as compared to the lysis treated slides in the liver, the brain and the lymphocytes of experimental diabetic rats (Table 4). The increase caused by Fpg and Endo III was 30% and 28% in the liver, 19% and 29% in the brain, 34% and 73% in the lymphocytes of diabetic group. When comparison was made with the Fpg and Endo III treated control group, increased damage index was found in the liver, brain and lymphocytes and it increase was 184%, 713% and 434%, respectively, by Fpg treatment and 425%,

Table 2 — Effect of oral administration of *Trigonella foenum graecum* seeds and *Cinnamomum zeylanicum* bark powder for two weeks on NADPH, $NADP^+$ and their ratio NADPH/$NADP^+$ in the liver and the brain of control and diabetic rats

Groups	NADPH	$NADP^+$	NADPH/$NADP^+$
Liver			
Control	2.80 ± 0.05	1.60 ± 0.03	1.75
Diabetic	2.15 ± 0.04***	5.43 ± 0.17***	0.39
Diabetic + *Tfg* (1740 mg/kg)	2.52 ± 0.02**	2.97 ± 0.06**	0.84
Diabetic + *Cz* (200 mg/kg)	2.37 ± 0.02**	3.92 ± 0.11**	0.61
Diabetic + Gli (0.6 mg/kg)	2.64 ± 0.02***	2.18 ± 0.08***	1.21
Brain			
Control	0.81 ± 0.02	0.46 ± 0.01	1.76
Diabetic	0.45 ± 0.01**	1.33 ± 0.02***	0.33
Diabetic + *Tfg* (1740 mg/kg)	0.62 ± 0.01***	0.72 ± 0.01**	0.86
Diabetic + *Cz* (200 mg/kg)	0.55 ± 0.02**	0.84 ± 0.01**	0.65
Diabetic + Gli (0.6 mg/kg)	0.70 ± 0.01***	0.62 ± 0.02***	1.13

NADPH and $NADP^+$ levels are expressed as µg NADPH or $NADP^+$ g $tissue^{-1}$.
Results are mean ± S.E. of six set of observation. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$ and $^{\#}$ $P > 0.05$ when compared with control.
Diabetic rats were given aqueous suspension of powdered *Trigonella foenum graecum* seed and *Cinnamomum zeylanicum* bark orally at a dose of 1740 mg/kg body weight (*Tfg*) and 200 mg/kg body weight (*Cz*), with the help of cannula, daily for two week.
Glibenclamide (Gli) is taken as positive control and 0.6 mg/kg body weight was given.

Table 3 — Effect of oral administration of *Trigonella foenum graecum* seeds and *Cinnamomum zeylanicum* bark powder for two weeks on NADH, NAD^+ and their ratio NADH/NAD^+ in the liver and the brain of control and diabetic rats

Groups	NADH	NAD^+	NADH/NAD^+
Liver			
Control	6.35 ± 0.13	2.17 ± 0.05	2.92
Diabetic	2.28 ± 0.05***	12.03 ± 0.32***	0.19
Diabetic + *Tfg* (1740 mg/kg)	4.15 ± 0.20***	5.85 ± 0.13**	0.59
Diabetic + *Cz* (200 mg/kg)	3.45 ± 0.10**	8.35 ± 0.22*	0.49
Diabetic + Gli (0.6 mg/kg)	5.42 ± 0.19***	4.03 ± 0.09***	1.34
Brain			
Control	2.05 ± 0.06	1.51 ± 0.03	1.35
Diabetic	0.86 ± 0.02***	2.83 ± 0.02**	0.30
Diabetic + *Tfg* (1740 mg/kg)	1.48 ± 0.03**	1.95 ± 0.03**	0.75
Diabetic + *Cz* (200 mg/kg)	1.24 ± 0.02**	2.28 ± 0.06**	0.54
Diabetic + Gli (0.6 mg/kg)	1.88 ± 0.02***	1.86 ± 0.02***	1.01

NADH and NAD^+ levels are expressed as µg NADPH or $NADP^+$ g $tissue^{-1}$.
Results are mean ± S.E. of six set of observation. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$ and $^{\#}$ $P > 0.05$ when compared with control.
Diabetic rats were given aqueous suspension of powdered *Trigonella foenum graecum* seed and *Cinnamomum zeylanicum* bark orally at a dose of 1740 mg/kg body weight (*Tfg*) and 200 mg/kg body weight (*Cz*), with the help of cannula, daily for two week.
Glibenclamide (Gli) is taken as positive control and 0.6 mg/kg body weight was given.

Table 4 — Effect of oral administration of *Trigonella foenum graecum* seeds and *Cinnamomum zeylanicum* bark powder for two weeks on oxidative DNA damage in the liver, the brain and lymphocytes of control and diabetic rats

	Groups	Buffer	Lysis	Endo III	FPG
Liver					
	Control	0.22 ± 0.01	0.22 ± 0.02	0.47 ± 0.06	0.88 ± 0.02
	Diabetic	0.63 ± 0.02**	1.93 ± 0.01***	2.47 ± 0.12***	2.50 ± 0.21***
	Diabetic + *Tfg* (1740 mg/kg)	0.38 ± 0.01**	0.95 ± 0.02**	1.02 ± 0.03***	1.40 ± 0.01**
	Diabetic + *Cz* (200 mg/kg)	0.53 ± 0.02*	1.30 ± 0.03**	1.91 ± 0.06**	1.80 ± 0.01**
	Diabetic + Gli (0.6 mg/kg)	0.32 ± 0.01**	0.77 ± 0.02***	1.57 ± 0.01**	1.10 ± 0.01***
Brain					
	Control	0.13 ± 0.01	0.17 ± 0.01	0.43 ± 0.01	0.32 ± 0.01
	Diabetic	0.73 ± 0.01**	2.18 ± 0.06***	2.82 ± 0.02***	2.60 ± 0.05***
	Diabetic + *Tfg* (1740 mg/kg)	0.51 ± 0.01**	1.18 ± 0.03**	1.73 ± 0.02***	1.78 ± 0.03**
	Diabetic + *Cz* (200 mg/kg)	0.65 ± 0.02*	1.36 ± 0.03**	1.87 ± 0.03**	2.18 ± 0.04**
	Diabetic + Gli (0.6 mg/kg)	0.42 ± 0.01**	0.92 ± 0.03***	1.50 ± 0.05***	1.14 ± 0.31***
Lymphocytes					
	Control	0.13 ± 0.02	0.21 ± 0.01	0.59 ± 0.02	0.47 ± 0.01
	Diabetic	0.79 ± 0.02**	1.88 ± 0.02***	3.26 ± 0.07***	2.51 ± 0.03***
	Diabetic + *Tfg* (1740 mg/kg)	0.37 ± 0.02**	1.11 ± 0.04***	1.40 ± 0.02***	1.24 ± 0.02**
	Diabetic + *Cz* (200 mg/kg)	0.62 ± 0.03*	1.35 ± 0.03**	1.68 ± 0.03**	1.47 ± 0.02**
	Diabetic + Gli (0.6 mg/kg)	0.30 ± 0.01***	0.88 ± 0.05***	1.27 ± 0.02***	0.82 ± 0.05***

Values of DNA damage are expressed as damage index calculated as #0 + #1 + #2 + #3 + #4/ # of cell scored where # is the total number of nuclei counted.

Results are mean ± S.E. of six set of observation. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$ and $^{\#}$ $P > 0.05$ when compared with control.

Diabetic rats were given aqueous suspension of powdered *Trigonella foenum graecum* seed and *Cinnamomum zeylanicum* bark orally at a dose of 1740 mg/kg body weight (*Tfg*) and 200 mg/kg body weight (*Cz*), with the help of cannula, daily for two week.

Glibenclamide (Gli) is taken as positive control and 0.6 mg/kg body weight was given.

555% and 453%, respectively, by treatment of Endo III in diabetic group. The treatment of *Tfg*, *Cz* and glibenclamide for two weeks showed reduced oxidative DNA damage in experimental diabetic group when compared with untreated group. The decrease in the damage index observed was 51%, 33% and 60% in the liver, 46%, 38% and 58% in the brain and 41%, 28% and 53% in the lymphocytes of rats receiving treatment of *Tfg*, *Cz* and glibenclamide, respectively. Treatment of *Tfg*, *Cz* and glibenclamide also caused significantly marked decrease in the DNA damage index in Endo III and Fpg treated slides of the liver, brain and lymphocytes of diabetic rats when compared with respective control. The decrease in DNA damage index observed in Endo treated slides was 59%, 23% and 36% in the liver, 39%, 34% and 47% in the brain and 57%, 48% and 61% in the lymphocytes of diabetic rats on *Tfg*, *Cz* and glibenclamide treatment, respectively, when compared with untreated diabetic rats (Table 4). Similarly in Fpg treated slides showed 44%, 28% and 56% decrease in the liver, 32%, 16% and 56% decrease in the brain, 51%, 41% and 67% decrease DNA damage index observed in the lymphocytes of diabetic group when treated with *Tfg*, *Cz* and glibenclamide for two weeks, respectively, when compared with untreated diabetic group (Table 4).

Discussion

Oxidative stress refers to the imbalance between generation and removal of free radicals or the reactive oxygen species (ROS) in the biological system. The oxidative stress consequently results in damage to all the biomolecules including proteins, lipids and DNA of the cell. Oxidative stress is the known causative factor in several diseases in humans including atherosclerosis, heart failure, myocardial infarction, schizophrenia, bipolar disorder, fragile X syndrome, age related degenerative disorders like parkinson's disease and alzheimer's disease[21,22]. Various drugs, xenobiotics and environmental pollutants are known to cause oxidative stress. Reactive species such as free

radicals, one-electron oxidants, various chemicals, *etc*., can react with different components of DNA to produce a plethora of DNA lesions[23]. These reactive species can modify bases[20,23], induce inter and intra-strand crosslinks[24] promote DNA–protein crosslinks[23] and create strand breaks[20]. Several reactive species that contain oxygen such as superoxide radical anion ($O2^{\cdot}-$), hydroxyl radical ($OH^{\cdot}$), peroxynitrite ($ONOO-$), hypochlorous acid (HOCl), *etc*. are generated inside the living cells during normal metabolic activities[23]. For example, leakage of electrons to molecular oxygen (O_2) from mitochondrial electron transport chains consisting of flavoproteins, iron sulphur proteins, ubiquinone and cytochromes produces $O_2^{\cdot}-$ and $OH^{\cdot}$ radicals are very reactive and can damage structures of all components of the DNA[25]. Failure to repair damaged lesions of DNA is associated with serious consequences including genome instability, mitochondrial dysfunction, neurodegeneration, aging or cancer[26]. Identification of mechanism of damage is important and necessary for planning of effective preventive as well as therapeutic measures.

Considerable number of evidences exist showing hyperglycemia induced increase in generation of ROS, leading to increased oxidative stress in various tissues which plays an important role in development of diabetic complications[27]. Excessive levels of glucose can disrupt the electron transport chain in the mitochondria, leading to generation of excess of superoxide anions[28,29]. High glucose can also stimulate oxidative stress *via* its auto-oxidation and non-enzymatic glycation. Reactive oxygen species (ROS) is generated in the process of advanced glycation endproducts (AGEs) formation[28] and interaction between AGEs and their receptors, RAGE, can also lead to ROS formation[30]. Moreover, glycation can inactivate antioxidant enzymes, impairing antioxidant defense, as observed with glycation of superoxide dismutase[40]. Diabetes caused decrease in activity as well as expression of antioxidants enzymes namely, glutathione peroxidise (GPx), superoxide dismutase (SOD) and catalase (CAT) in the tissues of rats[5,11,31]. Hyperglycemia can also induce oxidative stress by the polyol pathway. There are mainly three potential mechanisms by which the polyol pathway contributes to oxidative stress. First, under hyperglycemic condition, approximately 30% of the glucose is channeled into AR-dependent polyol pathway, which depetes NADPH and consequently reduces GSH level. Second, oxidative stress is produced during the conversion of sorbitol into fructose by SDH. In this step, the co-factor NAD^+ is converted to NADH by SDH. NADH is a substrate of NADH oxidase leading to production of superoxide anions. Third, the polyol pathway converts glucose to fructose, and fructose can be further metabolized into fructose-3-phosphate and 3-deoxyglucosone, which is more potent non-enzymatic glycation agent than glucose. Thus, the flux of glucose through the polyol pathway would increase AGEs formation, ultimately leading to ROS generation[32].

Glutathione (GSH), an important nonenzymatic antioxidant, is a vital substance providing antioxidant defence and is involved in various other important physiological functions like detoxification of xenobiotics and also act as substrate and co substrate in many enzymatic reactions. GSH plays important role in protecting cells against free radical *i.e*. reactive oxygen species (ROS) induced injuries. The cellular levels of GSH are very delicately balanced at the expense of NADPH. It is reversibly converted to GSSG after donating its reducing equivalents in redox reactions. Thus the shift of GSH ⇌ GSSG equilibrium towards right indicates the disturbance of the redox balance in the cells which should be maintained to keep the normal cellular physiological state by keeping the enzymes and proteins in the active state. The present study showed an elevated level of superoxide anion and simultaneously decreased TAC which is observed in oxidative stress and this observation supports the previous studies. Results of the present study also showed significantly decreased ratios of GSH/GSSG along with $NADPH/NADP^+$ and $NADH/NAD^+$ in the tissues of diabetes rats, indicating the induction of oxidative stress because the ratios of GSH/GSSG, $NAD(P)H/NAD(P)^+$ and $NADH/NAD^+$ are the indicators of redox status in the tissues. Decrease in the GSH level and impairment in GSH metabolism have been previously reported in the diabetics[33,34]. Decrease level of GSH found due to the low level of NADPH and consequent increased oxidative stress. Relatively high GSH/GSSG ratios are maintained by the enzyme glutathione reductase using NADPH[34]. In addition to controlling the activity of redox sensitive enzymes, the $NAD(P)H/NAD(P)^+$ ratio can regulate the activity of various transcription factors, leading to changes in gene expression.

Results of the present study showed that diabetes caused extensive DNA damage in rat tissues and lymphocytes. DNA oxidation is known to be the most common type of DNA damage to human and other species in condition of oxidative stress[35]. The elevated ROS in diabetes can cause strand breaks and base modifications including oxidation of guanine residues to 8-OHdG, an oxidized nucleoside of DNA, which is the most frequently detected and studied DNA lesion[36]. An increased extent of DNA damage in T2DM patients compared to controls has also been reported in several studies[37]. On the other hand, several studies showed the lack of association between diabetes and increased DNA damage[37] while some reports are found on a significant positive correlation between 8-OHdG, a biomarker of oxidative DNA damage[38], and fasting blood glucose and HbA1c[37-40]. Hyperglycemia causes glucose auto-oxidation, glycation of proteins, activation of polyol metabolism and subsequent formation of ROS. The present study also revealed increase in damage to purines and pyrimidines bases in the tissues of diabetic rats. It was observed that damage to purines was higher than pyrimidines as Fpg treatment caused more increase in DNA damage index in the brain and the lymphocytes while in the liver, Endo III treatment caused higher damage of pyrimidimes than to purines in diabetic group. There are many reports describing elevated levels of oxidatively modified DNA lesions, in various biological matrices, in a plethora of diseases; however, for the majority of these the association could merely be coincidental, and more detailed studies are required. DNA damage due to generation of oxidative stress caused diseases including cancer. ROS have influence on almost all kinds of cellular activities. Organisms have evolved to deal with it, and put the ROS generation into intra- and inter-cellular regulatory system that needs to coordinate the balanced cell growth that needs to involve autophagy and cell death[41].

Treatment with *Tfg* and *Cz* caused significant reduction in the level of superoxide anion with simultaneous increase in the TAC as well as ratios of GSH/GSSG, NAD(P)H/NAD(P)$^+$ and NADH/NAD$^+$ in the diabetic group. The results of our study showed that the indices of oxidative stress were significantly restored to normal levels on *Tfg* and *Cz* treatment, while *Tfg* was more effective than *Cz*. Results of both treatments were also compared with standard drug, glibenclamide. The major constituents of *Tfg* are saponin glycoside, carbohydrates, and proteins. It is well-established that saponins are hypocholesterolemic and antioxidant studied in animal species[42]. Therefore, it is possible that the presence of saponins in *Tfg* is responsible for the protection against oxidative stress generated complications. Similarly the two main components of bark of *Cz* namely cinnamaldehyde and eugenol show antioxidant potential. Previous studies have also demonstrated that cinnamaldehyde decreased the levels of lipid peroxidation products and increased the activities of antioxidant enzymes[43] and eugenol (4-allyl-2 methoxyphenol) is natural antioxidant, which has phenolic structure, play an important role in protecting the tissues against free radical induced damage. The antioxidant activity of eugenol was evaluated by the extent of protection offered against free radical-mediated lipid peroxidation using both *in vitro* and *in vivo* studies[44]. Hence *Tfg* seed and bark of *Cz* seem to have therapeutic potential against diabetes induced oxidative stress and DNA damage which may be related to their free radical scavenging properties. *Tfg* and *Cz* are reported to have antidiabetic and antioxidative properties which have been confirmed in the present study[45,46]. There are many other plants available with antioxidative potential and are being used as remedies in diabetes, by reducing oxidative stress and consequently their complications such as redox imbalanced and DNA damage[47-49].

Conclusion

The present study concluded that *Tfg* and *Cz* have antihyperglycemic and antioxidative potential against diabetes induced redox imbalance. The protective effects of *Tfg* and *Cz* on DNA damage have also been observed in alloxan induced diabetic rats. This establishes the efficacy of both the plants which may be used as therapeutic agents in the management of diabetes. Further studies are required to find out the exact mechanism of hypoglycemic action of the *Tfg* seed and *Cz* bark powder.

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Conflict of Interest

All authors declare no conflict of interest.

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