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Original Article

Possible ameliorative role of green tea on chronic alcohol mediated renal toxicity of STZ -induced diabetic rats

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SUMMARY

This research was planned to assess the protective effect of aqueous green tea extract (AGTE) in alcohol treated diabetic rats. This study was examined, whether 20% v/v of 5 g/kg.b.w alcohol intake exacerbates diabetes-induced renal damage associated with an escalated nitroxidative stress and glucose homeostasis in comparison with diabetic condition alone. The present study is primarily focused to investigate the possible toxic effects of chronic alcohol intake on kidney of streptozotocin (STZ)-induced diabetic rats. Forty albino male Wistar rats were used and randomly allocated into five groups. Diabetes was induced by a single intraperitoneal injection of STZ (50 mg/kg) in rats. Alcohol was administered through orogastric tube once daily for a period of 60 days. Impact of chronic alcohol treatment on renal function and selected metabolites of catecholamines and antioxidant metabolisms have been investigated in the kidney of STZ-induced diabetic rats. Biochemical analysis of kidneys demonstrated that chronic alcohol induced alterations in *vitro* gluconeogenesis, significant decrease in the body weight, and activities of antioxidant enzymes in diabetic rats. A significant increase in the levels of epinephrine and sertonine was observed between alcohol treated, diabetic control and alcohol treated diabetic rat groups. Further,

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excessive chronic alcohol treatment caused additive deterioration of the kidney function as evidenced by increased inducible nitric oxide synthase (iNOS) protein expression and thus nitroxidative stress and histopathological aberrations leading to aggravation of diabetic nephropathy under alcohol intoxication. Treatment with aqueous green tea extract (300 mg/kg.b.w) to diabetic rats treated with alcohol renders the protection against the decreased body weight, increased hyperglycemia, hyperlipidemia, catecholamines, nitroxidative stress and further tissue damage.

1. Introduction

Alcohol abuse and diabetes are the two major independent challenges which affect various organs with no exception in health systems worldwide. 12% of the global population have diabetes mellitus (DM), a metabolic disorder of multiple etiology [1]. And ~ 2.5 billion people extensively being used as a component of the diet and relaxant. Low to moderate consumption of alcohol is beneficial to the mankind against diabetic risk [2] and also cardio vascular diseases [3], remains controversial due to inconsistent results across studies. On the contrary, an increased risk for DM was apparent when alcohol is consumed in excess. Diabetes and alcoholism induced hyperglycaemia plays a major role in long-term complications associated with DM which include weight loss [4], retinopathy and nephropathy that may lead to renal failure. Chronic excessive consumption of alcohol in well-nourished diabetics can result in hyperglycaemia [5]. However, the interplay between alcohol consumption and diabetes remain more controversial and complex. Alcohol misuse among those diagnosed with diabetes is often not addressed at all, or addressed only in clinical settings. Hence, this collective burden may thus be potentially harmful to human health [6,7]. Effects of alcohol on nitric oxide (NO) production and iNOS activity is important in the ethanol-induced alterations of cell or organ function [8]. But no studies have explored the consequence of chronic alcohol on NO levels through induction of iNOS in kidney of diabetic rats. Hence, our studies indicate that iNOS protein expression is correlated with increased free radical production in the kidney of diabetic-alcoholic rats.

High levels of NO may react with superoxide anion (O_2^-) to generate peroxynitrite radical ($ONOO^-$), which binds to proteins and thus affects their function [9]. Hyperglycaemia and ethanol exposure reduce antioxidant status and increase in the production of free radicals with subsequent activation of redox sensitive genes [10,11]. Although liver is the primary site for alcohol metabolism, kidney is also more susceptible to damage due to chronic alcohol abuse as well as diabetes. Although the effects of diabetes and alcohol abuse on nitro-oxidative stress, hyperglycaemia and renal toxicity have been well documented, their combined effects have not been studied so far in detail. Hence, the present study focuses on the effect of chronic, excessive alcohol consumption on STZ-diabetic rats in terms of nitroxidaative stress. Though the role of chronic use of alcohol under diabetes is more important for clinical research and practice [12], therapeutic strategies are warranted for which the precise mechanisms or targets related to the combined effects of alcohol and diabetes are to be identified, targeted and addressed to lessen the additive disease burden and also to reduce the mortality rate worldwide.

Green tea, a beverage commonly consumed in Asian countries, is a significant source of flavonoids called catechins. The green tea catechins include (−)-epigallocatechin gallate (EGCG) (−)-epigallocatechin (−)-epicatechin gallate, and (−)-epicatechin. EGCG is the most abundant of these catechins, and many healthful benefits, including anticarcinogenic, antioxidant, antiangiogenic, antidiabetic and antiviral activities, have been attributed to EGCG. The consumption of green tea is conscious health benefitable habit, and is the key marker to reduce many of the dangerous impacts with a focus on oxidative and nitrosative stress induced by diabetes [13] as well as by alcohol abuse [14]. EGCG

reduced hyperglycaemic condition disturbs all the metabolic pathways directly or indirectly, and also has proven that it ameliorates glucose intolerance [15]. Though green tea is reported to exert effect on ethanol metabolism, its role in amelioration of toxicity and related events and precise mechanisms under diabetic condition is not known. Limited information is available on the effect of native extract and/or the combination of constituents of green tea on joint effect of alcohol and diabetes induced damage, moreover, the studies available focused on liver without mentioning the damage of other tissues [14]. Hence, the current study is primarily aimed to investigate the possible protective effects of green tea on chronic alcohol induced exacerbation of renal tissue damage of STZ-diabetic rats.

2. Materials and methods

2.1. Chemicals

Streptozotocin (STZ), epinephrine, serotonin, dopamine standards, NADP, TBA, TCA were from Sisco Research Laboratories, Mumbai, India. Monoclonal iNOS primary antibody and Enhanced chemiluminescence (ECL) detection reagent kit were procured from Sigma–Aldrich Company (St. Louis, MO, USA) and Biorad respectively. Protease Inhibitor Cocktail (100X) was procured from (Bethyl Laboratories, Montgomery, TX, USA) Anti-iNOS rabbit antibody was procured from Santa Cruz biotechnology. Ethanol and all other chemicals were of analytical grade and obtained from Hi–Media Laboratories Ltd., Mumbai.

2.2. Experimental animals and maintenance

Adult male albino *Wistar* rats, weighing 140–160 g were procured from an authorized vendor (Sri Venkateswara Agencies, Bangalore, India) and used for all experiments. Animals were housed in clean polypropylene cages having 8 rats per group, maintained on a standard pellet diet (M/S. Hindustan Lever Ltd., Mumbai, India) and water *ad libitum* with 24 h light–dark cycle throughout the experimental period. Guidelines have been followed and experimentation was carried out under controlled conditions, in accordance with the guidelines (Reg. No. 1889/GO/Re/S/16/CPCSEA) and protocol approved by Institutional Ethical Committee (F. No. 25/30/2015 CPCSEA) for the care and use of laboratory animals and the consent was obtained for experimentation with animal subjects.

2.3. Experimental design

A total of 32 rats were divided into four groups, eight rats in each group ($n = 8$), treated as follows. Group I: Normal control rats (NC) received 100 μL citrate buffer. Group II: Alcohol treated rats (AT) received ethanol (20% v/v of 5 g/kg b. w/day) for 60 days. Group III: STZ induced diabetic control rats (DC) or diabetic rats. Group IV: Diabetic rats treated with alcohol (D + A) for 60 days or Alcohol treated diabetic rats. Group V: Alcohol treated diabetic rats supplemented with aqueous green tea extract (D + A + E) for 60 days.

2.4. Induction of diabetes

Diabetes (type 1) was induced to overnight fasted adult albino male *Wistar* rats by single intraperitoneal (i. p.) administration of a freshly prepared solution of STZ (50 mg/kg b. w) dissolved in 0.1 M cold citrate buffer (pH 4.5). The STZ-induced diabetic animals were allowed to drink 5% glucose solution overnight to overcome drug-induced hypoglycaemia. Rats having fasting blood glucose (FBG) above 250 mg/dL on the third day following STZ injection were confirmed as diabetic and used for further experimentation.

2.5. Preparation of aqueous extract

Green tea (*Camellia sinensis)* leaves were collected from Kanan Devan Hills Plantations Company (P) Limited, Munnar, Kerala, India. The aqueous extract of green tea leaves was prepared by soaking 100 g

of dried leaves in 1 L distilled water in a glass jar for 72 h at room temperature. And then, the collected aqueous residue by filtration was concentrated under reduced pressure in the Buchi Rotavapor R-20, freeze dried. The obtained extract was stored at 4 °C in airtight containers until needed for further studies.

2.6. Qualitative analysis for phytochemicals

Small quantities of green tea extract were taken for confirmation of the presence of following phyto constituents [16,17,20].

Drangendroff's test: To the GTE a few drops of Drangendroff's reagent were added along the walls of test tube. Formation of an orange red precipitate indicated the presence of alkaloids.

Shinoda test: To the extract, HCl and magnesium metal were added and boiled for 10 min. Formation of red colour indicated the presence of flavonoids.

Lead acetate test: GTE was diluted with distilled water and few drops of 1% aqueous solution of lead acetate were added. Formation of yellow precipitate indicates the presence of phenols.

Ferric chloride test: To the extract, a few drops of 5% aqueous ferric chloride solution were added. A bluish black which disappeared on addition of few ml of dilute sulphuric acid forming yellowish brown precipitate indicates the presence of tannins.

Keller-Killiani test: GTE was treated with chloroform and evaporated to dryness. Then glacial acetic acid containing trace amount of ferric chloride was added. The contents were transferred to a small test tube and concentrated sulphuric acid was added carefully, the formation of blue colour indicates the presence of glycosides.

Leiberman-Burchards's test: The extract was treated with a few drops of acetic anhydride, boiled and cooled. Then concentrated sulphuric acid was added from the side of the test tube. Formation of brown ring at the junction of two layers and formation of deep green colour at the upper layer indicates the presence of terpenoids.

2.7. Quantitative analysis for phytochemicals

Concentrations of specific phytoconstituents of aqueous GTE, in particular, total phenolics, flavonoids, and saponins were determined by following the methods adopted.

2.7.1. Estimation of total phenolics

The total content of phenolics in GTE was determined by following the method of Singleton [18]. Methanolic solution of the extract in the concentration of 1 mg/mL was used for the analysis. The reaction mixture was prepared by mixing methanolic solution of extract with Folin-Ciocalteu's reagent dissolved in water and $NaHCO_3$. The samples were incubated in a thermostat and the absorbance was read spectrophotometrically at 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid. The concentration of total phenolics was read and expressed in terms of gallic acid equivalent (mg of GAE/g of extract).

2.7.2. Determination of total flavonoids

Total flavonoid content of the sample was determined by following aluminum chloride colorimetric method [19]. For total flavonoid determination, quercetin was used to make the standard calibration curve. An amount of diluted standard quercetin solutions or extracts was separately mixed with 2% aluminum chloride. After mixing, the solution was incubated for 60 min at room temperature. The absorbance of the reaction mixture was measured at 420 nm using UV–Vis spectrophotometer. The concentration of total flavonoids was calculated and expressed as mg quercetin equivalent (QE)/g of green tea powder.

2.7.3. Evaluation of total saponins

Total steroidal saponins were determined by using the method [19]. GTE was dissolved in 50% aqueous methanol, from which an aliquot was subjected to boiling water bath treatment in order to

remove the excess methanol. After cooling, ethyl acetate followed by anisaldehyde–ethyl acetate reagent (0.5:95.5, v/v) and sulphuric acid–ethyl acetate reagent (50:50, v/v) were added and incubated in a water bath. After cooling, the absorbance was measured spectrophotometrically at 430 nm. The total steroidal saponin concentrations were expressed as sarasasapogenin equivalents (SE).

2.8. Alcohol administration and green tea extract treatment

Alcohol treated animals received 20% (v/v) ethanol of 5 g/kg b.wt/day for 60 days using orogastric tube. Alcohol treatment was started to the STZ-diabetic rats from 3rd day, which was considered as day one for the alcohol treated diabetic rat group. GTE was administered at a dose of 100–300 mg/kg b.w/day for 60 days. All animals were fasted overnight after experimental period and then sacrificed by cervical dislocation. Renal tissues were collected and used immediately for further studies. Clinical monitoring of the animals was performed for body weight, fasting blood glucose levels and histopathological changes.

2.9. Acute and chronic toxicity evaluation of the alcohol

The acute toxicity test of the ethanol was carried out by standard method with slight modifications [21]. Fifteen albino rats were fasted for 12 h but allowed access to water. They were divided into five groups of three rats each ($n = 3$) and orally administered ethanol at varied doses of 1, 2, 3, 4, 5 g/kg b. w (20% v/v) respectively. The animals were monitored for 24 h to check for convulsion, salivation, diarrhoea, lethargy, sleep, coma, nervousness and/or mortality.

Similarly Group II, Group IV (See experimental design 2.3) which were received 20% v/v of 5 g/kg b. w of ethanol for 60 consecutive days are considered under chronic study. The animals also monitored daily to check for convulsion, salivation, diarrhoea, lethargy, sleep, coma, nervousness and/or mortality.

2.10. Collection of blood and tissue isolation

At the end of experimental period (60 days), animals were fasted overnight, weighed and sacrificed using anaesthetic ether followed by cervical dislocation. Blood samples from all the experimental rats were withdrawn by cardiac puncture and blood glucose levels were determined. Serum was separated by centrifugation at 3000 rpm for 15 min after overnight storage at 4 °C, and was stored at −20 °C. Kidneys were immediately harvested, washed with ice-cold saline, weighed to the nearest milligram. The renal tissues were rinsed in HEPES buffer (pH 7.4) and stored in liquid nitrogen at −80 °C until further assays were carried out. A part of the tissue was stored in buffered formalin for histological examination.

2.11. Blood glucose, body weight and kidney weight measurements

From the first week, until the end of experimental period, fasting blood glucose (FBG) and body weights were assessed twice a week. For FBG measurements, all animals were fasted for 6 h and were analysed using AccuCheck glucometer (LifeScan, Wayne, PA, USA). All the experimental animals were weighed using a precision balance with an interval of one week and the kidney weight was measured at the end of experimentation period.Plasma and tissue analysis

2.12. Autoanalyser kit methods

Serum levels of urea, uric acid and blood urea nitrogen (BUN) were measured by monozyme enzymatic kit methods. Creatinine was determined according to the spectrophotometric method of Jaffe's which is based on the reaction of creatinine with picric acid in alkaline pH described by the method [22].

2.13. Biochemical analysis

Lipid peroxidation (LPO) was determined by estimating the level of thiobarbuturic acid reactive substances (TBARS) and measured as malondialdehyde (MDA) by following the method [23]. Protein carbonyls (PCO) content in the tissue samples was measured using DNPH method [24]. Nitric oxide levels in tissue samples were estimated by using Greiss reagent method [25], in terms of nitrates and nitrates. Peroxynitrite content was measured by using nitrophenol [26].

2.14. Measurement of anti-oxidant status

Antioxidant status of kidney and its mitochondria were measured. Total reduced glutathione (GSH) content was measured by using Ellman's reagent [27]. The activity of glutathione-S-transferase (GST, EC 2.5.1.18) was assayed by measuring the 1-chloro 2, 4 dinitrobenzene (CDNB)–GSH conjugates [28]. The activity of glutathione peroxidase (GPx, EC 1.11.1.9) was assayed by the 5, 5′- dithiobis-2-nitrobenzoic acid (DTNB) method [28]. Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by the method [29]. Catalase (CAT, EC 1.11.1.6) was assayed by following the method [30].

2.15. Extraction of lipids from renal tissue

Total lipids were extracted from the renal tissue by using chloroform-methanol method [31]. A known volume of sample was mixed with chloroform-methanol mixture (2:1 v/v) and vigorously shaken and then filtered. The filtrate was mixed with physiological saline and the mixture was kept overnight undisturbed. The lower phase containing the lipid was drained off into pre-weighed beakers. The upper phase was re-extracted with more of chloroform-methanol mixture and the extracts were pooled and evaporated to dryness. The lipids were re-dissolved in chloroform-methanol mixture and the aliquots were dried at room temperature to evaporate the solvent and used for the estimation of total cholesterol, total phospholipids, and triglycerides. Total cholesterol content in the kidney tissue sample was estimated by the method [32]. The total phospholipids in kidney tissue suspension were estimated by using amino napthosulphonic acid [33]. The levels of triglycerides in the tissue lipid extract were estimated by the method [34].

2.16. Constituents of aminergic system using HPLC

2.16.1. Apparatus

HPLC system (Shimadzu, Japan) is equipped with a binary gradient system with variable UV/VIS detector (SPD-20 A) and Rheodyne injector with a 20 μL loop and LC-20AD pumps and integrator. Reversed phase chromatographic analysis was performed in isocratic condition using C_{18} reverse phase column at 37°Cand a diode-array detector (DAD). A personal computer system running Chem Station Software (Agilent Technologies, Santa Clara, CA, USA) was used for data collection and processing.

2.16.2. Chromatographic conditions

Resolution of peaks was performed with the mobile phase consisting a mixture of potassium dihydrogen phosphate and sodium n-heptane sulfonate dissolved in 820 mL of water and 180 mL of methanol (HPLC grade). The pH of the mobile phase was adjusted by dropwise addition of ortho phosphoric acid (pH 3.2). The mobile phase solutions were pumped at a flow rate of 1.0 mL min^{-1}. Signals were accumulated in the wavelength range 190–450 nm using a DAD, and the chromatograms were recorded at 254 nm. The injection volume was 20 μL. The temperature of the column was kept at 40 °C.

2.16.3. Standard treatment

The stock standard solutions of each biogenic amine were diluted with ultra-pure water to the desired concentrations. Concentrations of 1 mg/mL were used as stock solution. The qualitative analysis was realized using the retention times. The quantitative analysis was performed using the

corresponding external calibration. The calibration curves were obtained by plotting the biogenic amines peak area values against the respective concentrations of biogenic amines standards.

2.16.4. Sample preparation

To the plasma sample, 0.4 M perchloric acid was added, then vortexed for 1 min, and centrifuged at 13,000 rpm for 10 min. The supernatant was aspirated into another test tube followed by the addition of 2.0 M NaOH, vortexed for 1 min and then saturated solution of sodium bicarbonate was added into the test tube to adjust the pH to 8.0–8.5. To a prepared sample 1 mL of 10 g L^{-1}dansyl chloride was added and the mixture was vortexed for 1 min, and left to react in a closed water bath at 40 °C for 45 min. Finally, 25% ammonium hydroxide was added to remove the excess derivatization reagent, and incubated for 30 min, at room temperature, in the dark. Before injection, all samples were filtrated through 0.40 μm Econofilters [35].

2.17. *Gluconeogenesis* in vitro

Cortical slices of kidney (0.3 mm) weighing 50 mg were incubated in of Krebs–Ringer bicarbonate (KRB) buffer (0.12 M NaCl, 0.005 M KCl, 0.001 M $CaCl_2$, 0.001 M $MgSO_4$.7 H_2O, 0.025 M $NaHCO_3$) in Erlenmeyer flask. These flasks were gassed with carbogen (O_2: CO_2 in 95: 5) for 2 min to replace the air inside. The contents were incubated for 1 h in a metabolic shaking (40–60 oscillations per min) water-bath at 38 °C. At the end of incubation, the slices were removed and dried at 110 °C to a constant weight. The slices were incubated under identical conditions along with the controls but containing the substrates (10 mM. Succinate, pyruvate, glutamate, glycerol and alanine), unless otherwise stated. Incubation procedures and other details of glucose estimations were essentially the same as described by Krebs method [36]. A measured amount of medium was deproteinized with 3 N $HClO_4$ and centrifuged. The glucose in the supernatant was measured by the glucose oxidase method [37]. Results are expressed as μmoles of extra glucose produced/g tissue/h.

2.18. *Histopathological analysis and scoring*

Paraffin blocks were prepared having kidney tissue samples fixed in 10% neutral buffered formalin solution after routine tissue monitorization process. From each tissue sample, 4 μm thick sections were obtained, and these tissue sections were stained with haematoxylin and eosin. Renal tissue samples were examined for histopathological abnormalities such as glomerular sclerosis, glomerular focal necrosis, dilation of Bowman's capsule, degeneration, and necrosis of tubular epithelium and tubular interstitial inflammation. In this study, scoring system was assessed and graded according to the scale introduced by [38] (Grade 0 = 0%, 1 + = 1–25%, 2 + =26–50%, 3 + = 51–75% and 4 + = 76–100%).

2.19. *Western blotting*

Western blot analysis was used to determine iNOS protein expression in renal cortex of normal control and as well as other experimental rats. Protein extraction and Western blot analysis were performed as described previously [39]. Tissue samples were homogenized with a Polytron homogenizer in RIPA buffer containing a mixture of protease inhibitors. After incubation at 4 °C for 30 min, samples were centrifuged at 14,000 rpm for 15 min. Cellular protein was mixed with SDS sample buffer subjected to electrophoresis and transferred onto PVDF membranes. After blocking the PVDF membrane in non-fat dry milk (5%) in Tris buffered saline (TBS) for 1 h at room temperature, membranes were probed overnight with iNOS and β-tubulin (Sigma, St Louis, MO, USA) primary antibodies. Then, the membranes were incubated with secondary antibodies (anti-rabbit Ig G conjugated to HRP) for 1 h at room temperature. Before and after incubation with secondary antibodies, membranes were washed with TBS containing 0.1% Tween-20. Western blot band intensities were quantified using the Image J program.

2.20. Statistical analysis

All the quantitative data were expressed as Mean ± SEM. One way ANOVA was used to determine the significance of the parameters between the groups. Pearson correlation coefficient is used to measure of the strength of the association between the two variables. using Graph Pad Prism version 6.01 for Windows. $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Qualitative and quantitative analysis of AGTE

Results of preliminary qualitative analysis confirmed the presence of certain phytoconstituents, in particular flavonoids, phenols and tannins in the aqueous extract. Information related to the details of biochemical tests performed and the phytoconstituents are listed in Table 1. Concentration of individual class of phytocompounds in AGTE (Table 2) revealed that it chiefly contained flavonoids (66%), polyphenols (85%), tannins (12%) and caffeine (4%).

3.2. AGTE effect on alcohol induced changes in FBG body weight of diabetic rats

At the end of the experimental period i.e., 8 weeks, the body weights showed significant changes in the diabetic as well as alcohol treated diabetic rats treated compared to

The alcohol treated and normal control rats. And no change was observed in body weight of D + A + E rats in comparison with normal control rats. Alterations in the FBG levels were observed in all the experimental groups and were compared with the normal rats. In diabetic rats and alcohol treated rats, the FBG levels were significantly higher than the control rats. FBG levels in alcohol treated diabetic rats were further elevated significantly ($P < 0.05$) as compared to diabetic control rats. The mean values of fasting blood glucose are significantly decreased to the level of normal control rats in diabetic alcoholic rats supplemented with EGCG. The percentage changes in the body weights of alcohol treated rats were increased by 65.7%. However, in the diabetic group, the body weight was decreased by 20.4% significantly ($P < 0.05$) whereas in alcohol treated diabetic rats, the decrease in body weights were more pronounced i.e., 27.6% as compared to diabetic rats. Supplementation of AGTE to D + A rats caused significant decrease in the FBG moreover, thereby restored the weight of the body close to the NC rats (Table 3).

Behavioural changes such as alertness, motor activity and aggravation were observed in rats at various doses such as 1–5 g/kg b. w. whereas; breathlessness, restlessness, diarrhoea, tremor, convulsion and coma were not observed at the administered doses of alcohol in oral toxicity study. The rats were physically inactive up to 6 h and no death was recorded even at the dose of up to 5 g/kg b. w (Table 4). Therefore, the LD_{50} is greater than 5 g/kg body weight. Similarly, behavioural changes such as alertness, motor activity, breathlessness, restlessness, diarrhoea, tremor, convulsion and coma were monitored in Group II and Group IV rats treated with ethanol for 60 consecutive days, a chronic study. The rats were physically inactive up to 6 h after oral dosage, tremor or convulsion was observed in some

Table 1
Qualitative analysis for phytochemicals in aqueous green tea extract.

S. No	Test for phytoconstituents	GTE
1	Alkaloids (Dragendroff's test)	+
2	Flavonoids (Shinoda test)	+
3	Phenols (Lead acetate test)	+
4	Tannins (Ferric chloride test)	+
5	Glycosides (Keller–Killiani test)	+
6	Terpenoids (Leiberman-Burchards's test)	+

'+' indicates the presence of class of phytoconstituents.

Table 2
Concentration of individual class of components in AGTE.

S.No	Name of the component	mg/g of AGTE
1.	Polyphenols	960.2 ± 0.12
2.	Flavonoids	850.1 ± 0.21
3.	Tannins	12.1 ± 0.11
4.	Caffeine	4.32 ± 0.36

Values are Mean ± 3 independent experiments.

rats. However, one diabetic rat went into a state of diabetic coma after 56thday treatment and no death was recorded even at the dose of 5 g/kg b.w. Therefore, the LD_{50} is greater than 5 g/kg b.w (Table 5).

3.3. AGTE effect on alcohol induced changes in serum markers and renal lipid profile of diabetic rats

Increased levels of cholesterol, phospholipid and triglycerides from alcohol treated diabetic rat groups are the important observations of the study in comparison with normal controls. No significant increments were observed in the levels of total cholesterol, and triglycerides in alcohol treated and diabetic control rats ($P > 0.05$), while there was more pronounced significance in the levels of phospholipids in all the experimental rat groups as compared to normal control rat group. AGTE supplementation to the D + A rats reverted the levels of cholesterol, triglycerides and phospholipids at the level of NC rats (Fig. 1).

In addition, relative to the AT, DC groups, rats in the D + A group had greater serum levels of urea, uric acid and creatinine at all time points ($P < 0.05$). While this hike was normalized to the levels of NC rats with the supplementation of AGTE. Increased serum levels of urea, uric acid and creatinine were in the order as, D + A > DC > AT > D + A + E as compared to control group (Fig. 1).

3.4. AGTE effect on alcohol induced changes in renal nitroxidative stress and antioxidant status of diabetic rats

Elevated levels of malondialdehyde (MDA), protein carbonyls, nitric oxide, and peroxynitrites were significant ($P < 0.05$) in renal tissue of alcohol treated diabetic rats when compared to normal control and other experimental rat groups. Concentrations of MDA and nitric oxide showed a significant difference between all the experimental groups in comparison with normal control rats except with D + A + E rats, as supplementation of AGTE reverted the above increased levels to NC rats. No significance was observed in the levels of protein carbonyls and peroxynitrite levels between alcohol treated and diabetic control rat groups. The mean values of malondialdehyde, protein carbonyls, peroxynitrites, nitric oxide were observed to be decreased in D + A + E rats in comparison with normal control rats.

Table 3
AGTE effect on alcohol induced changes in FBG and body weight of diabetic rats.

Groups	FBG (mg/dl)		Body weight (g)		% Change in b. w (g)
	1st week	8th week	1st week	8th week	
NC	103.2 ± 1.85^{a}	128.3 ± 3.4^{b}	159.2 ± 1.0^{a}	241.2 ± 3.8^{b}	51.5%
AT	136.2 ± 2.48^{a}	167.1 ± 2.9^{b}	157.2 ± 5.8^{a}	260.6 ± 2.7^{b}	65.7%
DC	332.8 ± 3.11^{a}	370.2 ± 4.1^{b}	157.2 ± 1.1^{a}	125.1 ± 1.8^{b}	−20.4%
D + A	341.1 ± 3.75^{a}	405.2 ± 3.3^{b}	167.5 ± 2.2^{a}	121.2 ± 2.2^{b}	−27.6%
D + A + E	110.7 ± 2.64 ^{b}	147.6 ± 3.86 ^{b}	158.8 ± 1.49^{a}	256.8 ± 4.52^{a}	61.7%

Values presented are the Mean ± SEM (n = 8). Significance was determined at a $P < 0.05$. Categories sharing a different alphabet between 1st and 8th week are statistically different.
Note: FBG-Fasting blood glucose; NC–Normal control; AT-Alcohol treated; DC-Diabetic control; D + A- Alcohol treated diabetic rats; D + A + E– Alcohol treated diabetic rats supplemented with green tea extract.

Table 4
Oral toxicity of ethanol in *Wistar* rats.

Dose of ethanol (g/kg b.w)	Alertness, Motar activity	Mortality
1	0/3	0/3
2	0/3	0/3
3	1/3	0/3
4	2/3	0/3
5	3/3	0/3

Table 5
Chronic oral toxicity of ethanol in Wistar rats.

Group	Dose of ethanol (g/kg b.w)	Alertness/motar/ activity	Breathlessness/ Restlessness	Diarrhoea/tremor/ convulsion	Coma	Death/ mortality
II	5	6/8	1/8	5/8	0/8	0/8
IV	5	8/8	2/8	6/8	1/8	0/8
V	5	2/8	0/8	3/8	0/8	0/8

It is clear from this study that, there was a significant decrease in antioxidant status of kidney tissue from all the alcoholic, diabetic, diabetic-alcohol rat groups in comparison with that of normal control group. GSH content and activities of antioxidant enzymes such as GPx, GST, SOD, and CAT were decreased in AT (15%, 29.2%, 11.4%, 26.4% and 20%), DC (22.6%, 51.9%, 17.8%, 24.4% and 40%), and (48.6%, 70.2%, 46.4%, 48.2% and 68%) rat groups as compared to the NC rats. GSH and the activities of antioxidant enzymes (GPx, GST, SOD and CAT) are diminished in the group of D + A with the supplementation of AGTE and hence D + A + E rats had no significant difference with normal control rats (Fig. 2).

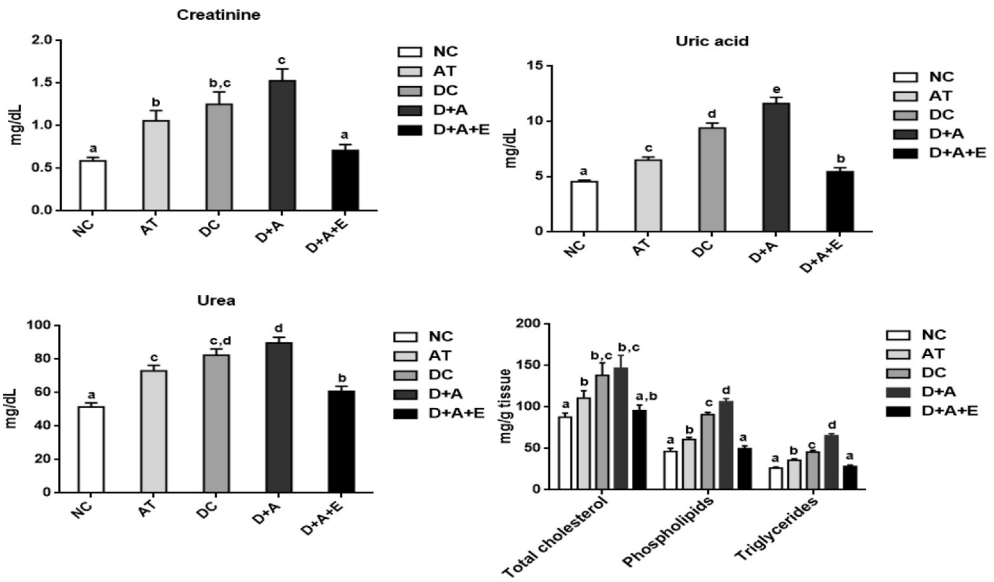

Fig. 1. Effect of green tea extract on alcohol exacerbated renal markers of serum and kidney lipid profile in diabetic rats Data are expressed as mean ± SEM. Superscript of different letters in each column differ significantly ($P < 0.05$) from each other.

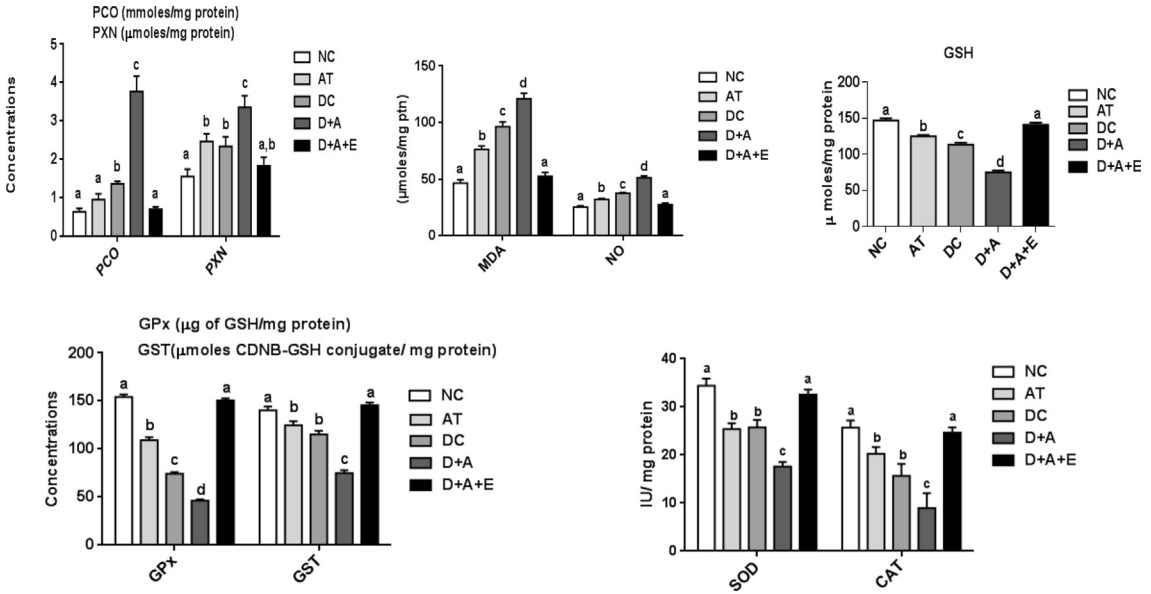

Fig. 2. Effect of green tea extract on alcohol induced alterations in nitroxidative stress and antioxidant status of diabetic rat kidney. Data are expressed as mean ± SEM. Superscript of different letters in each column differ significantly ($P < 0.05$) from each other. Note: PXN-Peroxynitrites; PCO-Protein carbonyls; MDA-Malondialdehyde; NO-Nitric oxide; GSH-Reduced glutathione; GPx-Glutathione peroxidase; GST-Glutathione S-Transferase; SOD-Super oxide dismutase; CAT-Catalase.

3.5. AGTE effect on alcohol induced changes on nitroxidative stress and antioxidant status of diabetic renal mitochondria

Elevated levels of MDA, nitric oxide and peroxynitrites were observed to be significant ($P < 0.05$) in renal mitochondria of alcohol treated, diabetic control, alcohol treated diabetic groups when compared to normal control rats. Levels of peroxynitrites showed a significant difference between all the experimental groups, but no such significance was observed between AT and DC rats in comparison with NC rats. The levels of protein carbonyls in AT rats showed a significant difference ($P < 0.05$) with all the other rat groups except DC rats, and DC rats were not significant with any rat groups except D + A rats. The concentrations of nitric oxide were not significant ($P > 0.05$) between AT and DC rats but showed significance with D + A rats in comparison with NC rats. The mean values of products of lipid peroxidation (MDA) were significantly increased among DC, AT and D + A groups and that were brought to NC levels by the supplementation of AGTE and D + A + E rats also showed no significant difference with DC rats (Fig. 3).

It is clear from this study that there was a significant decrease in antioxidant status in kidney mitochondria from alcohol treated, diabetic control, and alcohol treated diabetic groups in comparison with that of normal control group. GSH content and GST activity between AT and DC rats were not shown a significant difference between them, but significant change was observed with alcohol treated diabetic rats. Supplementation of AGTE to D + A rats increased these levels with the NC rats (except GSH). The mean values of SOD and GPx exhibit significant difference between the AT, DC and D + A groups, but these levels were observed to be increased to the levels of NC by the supplementation of AGTE. Levels of CAT were not significant between AT, DC, and D + A groups. The concentration of CAT was increased by the supplementation of AGTE to D + A rats but it was not significant even with the NC rats (Fig. 4).

3.6. AGTE effect on alcohol induced changes in the biogenic amines of diabetic rats

The peaks of the investigated biogenic amines in the serum samples could be separated within 20 min. Occasionally, the recording was spoiled by residues of the protein precipitate passing through

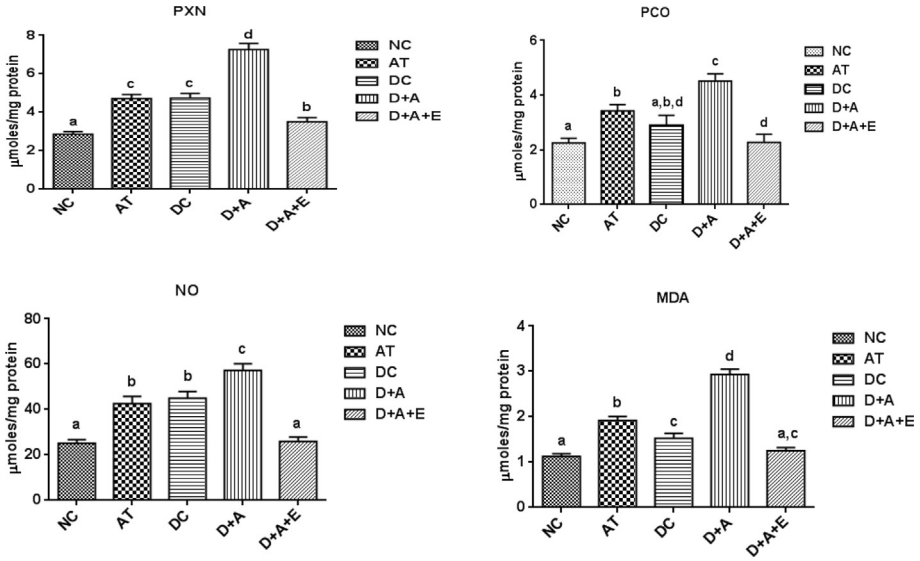

Fig. 3. Effect of green tea extract on alcohol induced alterations in concentrations of peroxynitrites, protein carbonyls, nitric oxide, and lipid peroxidation in kidney mitochondria Values presented are the Mean ± SEM (n = 8). Significance was determined at a $P < 0.05$. Categories sharing a different alphabet are statistically different.

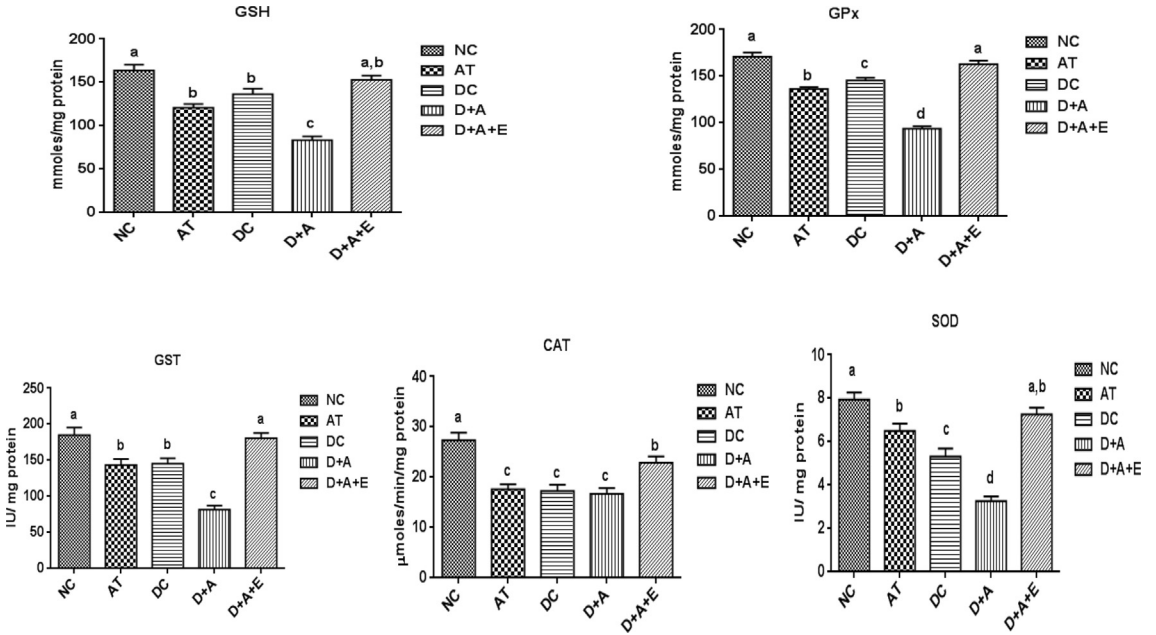

Fig. 4. Effect of green tea extract on alcohol induced alterations on antioxidant enzymes status in kidney mitochondria of diabetic rats Values presented are the Mean ± SEM (n = 8). Significance was determined at a $P < 0.05$. Categories sharing a different alphabet are statistically different.

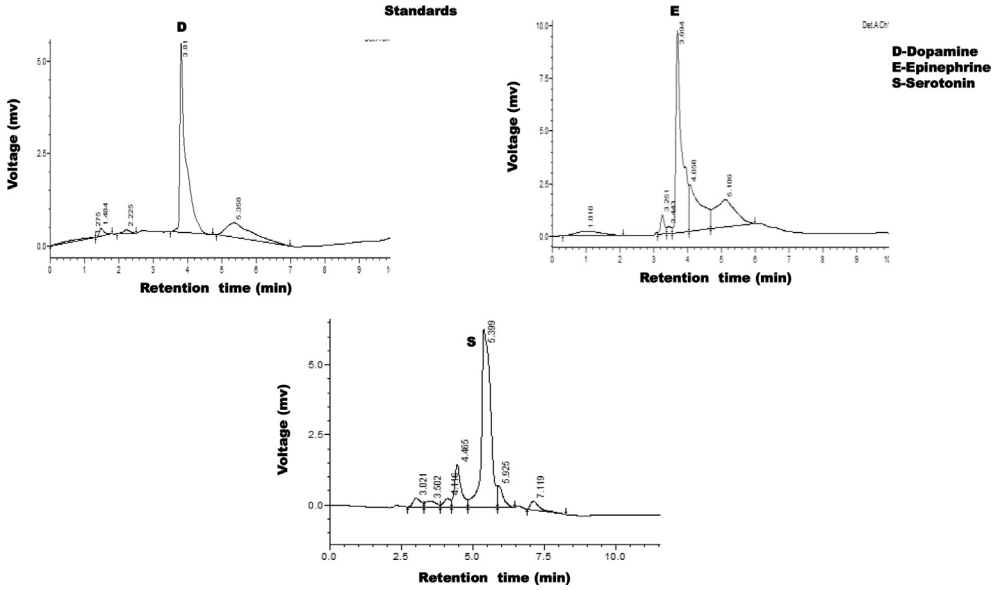

Fig. 5. HPLC chromatogram of standards of Dopamine, Epinephrine and Serotonin.

the filter. This was resolved by additional centrifugation of the sample. The range of retention time of standard epinephrine, dopamine and serotonin are 3.5–5.4 min, 3.6–6.2 min and 3.8–9.2 min respectively and showed a chromatogram peak at 3.6, 5.4 and 6.3 min respectively (Fig. 5).

Representative chromatograms of standards of epinephrine were at 4.9 min, dopamine at 6.3 min and serotonin at 6.5 min. The range of retention time of epinephrine was observed to be at 4.00 min, dopamine at 5.4 min, and serotonin at 6.2 min in group I control. In group II rats, the range of retention time of epinephrine at 4.9 min, dopamine at 5.4 min, and serotonin at 6.2 min, in group III rats the range of retention time of epinephrine was at 4.6 min, dopamine at 5.9 min, and serotonin at 6.0 min. The range of retention time of epinephrine at 4.8 min, dopamine at 5.4 min, and serotonin at 6.2 min in group IV and in group V, the range of retention time of epinephrine at 4.5 min, dopamine at 5.0 min, and serotonin at 6.5 min were observed (Fig. 6). No significant difference was observed in the concentrations of dopamine between all experimental groups (Group II, III, IV, V) and with that of group I rats, and there was no significant difference in the mean values of epinephrine between AT, DC and D + A rats, but significantly increased when compared to NC rats. The levels of serotonin were significantly increased in AT, DC and D + A rats in comparison with NC rats, and which were brought back to the level of NC rats by the supplementation of AGTE (Fig. 7).

3.7. AGTE effect on alcohol induced alterations in the kidney weight and BUN levels of diabetic rats

Rats in the AT group showed an increase in the kidney weight and also greater serum levels of blood urea nitrogen (BUN) relative to the NC group ($P < 0.05$; Fig. 8). In the current study, we observed that, there is no significant increase in the weight of kidney in DC and D + A rats. But the kidney weight of D + A rats showed a significant increase that was observed to be close to the NC rats by the supplementation with AGTE. The levels of BUN were observed to be significantly increased both in AT and D + A rats but decreased in DC rats which were brought back to the level of NC by the AGTE treatment.

3.8. AGTE effect on alcohol induced changes of in vitro gluconeogenesis

Glucose synthesis from gluconeogenic pathway in renal cortex was measured in different experimental rat groups using the substrates namely pyruvate, succinate, glutamate, glycerol and alanine as

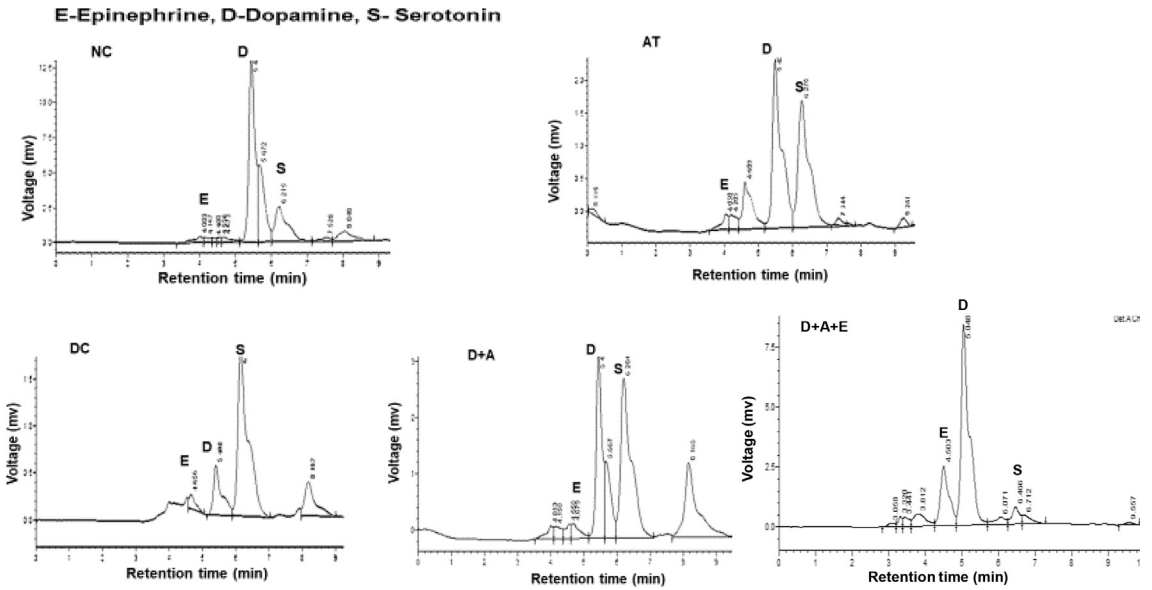

Fig. 6. HPLC chromatograms showing the effect of green tea extract on alcohol induced alterations in the levels of dopamine, epinephrine and serotonin in plasma of diabetic rats. Data are expressed as mean ± SEM ($P < 0.05$).

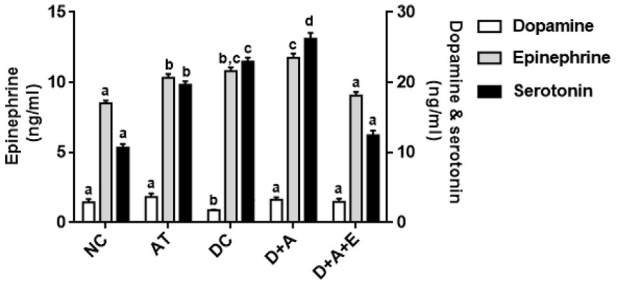

Fig. 7. Effect of green tea extract on alcohol induced alterations in the concentrations of epinephrine, dopamine, serotonin in diabetic rats. Superscript of different letters in each column differ significantly ($P < 0.05$) from each other.

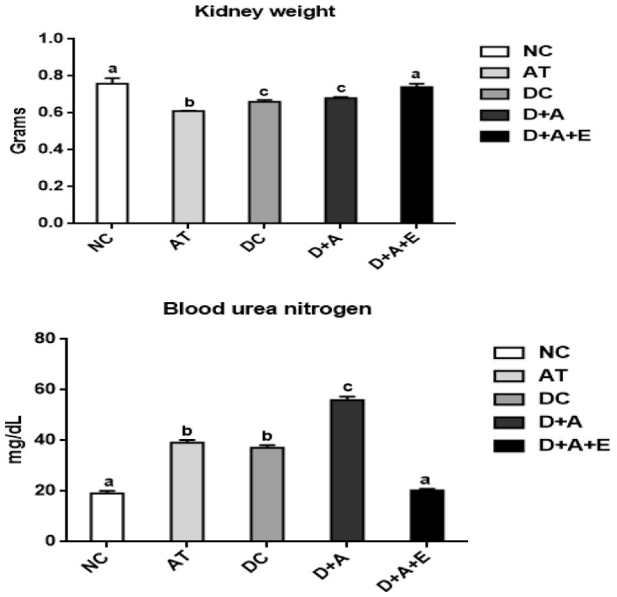

Fig. 8. Effect of green tea extract on alcohol induced changes in kidney weight and blood urea nitrogen levels of diabetic rats data are expressed as mean ± SEM. Superscript of different letters in each column differ significantly ($P < 0.05$) from each other.

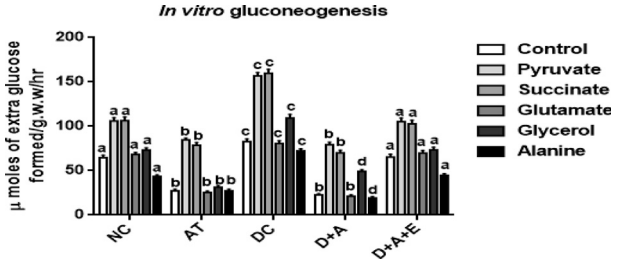

Fig. 9. Effect of green tea extract on alcohol induced alterations in *vitro* gluconeogenic potential of kidney cortical slices in the presence of 10 mM of substrates. Superscript of different letters in each column differ significantly ($P < 0.05$) from each other.

metabolites and the results were expressed in terms of extra glucose produced/g dry wt. tissue/h. Results indicate that renal cortical slices of diabetic rats possessed substantial *in vitro* gluconeogenic capacity which was found to be decreased in AT and D + A groups. In the present study it was observed that, supplementation of AGTE to the D + A rats normalized these alterations to the levels of NC rats (Fig. 9).

3.9. AGTE effect on alcohol induced changes in diabetic renal cortical iNOS protein expression and renal histopathological changes

We analyzed the NO production by measuring nitrate and nitrite levels as well as iNOS protein expression in renal cortex. Alcohol intoxication to the diabetic rats significantly increased iNOS protein expression and subsequently nitrate and nitrite levels, was observed in D + A rats. Together with increased formation of free radical intermediates at the end of experimental period, iNOS protein expression was considerably augmented in the kidney, a known target organ for both diabetes as well as alcohol toxicity. This data suggests that iNOS over expression may lead to protein nitration, which is consistent with peroxynitrite formation. The experimental groups showed significant increase in protein expression of iNOS compared to normal control group and immunoblot of iNOS was presented in Fig. 10. Taken together, our results strongly support a mechanism where detectable concentrations of radical adducts by alcohol intake in diabetic kidney are iNOS-dependent, possibly leading to increased nitroxidative stress in D + A rats, but there was no such significance between AT and Dc rats. These changes were reverted to normal levels by the supplementation of AGTE, observed in D + A + E group.

Histopathological changes in the cortical sections of kidney tissue of alcohol treated diabetic rats showed severe degenerative changes when compared with alcoholic, diabetic, control, and diabetic alcohol with supplementation of green tea extract groups (Fig. 11). In this study, Toblli scoring system (0–4) was used to report the histological damage in kidney injury. The scoring system consists of histological injury in glomerular, tubular, and interstitial components.

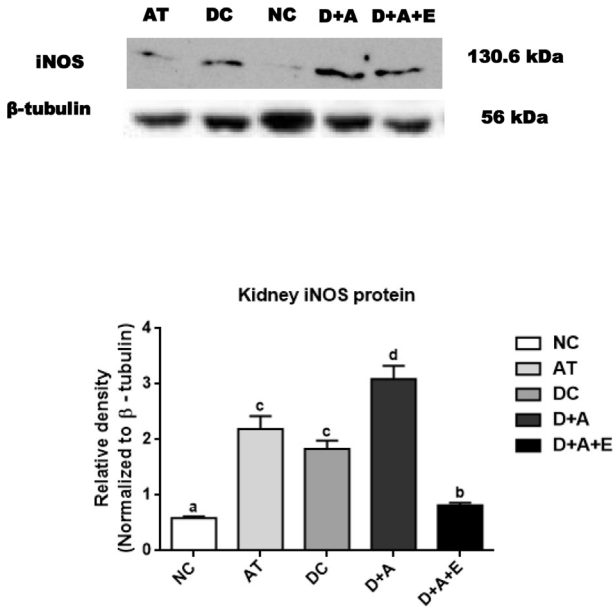

Fig. 10. The protein expression of iNOS in controls and experimental groups Values presented are the Mean ± SEM (n = 8). Significance was determined at a $P < 0.05$. Categories sharing a different alphabet are statistically different.

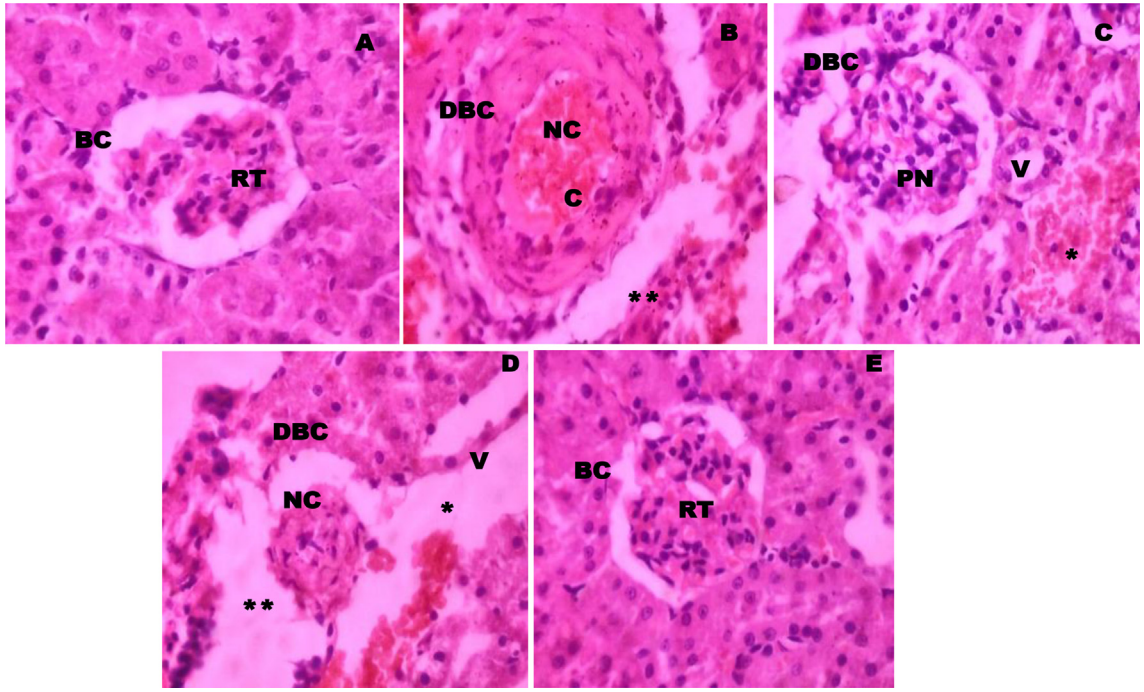

Fig. 11. Light micrograph of kidney sections (H&E x40). A) Normal control rats showed normal glomeruli in Bowman's capsule (BC) with normal baseline and renal tubules (RT). B) Kidney sections from chronic alcohol treated rats showed glomerular focal necrotic changes (NC) in more than 25% (score 2+), and glomerular sclerosis (**) with distended Bowman's capsule (DBC) and congestion (C) of tubular epithelial cells indicates damage of kidney tissue. C) Kidney sections from diabetic rats showed distended Bowman's capsule, glomerular focal necrotic changes, interstitial inflammatory infiltrates (*) with a score of more than 25% (score 2+), pyknotic nucleus (PN) and vacoulization (V). D) Kidney sections from alcohol treated diabetic rats showed vacuolization (V), with severe distended Bowman's capsule, glomerular necrotic changes, interstitial inflammatory infiltrates, and glomerular sclerosis in more than 75% (score 3+) E) Alcohol treated diabetic rats supplemented with AGTE showed normal structure without signs of pathology (score 0).

3.10. Correlation analysis between iNOS levels of various experimental groups and nitroxidative stress parameters

There was a strong positive correlation exist between iNOS and PC ($r = 0.50$) as well as $ONOO^-$ ($r = 0.59$) but not correlated with MDA and NO in alcohol treated rats. There was a strong positive correlation between iNOS and $ONOO^-$ ($r = 0.49$) but weak relationship between iNOS and MDA, PCO and NO in diabetic control rats. In alcohol treated diabetic rats, there was a moderate positive correlation between iNOS and PCO ($r = 0.13$) & $ONOO^-$ ($r = 0.20$). A weak negative correlation of iNOS with MDA ($r = -0.48$) and NO ($r = -0.23$) exist in alcohol treated diabetic rats. Though there was a moderate and strong correlation between iNOS and above nitroxidative parameters, there were no significant difference ($P > 0.05$) observed between them. There is a negative correlation of iNOS with nitroxidative stress markers observed in alcohol treated diabetic rats with supplementation of green tea extract ($D + A + E$) and a significant change ($P = 0.02$) was observed only in the levels of PCO (Table 6).

4. Discussion

The present study aimed to evaluate the potential protective effect of AGTE when used against the alcohol induced alterations in diabetic rats. This maintained a level of renal function similar to that observed in normal control animals. Nutraceuticals are the products with nutritive as well as therapeutic applications. Green tea, a popular beverage, is now considered as nutraceutical and received high interest and attention [40]. Green tea constituents, in particular, its catechins have ability to ameliorate the toxicities induced by a large number of chemicals and drugs etc., which is attributable to antioxidant and therapeutic properties of catechins [41]. In most countries, alcohol abuse is a clinical problem with great socio-economic repercussions. It is reported that moderate alcohol consumption may reduce the risk of type 2 diabetes and high alcohol consumption may increase the risk of type 2 diabetes in women [42]. Despite this positive outlook of alcohol consumption on DM, a very strong negative attribution still exists with an emphasis on chronic intake [43] as people with diabetes can't give up alcohol simply because of their diabetes. Though alcohol is a hypoglycaemic drug under acute conditions, when chronically used, it causes hyperglycaemia [44]. Fasting blood glucose in alcohol treated diabetic rats increased by 3.5-fold in the present study when compared to normal control rats which indicated severe hyperglycaemia in STZ-induced diabetic rats consuming alcohol. This finding appears to be a characteristic response to chronic alcohol treatment in diabetic rats.

Increase in FBG in diabetic and alcohol treated rat groups is well expected and agrees with earlier reports [45]. As alcohol increases the absorption of other food-stuff and nutrients from intestine, it results in increased gain in the total body weight of alcoholic rats in the present study. This finding coincides with the previous studies [46]. This may be due to the deposition of lipids in adipose tissue and fluid accumulation in the organs. Another factor could be the osmotic diuresis resulting in hyper osmotic dehydration. Due to excess utilization of proteins for the energy purpose, alcohol treated diabetic rats showed a significant decrease in body weight ($P \leq 0.05$) compared to diabetic rats. Furthermore, alcoholic drinks contain calories, and therefore can lead to weight gain. Green tea catechins showed effects on body weight maintenance affecting energy expenditure, fat oxidation and fat absorption causing weight loss in a beneficial way i.e., near to the NC rats. Moreover, catechins

Table 6
Correlation matrix of iNOS with oxidative and nitrosative stress parameters.

iNOS	AT		DC		D + A		D + A + E	
	r	P	r	P	r	P	r	P
MDA	−0.082	0.84	0.16	0.71	−0.48	0.22	−0.57	0.13
PCO	0.50	0.20	0.17	0.69	0.13	0.76	−0.79	0.02
NO	−0.001	0.99	0.23	0.57	−0.23	0.57	−0.15	0.72
$ONOO^-$	0.59	0.12	0.49	0.21	0.20	0.63	−0.27	0.51

Note: MDA- Malondialdehyde; PCO- Protein carbonyls; NO-Nitric oxide; $ONOO^-$ – Peroxynitrites; r = Correlation coefficient ($P < 0.05$).

upregulate lipid metabolizing enzymes via nuclear transcription factor kB (NF-kB) thereby stimulating fat oxidation [47]. And the rats were physically inactive up to 6 h and no death was recorded even at the dose of up to 5 g/kg b.w (20% v/v), with some behavioural changes in Group II and Group IV rats treated with ethanol for 60 day's chronic study. However, one diabetic rat went to a stage of coma in the last week of experimental period without any mortality.

Prolonged and persistent hyperglycaemia, a principle factor responsible for various alterations at the renal level in diabetic as well as alcohol treated diabetic group reflects the aggravating potential of alcohol on hyperglycaemia. Destruction of insulin producing pancreatic β-cells in STZ-induced diabetic rats with and without alcohol consumption contributes to the observed hyperglycaemia in these groups and alcohol consumption caused further escalation of blood glucose levels resulting in higher glycosylated haemoglobin (HbA1c) content (not shown in this study). Another important source of free radicals in diabetes is the interaction of glucose with proteins leading to the formation of an amadori product and then advanced glycation end products (AGEs). By increasing intracellular oxidative stress, AGEs might activate the transcription factor NF-kB, thus promoting up-regulation of various NF-kB controlled target genes [48]. NF-kB thus, enhances production of nitric oxide, which is believed to be a mediator of islet beta cell damage. Hyperglycaemia is directly linked to diabetic complications, particularly in the kidney; the FBG level of diabetic-alcohol rats showed approximately a 3.5-fold, significant increase; therefore, glycemic control remains the major target of therapy and however, AGTE inhibited this increase dose-dependently.

Our results showed elevated concentrations of cholesterol, phospholipids and triglycerides in kidney of alcohol treated diabetic group when compared with other experimental groups. Furthermore, this study demonstrated that alcohol administration further increased the lipid profiles in STZ-induced diabetic rats. Hyperlipidaemia and hypertriglyceridemia are the characteristic responses observed in diabetes and alcohol treated rats in agreement with the earlier reports [49,50]. Further, the levels of glutathionyl haemoglobin with high oxygen affinity and low cooperatively and hyperlipidemia might lead to reduced tissue oxygen delivery in diabetes which might be useful as a clinical marker of oxidative stress [51]. When both alcoholism and diabetes coexist, the lipid abnormalities are increased which might make the animals more susceptible even for cardiovascular risk. Actually, insulin has an inhibitory action on HMG CoA reductase. The increased production of cholesterol rich low-density lipoprotein (LDL) is mainly due to elevated levels of HMG Co A reductase in the absence of insulin action under diabetes. And higher phospholipid content in the kidney of alcohol treated diabetic rats may be due to augmented synthesis, availability, oxidation and/or decreased turnover of free fatty acids that can impair insulin action and glucose metabolism leading to the development persistent hyperglycaemia [52]. Though the precise mechanism by which AGTE rectifies the above changes is not known, it is pertinent from findings of the present study to note that all abnormalities, if not majority of alcohol induced abnormal changes, are corrected upon AGTE supplementation to alcohol receiving diabetic rats. Probably action of green tea catechins related to signaling processes, in particular inactivating NF-kB, may play a major role in this beneficial effect.

Ethanol consumption produces a wide variety of pathological conditions including diabetes that affect several organs. Mechanisms might be involved in the genesis of oxidative and nitrosative stress in both diabetic and alcoholic rats due to glucose auto-oxidation, protein glycation, formation of advanced glycation end products, polyol pathway, nitration of proteins, and binding of peroxynitrites to DNA. Measurement of both the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) discern the renal toxicity. The direct toxicity of nitric oxide is modest but is greatly enhanced by reacting with O_2^- to form peroxynitrite, which is a transition metal-independent source of hydroxyl radicals (•OH). Peroxynitrite reacts relatively slowly with most biological molecules, making them a selective oxidant. Protein oxidation and lipid peroxidation are widely used as biomarkers of tissue damage or oxidative stress. In the present study, amounts of oxidized proteins and lipids in the kidney of the alcohol treated diabetic rat group were higher than in other experimental groups and normal controls. Oxidized proteins are often functionally inactive and may be associated with enhanced susceptibility to ubiquitination.

In this study, for the first time, lipid and protein oxidation, carbonylation levels in the kidney were measured in diabetic rats exposed to chronic alcohol over a long period. Taken together, the results of the study showed that chronic excessive ethanol intake under diabetes induces double jeopardy in rat

kidney, mainly through increased lipid peroxidation and protein oxidation in the tissues. Oxidative stress induced by alcohol and has been implicated in β-cell dysfunction [52] which can be similar in diabetes. Elevated endogenous rates of ROS production and the impairment of tissue free-radical defence machinery create a milieu that inactivates NO mediated vascular relaxation and generates secondary reactive species that also impair vascular function. Observed decrease in GSH content and reduced activities of antioxidant enzymes in kidney of D + A rats suggested decreased antioxidant status including glutathione peroxidase, glutathione S-transferase, superoxide dismutase and catalase (GPx, GST, SOD, and CAT respectively). GSH is almost uniquely present in a quite high concentration which allows scavenging ROS either directly or indirectly.

In the present study, no significant differences were observed in GST, CAT and Cu/Zn SOD activities between alcohol treated and diabetic control rats compared with these parameters in controls but we noted significant decrease in GPx and GSH levels. The reported decrease in GPx activity may have been a result of non-enzymatic glycation of the enzyme which can ultimately lead to catalytic alterations in the activity, representing a decline in renal function. ROS and acetaldehyde can react with GSH and decrease its content to diminish GSH-dependent cellular defences against oxidative stress [53]. The presence of AGTE reduced the ROS formation and restored glutathione levels and other antioxidant enzymes to baseline levels, which prevented the oxidative stress induced by hyperglycaemic conditions, suggesting the tremendous therapeutic modulation of AGTE.

To identify the anti-hyperglycemic activity of AGTE involved in renal protection, *in vitro* studies were carried out using renal tissue. Earlier studies clearly demonstrated that hyperglycaemia is the chief cause for the adverse health effects and sequelae of diabetes, and further, alcohol consumption escalates hyperglycaemia making it more pronounced [49]. Observed prolonged and persistent hyperglycaemia in diabetic as well as alcohol treated diabetic rats is due to increased glycogenolysis and/or gluconeogenesis and/or decreased utilization of glucose by peripheral tissues. Moreover, the action of other hyperglycaemic hormones such as epinephrine plays a key role in the absence of insulin. Insulin dependent enzymes are also less active with inhibition of glycolysis and stimulation of gluconeogenesis leading to hyperglycaemia. It is evident from the results of this study that diabetes coupled with alcohol intake synergistically modulates the gluconeogenic potential and decreased utilization of glucose in peripheral tissues.

Glucose formation from glutamate and pyruvate requires the uptake and entry of these substrates into mitochondria where they are converted into oxaloacetate leading to the generation of ATP. The regulatory mechanisms that determine the flux rate through gluconeogenic pathway depends on the concentrations of gluconeogenic substrates which modulate the rate limiting enzymes of the pathway, and on acid-base status of the animal [54]. Alcohol treated rats showed a significant decrease in the *in vitro* gluconeogenic capacity and this might be due to the inhibitory effect of alcohol on gluconeogenesis. There is a significant increase in the activities of gluconeogenic enzymes such as glucose 6-phosphatase and fructose 1, 6-bisphosphatase in diabetic rats, which could be due to low/no levels of insulin in diabetic state. Insulin deficiency results in the activation of gluconeogenic enzymes during diabetes and, in normal conditions, insulin functions as a suppressor of gluconeogenesis [55]. Surprisingly, from this study, it is well known that the *in vitro* gluconeogenic capacity of kidney in alcohol treated diabetic rats was significantly decreased and this might have been due to the decreased activities of the above enzymes by the counter action of alcohol in diabetic state. However, the results of the studied tissues investigating the effect of green tea extract on glucose control with an emphasis on increased renal *in vitro* gluconeogenesis have been inconsistent with the earlier studies (hepatic gluconeogenesis) that suggest that an exposure to EGCG or tea extract causes decreased glucose production by inhibiting the expression of hepatic key gluconeogenetic enzymes namely phosphoenol pyruvate carboxykinase and glucose- 6-phosphatase in a parallel manner to insulin. EGCG activates AMPK which is necessary for the inhibition of gluconeogenesis through the calmodulin-dependent protein kinase. But, the present study suggests that EGCG may inactivate AMPK in the kidney tissue which further leads to increased renal *in vitro* gluconeogenesis. However, the exact underlying mechanisms in this regard are yet not very clear which needs further research.

Among a wide panel of analytical methods developed for the quantification of biogenic amines, HPLC separation after derivatization with dansyl chloride remains the most commonly used method. The present study clearly demonstrated that the increased levels of plasma epinephrine and

indolamine like serotonin in all the experimental groups when compared to normal rats. The changes were more pronounced in the alcohol treated diabetic group as compared to alcohol treated and diabetic control groups, suggesting an additive effect of alcohol on diabetic damage. Levels of dopamine in the plasma also increased, but to a much lesser degree. Our study demonstrated that blood plasma levels of neurotransmitters can be employed to evaluate the mood changes associated with alcohol treatment and more importantly, provided an important clue for understanding of the relationship between alcohol treatment to diabetic rats and mood disorders. Fluctuations in tryptophan levels in alcohol treated diabetic rats may also affect the biosynthesis of serotonin. Dysregulation of mood, aggression, impulsive behaviour, sleep, anxiety disorders were highly observed in D + A rats and AT rats compared with the corresponding DC and NC rats, might be due the hike in the serotonin levels. Our results suggested that AGTE could improve these abnormal changes by modulating and normalizing the levels of dopamine and epinephrine.

Since kidney is one of the important organs, next to the liver, for storage, detoxification, metabolism, and excretion of many metabolites, it is also more vulnerable to nitroxidative damage. The mechanisms of kidney destruction are associated with the oxidative stress involving the secretion of cytokines, mainly tumour necrosis factor (TNF-α), interleukin (IL-1), and interferon gamma (IFN-γ). These alterations might cause an abnormal production of cytokines and growth factors [56] and subsequently, they might facilitate the synthesis of extracellular matrix proteins and the depositions in the glomerular level that finally lead to mesangial expansion, glomerular shrinkage, and glomerular basement thickening. High glucose levels directly increase hydrogen peroxide production in the murine mesangial cells, and lipid peroxidation of the glomerulus. Therefore, oxidative stress can be the common pathogenic factor for diabetic nephropathy which is similar to other complications including alcohol abuse. The functional consequences and clinical applicability and treatment for these observations require further study.

Histopathological examination of the normal kidney tissue of rats showed a normal structure of glomerulus surrounded by the Bowman's capsule, with proximal and distal convoluted tubules without any inflammatory alterations. Kidney sections from alcohol treated rats showing necrotic changes, distended Bowman's capsule and congestion of tubular epithelial cells causing significant damage to the kidney. Diabetic rats showed morphological changes such as atrophy of the glomeruli, necrotic tubular epithelial cells, and dark pyknotic nuclei. These findings were more prominent in group IV with an extensive shrinkage of glomeruli, infiltration of red cells and tubular inflammation. Haemorrhage is evident within the Bowman's space of both diabetic and alcohol treated diabetic rats that might be due to glomerular damage, degeneration of glomeruli with wider Bowman's spaces and diffused vacuolation of the tissues. Severe glomerular and tubular degeneration, focal necrosis of tubules, fatty infiltration and dilatation of Bowman's capsule might also be associated with increased diuresis and renal hypertrophy. This is in agreement with previous studies [57] who reported that excessive consumption of alcohol has been linked to numerous renal complications as well as the deadly end-stage renal disease (ESRD) by 4-fold. Hyperglycemia is the principal factor responsible for structural alterations at the renal level. These histopathological changes induced by diabetes and alcohol may be due to disturbances in lipid accumulation [58]. Administration of AGTE to alcohol treated diabetic rats surprisingly improved the kidney function as a result of its anti-hyperglyceamic and anti-thrombogenic actions that may control the arachidonic acid cascade system and thus inflammatory reactions in kidney tissue. Hence, in these animal experiments, AGTE was able to ameliorate the structural lesions present in D + A rats. It is noteworthy that there is also abundant evidence indicating independently that iNOS becomes responsible for strikingly enlarged glomerular basement membranes and kidney swelling and filtering in capacitance in diabetic state [59]. Heavy alcohol drinking might further exacerbates similar alterations induced by diabetes [60]. Neither xanthine oxidase, cytochrome neither P450 nor macrophage activation were required for the production of radical adducts. Interestingly, inducible nitric oxide synthase was identified as the major source of peroxynitrite which acts as a source of hydroxyl radical production [61].

Over expression of iNOS in the kidney of diabetic-alcoholic animals was closely correlated with the lipid radical generation and 4-hydroxynonenal-adducted protein formation, indicating lipid peroxidation and protein oxidation respectively. Taken together, our studies support chronic, excessive alcohol induced iNOS as a significant source of highly reactive intermediates, which leads to lipid

peroxidation, formation of peroxynitrites and protein carbonyls may contribute to diabetes progression as well. Lipid and protein damage caused by alcohol metabolites paralleled iNOS over expression in the kidney of diabetic animals. Such results clearly link iNOS over expression with increased free radical formation, lipid peroxidation, disturbed NO metabolism and protein oxidation in this geminate. Hence our study for the first time, also observed that a positive correlation exists between iNOS protein expression and the nitroxidative stress in alcohol treated, diabetic and alcohol treated diabetic rats. There is also abundant evidence, indicating that iNOS mediates insulin resistance where inflammatory processes are involved and markedly expressed in insulin-sensitive organs such as liver, skeletal muscle, and adipose tissue [61]. As suggested by the present observations, the capacity of green tea in modulating alcohol and diabetes-induced iNOS upregulation may also implicit beneficial interference in the deleterious effects of excessive iNOS signaling, which is central in mediating elevated tissue remodeling and alterations in renal hemodynamic and tubular functions, manifested by the development of diabetic nephropathy (DN).

Though previous findings suggest that green tea decreases theactivity and protein levels of iNOS [62] by reducing the expression of iNOS mRNA, via the blocking of NF–kB binding to the iNOS promoter, thereby inhibiting the induction of iNOS transcription, results of the study presented here demonstrate that alcohol induced free radical production arising from peroxynitrite formation as a result of iNOS over expression may potentially be a primary event which leads to lipid peroxidation, protein oxidation, over production nitrite and nitrates which in turn involved in the key mechanisms for the development of ESRD under diabetes. This observation of alcohol related damage provides a new insight necessary to understand free radical mechanisms behind diabetic complications.

In conclusion, our present study has been carried out to know the effect of AGTE chronic alcohol consumption on all the above discussed parameters during diabetic state in continuum with the earlier supporting studies. Green tea, rich in antioxidants maintained proper redox balance in the tissues. Thus, consumption of green tea acts as a key player for the reduction of ROS and RNS related pathological disturbances induced by diabetes and alcohol. Uncontrolled hyperglycemia, hyperlipidemia, systemic and intra-renal hypertension and activation of renin-angiotensin might be the most important factors that have been proposed for the pathogenesis of DN. Hence, treatment with AGTE fully restored all the above-mentioned alterations induced by alcohol in diabetic animals as well as in renal cells.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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