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Phyllanthus virgatus forst extract and it's partially purified fraction ameliorates oxidative stress and retino-nephropathic architecture in streptozotocin-induced diabetic rats

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Abstract: Diabetic retinopathy and nephropathy are questionably the most dreaded complications of diabetes; contribute to serious morbidity and mortality. The current study was undertaken with the aim of exploring the anti-lipoperoxidative and antioxidant status including nephroprotective and retinoprotective potential of *Phyllanthus virgatus* methanolic extract and its partially purified fraction in streptozotocin (STZ)-induced diabetic stressed rats. Diabetes was induced by intraperitoneal injection of *Streptozotocin* (60mg/kg B. Wt of rat). Among all the treatment groups, *P. virgatus* methanolic extract and its partially purified fraction at a dose of 50mg/kg (PET-1) and 0.5mg/kg (CT-1), respectively, showed significant protection against STZ-induced diabetic oxidative stress in rats with marked amelioration in lipid peroxidation byproducts level, antioxidant enzymes, nephroprotective and retinoprotective effects and plasma total antioxidant levels after treatment of 28 days. The combined results demonstrated significant protection against STZ-induced oxidative stress, nephropathy and retinopathy condition by *P. virgatus* methanolic extract and its bioactive compound.

Keywords: *Phyllanthus virgatus*, oxidative stress, nephropathy, retinopathy, CD and TBARS.

INTRODUCTION

It is well reckoned that persistent hyperglycemia is associated with increased production of free radicals that leads to damage and dysfunction of various organs (Baynes, 1991). Several hypotheses have been reported to explain the generation of free radicals in diabetes that include gluco-oxidation and glycation of proteins which intern leads to the formation of advanced glycation end products (AGEs) (Vlassara and Palace, 2001). Elevated glucose level in diabetes leads to protein oxidation and lipid peroxidation of membrane which clearly signifies augmented free radical production (Gallou *et al*., 1993). Increased free radical levels or inefficient free radical scavenging leads to tissue damage and that is assessed by the measurement of lipid peroxide and protein carbonyl level (Maxwell *et al*., 1997). Moreover, disturbance of antioxidant defense system lead to alteration in enzymatic and non-enzymatic antioxidants like impaired glutathione metabolism (McLennan *et al.,* 1991) that are the major cause of both micro vascular and macro vascular complications including accelerated progression of atherosclerosis due to endothelial dysfunction and micro angiopathy of retinal vessels (Foyer and Noctor, 2005). Diabetes also leads to distortion of the enzymatic antioxidants (Genet *et al.,* 2002) and its management is needed to avoid the progression of diabetes and its complications like diabetic nephropathy, neuropathy, retinopathy and cardiomyopathy (Wolff, 1993).

Despite the availability of well known hypoglycemic agents in the pharmaceutical sector, diabetes and its associated snag continues to be the foremost medicinal problems. All oral hypoglycemic drugs are accompanied with a host of serious and undesirable adverse effects (Moller, 2001). Therefore, herbal medicines have gained much importance in the discovery of new therapeutic agents in the form of biologically active secondary metabolites including antioxidants, hypoglycemic and hypolipidemic compounds (Mohamed *et al.*, 2006). The significant hypoglycemic activities of these plants are owed to their ability to restore the pancreatic insulin secretion or decrease in the intestinal glucose absorption (Srinivasan and Pari, 2012). Thus, the treatment approach with herbal medicines has a great impact on pancreatic β-cells protection and smoothing out fluctuations in glucose levels (Elder, 2004). In addition, oxidative stress induced complications are treated and managed by supportive therapies that include the use of antioxidants and natural products (Alvi *et al*., 2016; Alvi *et al*., 2017). Therefore, we have focused on plant sources for new therapeutic agents with minimal side effects (Khan *et al.,* 2011b; Alvi *et al*., 2015; Iqbal *et al.*, 2014; Akhter *et al.,* 2013; Khan *et al.,* 2011a). Previously our lab showed that *Phyllanthus virgatus* Forst (Euphorbiaceae) commonly known as Bhuiamla, has great potential as an antioxidant and antihyperglycemic agent *in vitro* (Hashim *et al.,* 2013; Hashim *et al.,* 2014) and has been previously used in the treatment of intestinal, liver, kidney and bladder problems (Shabeer *et al.*, 2009). Based on our previously published data (Hashim *et al.,* 2013; Hashim *et al.*, 2014), which delineated the *in vitro* and *in vivo* anti-diabetic efficacy of

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methanolic extract of *P. virgatus* and its partially purified fraction, the current study mainly intend to evaluate the preventive role of *P. virgatus* methanolic extract and its partially isolated fraction in ameliorating the lipid per oxidation products and antioxidant enzyme activity, as well as in protecting the nephropathy and retinopathy in experimental diabetes in rats.

MATERIALS AND METHODS

Collection of plant material and extract preparation

The whole plant of *P. virgatus* was collected from the region, nearby Integral University, Lucknow, India, in the months of July-August. The identification and authentication of plant was carried out by Dr. Mohd. Tariq, National Botanical Research Institute (NBRI), Lucknow, India and a voucher specimen (98195) was assigned and submitted there. The plant was processed and extracted according to our previously published reports (Hashim *et al.*, 2013; Hashim *et al.*, 2014). The resulting extracts were filtered and left till dryness at room temperature and were stored at -20^{o}C for future use (Hashim *et al.*, 2013). Further, the bioactive compound was fractionated from methanolic extract of *P. virgatus* via repetitive preparatory thin layer chromatography (TLC) as discussed earlier (Hashim *et al.*, 2014).

Dose preparation

Sequentially extracted *P. virgatus* methanolic extract and its partially purified fraction with different concentrations were solubilized in 5% dimethyl sulfoxide (DMSO) and homogenized with saline. The doses of extract were selected on the basis of previously published reports (Shabeer *et al.*, 2009). Glibenclamide, the standard drug, was dissolved in distilled water and administered orally. The animals in normal control (NC) group received buffer only.

Animals

Male wistar rats (weighing between 180-220g) were procured from the Indian Institute of Toxicology Research (IITR), Lucknow, for this study and this in-vivo study was dully approved by Institutional animal ethics committee (IAEC) vide registration number: IU/Biotech/project/CPCSEA/13/14. Animals were fed on normal standard pellet diet with water *ad libitum*, and acclimatized to the laboratory conditions for a week.

Induction of diabetes and hyperglycemic-stress

In order to induce experimental diabetes, thirty overnight fasted rats (free access to water) were injected with STZ, freshly dissolved in 10mM citrate buffer, pH 4.5, (60 mg/kg B. Wt) intraperitonially (Zhang *et al.*, 2008). The rats were injected with buffer only in NC group. The fasting blood glucose (FBG) level was recorded after 48 h of STZ injection. The rats depicting FBG≥230 mg/dl were considered to be diabetic for investigating the hypoglycemic and hypolipidemic impacts of *P. virgatus* and its partially purified fraction and were divided randomly and equally (5 rats in each group) in groups as illustrated in table 1. Plant extract, its isolated fraction and glibenclamide suspension were administered through gastric intubation in two divided doses (morning and evening) of 0.5ml each /rat/day for 28 days.

Analytical procedures

At the end of 28 days treatment, overnight fasted rats were anaesthetized and blood, liver and kidney tissues were collected and processed further as described earlier (Khan *et al.*, 2011b). The ferric reducing ability of plasma, the FRAP assay, which estimates "total antioxidant power" was measured as described by Benzie and Strain (1996) with minor changes (Hashim *et al.*, 2014). The malondialdehyde (MDA) release from intact erythrocytes and MDA content was assayed according to the protocol of Cynamon *et al.* (1985). In order to estimate lipid per oxidation byproducts from plasma and tissues initially the lipid was extracted according to the procedure of Folch *et al.* (1957). For the estimation of conjugated diene (CD) in plasma and liver the method of Beuje and Aust (1978) was employed. In another experiment, lipid hydro peroxide (LHPO) concentration was estimated according to the pocedure of Nourooz-Zadeh *et al.* (1995). Moreover, Yagi's method (1987) was used for the estimation of lipid peroxide (MDA) contents in plasma while the protocol of Ohkawa *et al.* (1979) was followed for the quantification of MDA in liver and kidney homogenates.

Measurement of antioxidant enzyme activities

The enzymatic activities of catalase (CAT) and super oxide dismutase (SOD) in PMS of liver and kidney were measured by the procedure of Sinha (1972) and Kakkar *et al*. (1984). Glutathione per oxidase (Gpx) activity in kidney and liver homogenate was determined by a modification of Mill's procedure (1957). Glutathione reductase (Gred) activity was evaluated according to the method of Carlberg and Mannervik (1975). Method of Habig *et al*. (1974) was used to measure the Glutathione-S-transferase (GST) activity in PMS fraction of liver and kidney. The glutathione content in liver homogenate was determined by the procedure of Sedlack and Lindsay (1968).

Histopathological studies

For the histopathological studies, 10% of phosphate buffered (neutral) formalin was used to fix the removed organs i.e. kidney and eyes. Further, washed through running water, and then the sections were dehydrated by steadily increasing the percentage of alcohol and the extra alcohol was removed by xylene and embedded into paraffin. A microtome was used to make sections of paraffin blocks with the thickness of 4-5µm and slides were prepared with hematoxylin-eosin and examined under a light microscope and photographs were taken.

Table 1: Protocol for the treatment of STZ-induced diabetic-stressed rats.

NC	Normal control
NC+PE	Normal control + Plant extract treated (40 mg/rat/day)
DC	Diabetic control
CT-1	Diabetic + partially purified compound (0.5 mg/rat/day)
CT-2	Diabetic + partially purified compound (0.1 mg/rat/day)
PET-1	Diabetic + plant extract (50 mg/rat/day)
PET-2	Diabetic+ plant extract (10 mg/rat/day)
D-GT	Diabetic + Glibenclamide (0.1 mg/rat/day)

Table 2: Impact of *P. virgatus* methanolic extract, it´s partially purified fractionglibenclamide on plasma total antioxidants, conjugated dienes, lipid hydroperoxides and thiobarbituric acid reactive substances in diabetic-hyperlipidemic rats after 28 days of treatment.

Group	Total antioxidants	Conjugated dienes	Lipid hydroperoxides	TBARS
NC[§]	1476.6±1.5	3.2±0.5	1.2±0.26	1.4±0.3
NC+PE[§]	1545.1±6.8	3.6±0.2	1.2±0.1	1.3±0.1
DC[§§]	988.2±4.7 (-33.1%) [a]	4.6±0.3 (+43.4%) [a]	1.9±3.7 (59.0%)[a]	2.1±0.8 (+51.1%)[a]
CT-1[§§]	1220.1±3.2 (+23.5%) [b]	3.0±0.5 (-34.5%) [b]	1.2±0.1 (-34.0 %)[b]	1.4±0.1 (-31.4%)[b]
CT-2[§§]	1112.2±3.6 (+12.5%) [b]	3.4±0.3 (-26.1%) [b]	1.3±0.1 (-30.0%)[b]	1.6±0.1 (-24.4 %)[b]
PET-1[§§]	1210.2±5.2 (+22.5%) [b]	3.1±0.5 (-31.7%) [b]	1.2±0.1 (-33.5 %)[b]	1.5±0.9 (-26.2%)[b]
PET-2[§§]	1085.4±1.5 (+9.8%) [b]	3.3±0.8 (-27.0%)[b]	1.3±0.1 (-28.9 %)[b]	1.7±0.7 (-15.9%)[b]
D-GT[§§]	1115.7±1.9 (+12.8%) [b]	3.7±0.6 (-18.4%) [b]	1.5±0.1 (-19.1 %)[b]	1.8±0.7 (-11.3 %)[b]

Table 3: Impact of P. virgatus methanolic extract, it´s partially purified fraction and glibenclamide on erythrocytes MDA release and thiobarbituric acid reactive substances in diabetic -hyperlipidemic rats after 28 days of treatment.

Groups	Erythrocytes MDA release (percent)	MDA content (nmole/g Hb)
NC[§]	13.5±0.2	6.1±0.1
NC+PE[§]	14.4±1.2	6.5±0.7
DC[§§]	57.5±0.8 (+76.5 %)[a]	15.4±0.6 (+152.0%)[a]
CT-1[§§]	25.9±0.3 (-54.9 %)[b]	6.5±0.2 (-57.2 %)[b]
CT-2[§§]	44.6±0.7 (-22.4 %)[b]	7.3±0.4 (-52.2 %)[b]
PET-1[§§]	46.2±0.8 (-19.7 %)[b]	9.6±0.1 (-37.7 %)[b]
PET-2[§§]	49.3±1.2 (-14.3 %)[b]	11.1±0.1 (-26.6 %)[b]
D-GT[§§]	48.1±0.4 (-16.3 %)[b]	12.9±0.2 (-15.9 %)[b]

[§]Values are mean (µmole/dl) ± SD from 5 rats in NC and NC+PE. [§§]Values are mean (µmole /dl) ± SD from 4 rats in DC, CT-1, CT-2, PET-1, PET-2 and D-GT. Significantly different from NC at [a]p<0.001, Significantly different from DC at [b]p<0.001

Table 4: Impact of *P. virgatus* methanolic extract, it's partially purified fraction and glibenclamide on liver and kidney conjugated dienes, lipid hydroperoxiodes and thiobarbituric acid reactive substances in diabetic-hyperlipidemic rats after 28 days of treatment.

Group	Conjugated dienes		Lipid hydroperoxides		TBARS	
	Liver	Kidney	Liver	Kidney	Liver	Kidney
NC[§]	5.4±0.2	2.3±1.2	2.1±0.3	4.9±0.7	4.7±0.8	2.1±1.1
NC+PE[§]	5.5±0.1	2.3±0.9	2.2±0.6	4.6±0.8	4.1±0.2	2.0±0.9
DC[§§]	11.5±0.4 (+112.6 %)[a]	2.9±0.7 (+27.9 %)[a]	4.2±0.7 (+97.7 %)[a]	6.6±0.6 (-36.6%)[a]	6.7±1.2 (+44.0 %)[a]	3.5±0.4 (+67.3 %)[a]
CT-1[§§]	5.7±0.8 (-50.3 %)[b]	2.4±0.4 (-29.5 %)[a]	3.3±0.3 (-22.2 %)[b]	4.7±0.9 (-30.0 %)[b]	2.5±0.7 (-62.3%)[b]	2.0±0.7 (-44.9%)[b]
CT-2[§§]	6.2±0.3 (-45.6 %)[b]	2.4±0.4 (-16.8 %)[b]	3.6±0.5 (-14.7 %)[b]	4.9±0.9 (-25.3 %) [b]	3.3±0.4 (-50.4%)[b]	3.8±0.8 (-7.6%)[b]
PET-1[§§]	5.9±0.6 (-48.6 %)[b]	2.3±0.5 (-27.9 %)[b]	3.4±0.7 (-17.5 %)[b]	4.8±1.4 (-26.8 %)[b]	3.0±0.7 (-55.4 %)[b]	3.0±0.7 (-15.2%)[b]
PET-2[§§]	6.7±0.8 (-41.1 %)[b]	2.6±0.4 (-11.1 %)[b]	3.8±0.4 (-9.7 %)[b]	5.4±0.2 (-19.2 %)[b]	4.4±0.4 (-34.1 %)[b]	4.2±0.9 (-18.9 %)[b]
D-GT[§§]	8.9±0.4 (-22.3%)[b]	2.6±0.6 (-12.4%)[b]	3.4±0.6 (-19.6%)[b]	5.8±0.9 (-11.8%)[b]	5.7±0.5 (-14.8%)[b]	3.7±1.0 (-7.1%)[b]

Table 5: Regulatory effect of *P. virgatus* methanolic extract, it´s partially purified fraction and glibenclamide on hepatic and kidney glutathione per oxidase, glutathione reductase and glutathione-s-transferase activities in diabetic-hyperlipidemic rats after 28 days of treatment.

Group	GPx (U/mg of protein)#		Gred (nM/mg of protein)‡		GST (U/mg of protein)^	
	Liver	Kidney	Liver	Kidney	Liver	Kidney
NC[§]	13.4±0.1	7.4±0.2	7.6±0.2	51.4±0.1	8.0±0.9	6.0 ±2.4
NC+PE[§]	14.1±0.4	7.8±0.3	7.7±0.9	52.7±0.5	8.8±0.5	6.1±0.9
DC[§§]	4.4±0.7 (-66.6 %) [a]	6.4±0.9 (-13.6 %)[b]	5.0±0.2 (-34.1 %)[a]	38.2±0.7 (-25.6%)[a]	4.2±0.3 (-47.2 %) [a]	3.4 ±0.5 (-44.2%)[a]
CT-1[§§]	6.7±0.9 (+51.1 %)[c]	7. 3±0.4 (+13.4 %)[c]	7.5±0.5 (+50.1 %)[c]	48.5±0.8 (+26.0%)[c]	6.2±0.5 (+48.5 %)[c]	5.8±0.4 (+73.5%)[c]
CT-2[§§]	6.2±0.6 (+39 %)[c]	6.9±0.5 (+7.5 %)[d]	6.9±0.1 (+38.9 %)[c]	45.3 ±0.4 (+18.5%)[c]	4.8±0.6 (+15.6 %)[c]	4.5±0.3 (+33.03%)[c]
PET-1[§§]	4.9±0.7 (+10.9 %)[d]	7.1±0.4 (+10.6 %)[c]	6.6±0.2 (+33.5 %)[c]	44.2±0.7 (-15.6%)[c]	5.5±0.5 (+30.7%)[c]	4.8±0.2 (+43.7%)[c]
PET-2[§§]	4.9±0.5 (+9.7 %)[d]	6.5±0.7 (+1.8 %)[d]	6.2±0.2 (+23.1%)[c]	41.6±0.6 (-8.8%)[d]	4.9±0.2 (+16.1%)[c]	4.0±0.1 (+18.98%)[c]
D-GT[§§]	5.1±0.3 (+15.8 %)[c]	6.6±0.2 (+2.5 %) [d]	5.9±0.3 (+19.1 %)[c]	41.5±0.3 (+8.6%)[d]	4.7±0.1 (+10.9%)[d]	3.6±0.1 (+7.59%)[d]

#One unit of enzyme activity is defined as nmole oxidized glutathione formed/min/mg homogenate protein. ‡One unit of enzyme activity is defined as nmole NADPH oxidized/min/mg PMS protein. ^One unit of enzyme activity is defined as the nmole of 1-chloro 2, 4-dinitrobenzene (CDNB) conjugate formed/min/mg PMS protein. [§]Values are mean ± SD from liver/kidney PMS fraction of 5 rats in NC and NC+PE. [§§]Values are mean ± SD from liver/kidney PMS fraction of 4 rats in DC, CT-1, CT-2, PET-1, PET-2 and D-GT. Significantly different from NC at [a]$p<0.001$ and [b]$p<0.01$. Significantly different from DC at [c]$p<0.001$ and non-significant from DC at [d]$p>0.05$.

STATISTICAL ANALYSIS

For all assays, samples were analyzed in triplicate and the results were expressed as mean ± S.D. The results were evaluated by using one-way analysis of variance (ANOVA) and two tailed Student's t-test (Statgraphics, Origin6.1). Statistical significance were expressed as *P<0.05, **P<0.01 and ***P<0.001.

RESULTS

In vivo Antioxidative and antilipoperoxidative effect of P. virgatus methanolic extract and its partially isolated fraction in STZ-induced diabetic stressed rats

The data summarized in table 2 demonstrated the impact of *P. virgatus* methanolic extract, it´s partially purified fraction and glibenclamide on total antioxidants, CD, LHPO and TBARS in the plasma of diabetic-hyperlipidemic rats. Twenty eight days after induction of diabetes in rats, total plasma antioxidant levels were reduced from a control value of 1476 to 988 (33%) µmole/dl. Treatment of diabetic-hyperlipidemic rats with *P. virgatus* methanolic extract and it´s partially purified fraction for 28 days resulted in an increase of total antioxidant levels in CT-1, PET-1, CT-2 and PET-2 groups by 24%, 13%, 23% and 10%, respectively, which is significantly higher than values in DC. The oxidative stress induced by diabetes significantly elevated the lipid per oxidation products in plasma (i.e. CD, LHPO and MDA) from 3.23, 1.22 and 1.40 to 4.36 (43%), 1.94 (59 %) and 2.13 (51%) µmole/dl, respectively, in DC rats. A significant decline of 35%, 34% and 31.4% was observed in the formation of CD, LHPO and MDA, respectively, in CT-1, when compared to DC rats. Similarly, a reduction of 26%, 30% and 24% in CT-2, 32%, 34% and 26% in PET-1 and 27%, 28% and 16% was seen in PET-2. These results demonstrate that CT-1 act as a potent antioxidant in diabetic-hyperlipidemic rats. In addition, the results also show that the feeding of glibenclamide (D-GT) to diabetic rats, has contributed an increase in plasma total antioxidant levels.

Table 6: Impact of *P.virgatus* methanolic extract, it´s partially purified fraction and glibenclamide on hepatic glutathione in diabetic-hyperlipidemic rats after 28 days of treatment.

Group	GSH (nmole/mg of protein) Liver
NC[§]	13.3±1.2
NC+PE[§]	11.5±0.5
DC[§§]	3.4±0.8 (-74.3%) [a]
CT-1[§§]	6.7±0.4 (+98.2%) [b]
CT-2[§§]	5.4±0.4 (+58.5%) [b]
PET-1[§§]	5.2±0.1 (+52.1%) [b]
PET-2[§§]	4.4±0.2 (+29.2%) [c]
D-GT[§§]	4.4±0.9 (+27.8%) [c]

[§]Values are mean (nmole SH group/mg protein)± SD from liver homogenate of 5 rats in and NC+PE. [§§]Values are mean (nmole SH group/mg protein)± SD from liver homogenate of 4 rats in D-C, CT-1, CT-2, PET-1, PET-2 and D-GT. Significantly different from N-C at [a]$p<0.001$. Significantly different from D-C at [b]$p<0.001$and [c]$p<0.01$.

Since, it is well known that diabetes leads to membrane lipid per oxidative damage in erythrocytes *in vivo*, we examined the basis of this membrane damage by the estimation of MDA, an end product of fatty acid per oxidation. As depicted in table 3, erythrocytes from DC group showed a greater susceptibility to hydrogen peroxide (H_2O_2) induced lipid per oxidation when compared to NC group. A significant increase of 77 % in the release of MDA in DC was observed, when compared to NC value. The MDA formation was markedly decreased by 55%, 22%, 20% and 14% after the treatment of CT-1, CT-2, PET-1and PET-2 for 28 days, when compared to the value in DC. Similarly, MDA content of erythocytes was increased from 6.1 in NC to 15.4 (153%) in DC. A decrease of 57%, 52%, 38% and 27% in MDA content was seen in diabetic rats treated with CT-1, CT-2, PET-1and PET-2 respectively, for 28 days, when compared to DC rats. The above result illustrate that diabetic rats showed an increase in erythrocytes membrane lipid per oxidation which was significantly reduced by the administration of partially isolated fraction of *P. virgatus* methanolic extract.

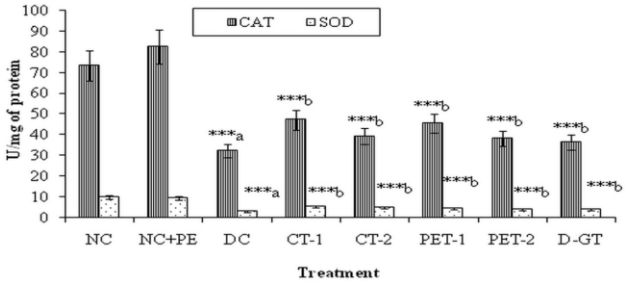


Fig. 1: Effect of *P. virgatus* methanolic extract, it´s partially purified fraction and glibenclamide on the regulation of hepatic CAT and SOD activities in diabetic-stressed rats after 28 days of treatment.

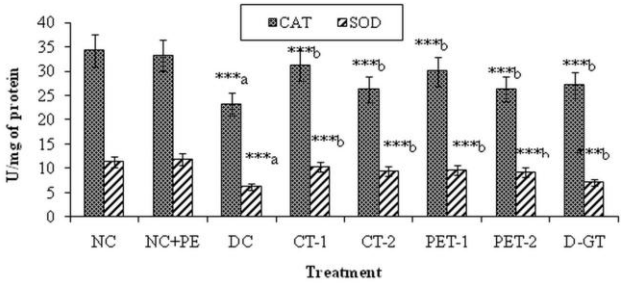


Fig. 2: Impact of *P. virgatus* methanolic extract, it´s partially purified fraction and glibenclamide on the regulation of kidney CAT and SOD activities in diabetic-stressed rats after 28 days of treatment.

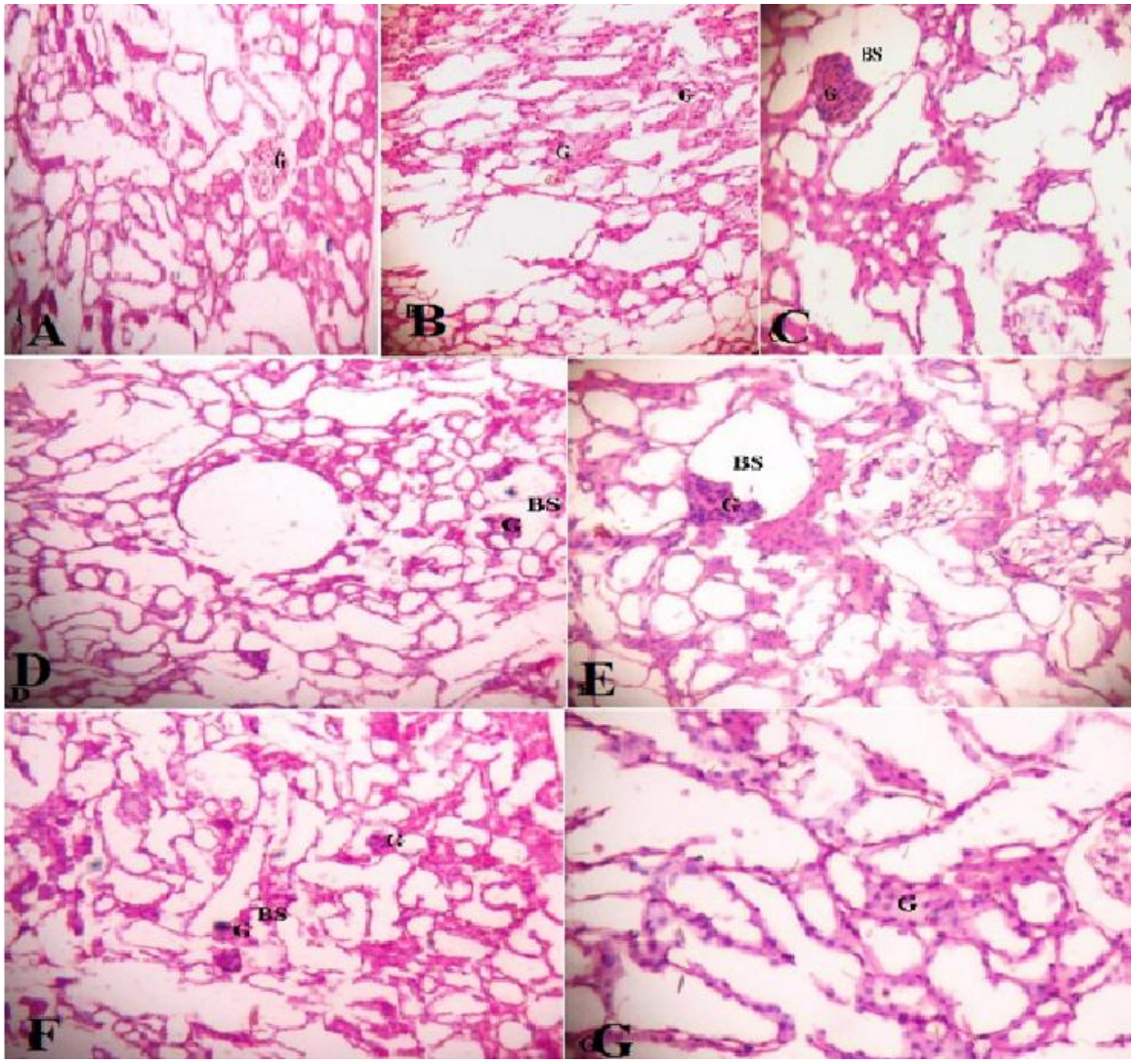
Further, as represented in table 4, formation of CD in liver and kidney was increased from 5.40 and 2.33 units/mg in NC to 11.5 (113%) and 2.98 (28%) units/mg, respectively, in DC. These values in liver and kidney were reduced by 50% and 30 % in CT-1, 47% and 17% in CT-2, 49% and 28% in PET-1 and 41% and 11% in PET-2 respectively, when compared to the values in DC rats. Similarly, in DC, the LHPO and TBARS formation in liver were elevated by 98% and 44 %, respectively, whereas, kidney LHPO and TBARS levels were elevated by 37% and 67%, respectively, in comparison to NC values. Subsequent to treatment of diabetic rats with *P. virgatus* methanolic extract and its partially isolated fraction, formation of LHPO and TBARS levels in liver and kidney was significantly decreased with maximum reduction observed in CT-1, when compared to the corresponding values in DC rats. Above results demonstrated that in diabetic-hyperlipidemic rats there was a significant increase in the formation of lipid per oxidation products in liver and kidney, which was markedly attenuated after treatment with partially isolated fraction of *P. virgatus*.

Impact on the regulation of hepatic catalase, super oxide dismutase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase activities

Since, it is well known that diabetes leads to excessive generation of ROS such as super oxide radical, hydroxyl radical, hydrogen peroxide and/or deficiency in the antioxidant defense systems, which has been attributed to protein glycation and/or glucose auto oxidation due to hyperglycemic environment. Due to insufficient radical scavenger function there has been decreased activity of enzymatic and nonenzymatic scavengers of free radicals. Thus, the determination of antioxidant enzymes, such as CAT, SOD, Gpx, GST and Gred including GSH concentrations in liver and kidney of experimental diabetic-hyperlipidemic rats are highly important.

Hepatic CAT, SOD, Gpx, GST and Gred regulation after the administration of *P. virgatus* methanolic extract and its partially isolated fraction showed a significant change in comparison to NC as shown in fig. 1 and table 5. An increase of 47% in CT-1, 22% in CT-2, 41% in PET-1 and 19% in PET-2 was observed in the hepatic CAT activity of diabetic rats after 28 days of repeated administration of *P. virgatus* methanolic extract and its partially isolated fraction. However, in comparison to NC values, the decline in hepatic SOD activity in diabetic rats was 69%. Administration of *P. virgatus* methanolic extract and its partially isolated fraction to diabetic rats resulted in a marked increase in hepatic SOD activity by 68%, 54%, 34% and 32% in CT-1, CT-2, PET-1 and PET-2 respectively, when compared to DC value (fig. 1).

In diabetic rats, the enzymatic activities of Gpx, Gred and GST were significantly decreased by 67%, 34% and 47 %, respectively, in comparison to NC values (table 5). As evident, after 28 days of treatment with partially isolated fraction (CT-1) resulted in a significant increase of 51 %, 50% and 49% in Gpx, Gred and GST activities, respectively, when compared to the corresponding activities in DC rats. Further, CT-2 rats showed increase of 39%, 39% and 16%. Similarly, in PET-1 and PET-2



(A) In normal rat, the kidney architectures were well preserved. Normal glomerular (G) lobules with no thickening of basement membrane were observed. (B) In DC rat, progression of diffused nodular glomerulosclerosis and capillaries with thick basement membrane with enlarged Bowman space (BS). (C) In CT-1 treated rat, the kidney architectures were relatively well preserved and regression in glomerulosclerosis and normalization of basement membrane. (D) In CT-2 treated rat showed restoration to the normal form with moderate thinning of the glomerular basement membrane. (E and F) PET-1 and PET-2 treated rat exhibited partial regression in glomerulosclerosis and normalization of Bowman space (G) In D-GT rat, the kidney architectures were well preserved.

Fig. 3: Histopathologic changes of kidney (H&E stain) under light microscope at 40X.

rats, the increase in Gpx, Gred and GST were significantly increased by 11%, 34%, 31% and 10%, 23% and 16% respectively, in comparison to DC values. Further, after 28 days of treatment with glibenclamide resulted in a significant increase of 16%, 19% and 11% in Gpx, Gred and GST activities, respectively, when compared to the corresponding activities in DC rats. In summary, hepatic CAT, SOD, Gpx, Gred and GST enzymes, which constitute an antioxidant defence system, are significantly decreased in diabetic-hyperlipidemic rats. However, feeding of *P. virgatus* methanolic extract and its partially isolated fraction substantially scavenges these free radicals, which intern normalizing the above enzyme levels closed to normal values.

Impact on the regulation of Kidney catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase activities

Similarly, kidney is one of the vital organs, which gets affected due to uncontrolled regulation of blood glucose level, undergoes oxidative stress in a similar fashion as in the hepatic antioxidant enzyme system. After 28 days of oral administration of *P. virgatus* methanolic extract and its partially isolated fraction kidney showed a significant change in CAT and SOD activity comparison to NC as shown in fig. 2. An increase of 35% in CT-1, 13% in CT-2, 29% in PET-1 and 13% in PET-2 was observed in the activity of CAT in kidney of diabetic rats after 28 days of repeated administration of *P. virgatus* methanolic extract

and its partially isolated fraction. However, in comparison to NC values, the decline in kidney SOD activity in diabetic rats was 44%. Administration of *P. virgatus* methanolic extract and its partially isolated fraction to diabetic rats resulted in a significant increase in kidney SOD activity by 66%, 53%, 55% and 48% in CT-1, CT-2, PET-1 and PET-2, respectively, when compared to DC values. table 5 summarizes the results of Gpx, Gred and GST activities in kidney of diabetic rats after 28 days of treatment. In diabetic rats, the enzymatic activities of Gpx, Gred and GST were significantly decreased by 1%, 26% and 44%, respectively, in comparison to NC values. As evident, after 28 days of treatment with partially isolated fraction (CT-1) resulted in a significant increase of 13 %, 26 % and 74% in Gpx, Gred and GST activities, respectively, when compared to DC group. Further, CT-2 rats showed increase of 8%, 19% and 33%. Similarly in PET-1 and PET-2 rats, the increase in Gpx, Gred and GST were decreased by 1%, 1%, 44% and 2%, 9% and 19 % respectively, in comparison to DC values. Further, after 28 days of treatment with glibenclamide resulted in an increase of 3%, 9% and 8% in Gpx, Gred and GST activities, respectively, when compared to the corresponding activities in DC rats.

Effect on hepatic glutathione content

The content of glutathione (GSH) was determined after repeated oral administration of *P. virgatus* methanolic extract and its partially isolated fraction to diabetic rats for 28 days as shown in (table 6). The results demonstrated that the GSH content was drastically reduced in STZ induced rats when compared to normal rats and the reduction was up to 73%. After 28 days of treatment, the content of glutathione in liver was markedly increased by 98% in CT-1 group followed by CT-2 (59%), PET-1 (52%) and PET-2 (29%).

Histological studies in kidney and eyeball of STZ-Induced diabetic rats without and with 28 days of P. virgatus methanolic extract, it´s partially purified fraction and glibenclamide treatment

Histopathological studies of kidney was determined after 28 days of oral administration of different doses of *P. virgatus* methanol extract and its partially purified fraction and glibenclamide in STZ-induced diabetic rats. As depicted in fig. 3A, photomicrograph of kidney from NC rats showed normal glomerular lobules with no thickening of basement membrane. The photomicrograph of kidney from diabetic rats depicted progression of diffused nodular glomerulosclerosis and capillaries with thick basement membrane. In addition, an increase in the number of mesengial cells and enlarged bowman space was also seen, when compared to the kidney from normal rats (fig. 3B). After 28 days of *P. virgatus* methanol extract and its partially purified fraction treatment, diabetic kidney showed a significant regression in glomerulosclerosis and normalization of basement membrane in CT-1 at a dose of 0.625 mg/kg rat (fig. 3C), while, rats in CT-2 group (0.125mg/kg) showed partial restoration to the normal form with moderate thinning of glomerular basement membrane (fig. 3D).

Moreover, PET-1 (50 mg/rat/day) and PET-2 (fig. 3E, 3F) treated rat exhibited partial regression in the glomerulosclerosis and normalization of bowman space. Kidney histopathology of glibenclamide treated group rats did not show any inflamed blood vessel and the bowman capsule is similar to the normal control group rat (fig. 3G). Further, a NC retina showed intact retinal membrane with normal epithelium (Ep), stroma (St) and endothelium (fig. 4A). In contrast, cytoplasmic vacuolations of the epithelial cell and separation of collagen fibrils (s) of retina from diabetic rats was seen (fig. 4B). Feeding of CT-1, CT-2, PET-1 and PET-2, respectively, to diabetic rats prevented these changes (fig. 4C,D,E and F). Treated diabetic group shows more or less normal histological appearance of the corneal epithelium (E), stroma (St) and endothelium. As depicted in fig. 4D, CT-2 treated diabetic rats, shows collagen separation while PET-1 and PET-2 treated diabetic rats exhibited normal epithelium and slight separation of collagen. In glibenclamide administrated rats eye architecture was well preserved (fig. 4G).

DISCUSSION

Since, we previously demonstrated the role of *P. virgatus* methanolic extract and its partially isolated fraction in alleviating the condition of hyperglycemia in STZ-induced diabetic rats, this current study was intended to evaluate the protective role of these fractions in STZ-induced oxidative stressed-diabetic rats. STZ has been chosen as an agent that induces hyperglycemia due to its selective destruction of pancreatic β-cell and DNA alkylation by entering into β-cell via GLUT-2 transporter, thus induces the activation of poly ADP-ribosylation which in turn leads to depletion of cellular NAD^+ and ATP. Therefore, increased dephospholrylation of ATP supplies substrate for xanthin oxidase resulting in generation of free radicals (Lukic *et al.*, 1998). Hyperglycemia has been known to depress natural antioxidant system and enhance oxidative stress via increased lipid peroxidation and carbonyl stress that has been well documented in experimental and human DM (Baynes, 1991; Baynes and Thorpe, 1997). Thus, oxidative stress induced by lipid peroxidation is one of the characteristic features of chronic uncontrolled diabetes. Concomitant with these reports, our data also showed an increase in plasma CD, LHPO and MDA levels in STZ-induced diabetic-stressed rats. The increase in plasma lipid peroxidation product is closely associated with significant decline in plasma total antioxidant level. Administration of *P. virgatus* methanolic extract and its partially purified fraction significantly decreased the level

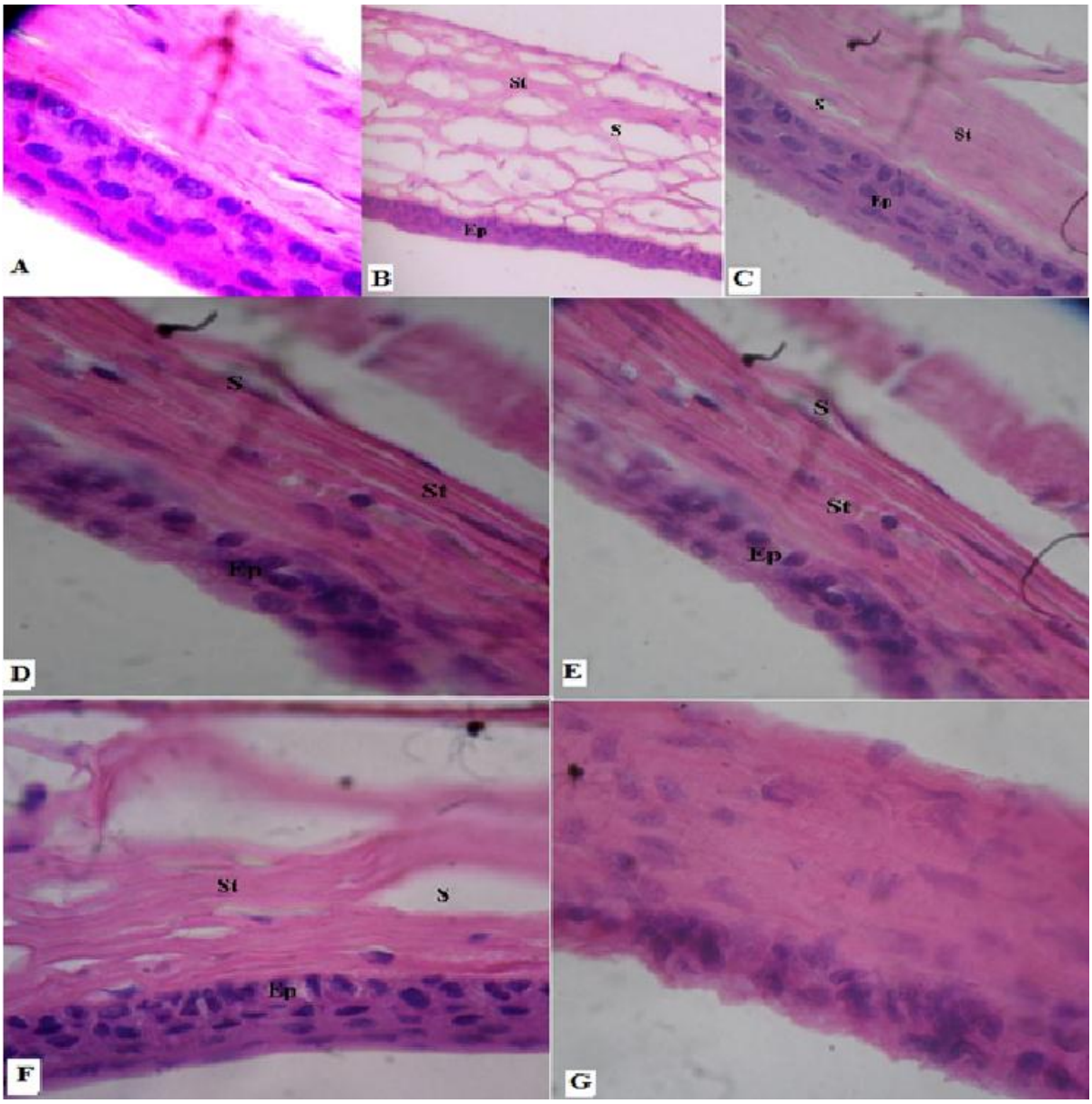


Fig. 4: Histopathologic changes of eyeball (H&E stain) under light microscope at 40X. A) Corneal sections of control group showing normal epithelium (Ep) and stroma (St). B) diabetic group showing cytoplasmic vacuolations of the epithelial cells and separation of collagen fibrils (S). C) CT-1 treated diabetic group showing more or less normal histological appearance of the corneal epithelium (Ep) and stroma (St). D) CT-2 treated diabetic group shows separation of collagen. (E and F) PET-1 and PET-2 treated exhibited normal epithelium and slight separation of the collagen after induction of DM with mild epithelial cell vacuolation and (G) eyeball architecture were well preserved.

of lipid peroxidation byproducts and increased the total antioxidant power in treated diabetic rats which could be due to improved glycemic control (Seghrouchi *et al.*, 2002).

Erythrocytes of diabetic patients are exposed to continuous oxidative stress because oxygen radicals are continuously generated by auto-oxidation of hemoglobin (Mills, 1957) and in turn attack the polyunsaturated fatty acid (PUFA) of lipid membrane, which results in MDA accumulation. Our results show a greater susceptibility of H_2O_2 induced lipid per oxidation in erythrocytes of diabetic rats than those from normal rats indicating massive oxidative stress in STZ-induced rats (Rajeswari *et al.*, 1991). Furthermore, *in vitro* MDA release from erythrocytes were significantly blocked in all the treated groups, with the maximum reduction observed in CT-1 group followed by CT-2, PET-1 and PET-2 groups. Similar to increase in plasma lipid peroxidation products, liver and kidney lipid peroxidation products were also

significantly increased in STZ-induced diabetic-stressed rats. These results illustrated that treatment with partially purified fraction at higher dose (CT-1) causes significant reduction in the level of MDA, CD and LHPO formation in liver and kidney tissues followed by CT-2, PET-1 and PET-2 groups. *P. virgatus* and its partially purified compound are known previously for its antioxidant (Hashim *et al*., 2013; Hashim *et al.*, 2014; Shabeer *et al*., 2009) and antiperoxidative properties which might be due to presence of hexadecanoic acid, 9,12-octadecinoic acid and linolenic acid (Hashim *et al.*, 2013; Hashim *et al.*, 2014; Sermakkani and Thangapandian, 2012). In agreement with these reports, the decreased level of lipid per oxidation products in this study was apparently due to increase consumption of antioxidant. Moreover, our results showed inverse association between lipid peri oxidation product and total antioxidant level in various treated group (CT-1, CT-2, PET-1 and PET-2), which further supports the potent antioxidant role of *P. virgatus* methanolic extract and its partially purified fraction (Shabeer *et al.*, 2009).

Our results indicating the erythrocytes membrane and tissue damage of STZ-induced diabetic rats are in consensus with previously published reports (Gutteridge, 1995; Kolanjiappan *et al.*, 2002). However, the exact mechanism by which increased blood glucose leads to lipid per oxidation of erythrocytes and tissues of diabetic subject is not well known. Though based on our results it seems plausible that free radicals formed over and above the detoxification capacity can attack the membrane phospholipid and causes lipid per oxidation. This free radical mediated per oxidation of membrane fatty acid is significantly blocked in STZ-induced diabetic rats treated with antioxidant *P. virgatus* and its bioactive compounds. The mechanism that relates diabetes and ROS generation is the increased oxidative stress due to high glucose concentration that includes auto oxidation of glucose, protein glycation as well as enhanced production of advanced glycosylation end products and activation of polyol pathway (Lorenzi, 2007). Vital organs are capable for antioxidant defense mechanism by their concerted action of both antioxidant enzyme and nonenzymatic antioxidants. Consensus with these reports, we observed a decrease in level of enzymatic (SOD, CAT and GSH-Px) and non-enzymatic (GSH) antioxidants in STZ-induced diabetic stressed rats with the subsequent alleviation of lipid peroxide level in liver and kidney tissue (Baynes and Thorpe, 1997).

In diabetes, the activity of GPx is significantly decreased by superoxide radical and by glycation reactions. During diabetes, there is a decrease in the concentration of GSH that reduced the activities of GPx. As NADPH, necessary for GSH regeneration, was utilized by the polyol pathway which is prominent in chronic hyperglycemic conditions, there occurs a depletion of GSH resulting in lowered GPx activity (Rathore *et al.*, 2000). Similarly, GST activity was reduced due to the reduction of GSH because it acts as a substrate for the GST activity (Srinivasan and Pari, 2012). In addition, SOD and CAT eliminate the toxic free radicals induced by STZ. The reduced activity of hepatic and renal SOD and CAT in diabetic rats is in agreement with previous report (Onozato *et al.*, 2002). Activities of enzymatic antioxidants have been observed to decrease during diabetes and this may result in a number of deleterious effects due to the accumulation of free radicals. The activities of hepatic and renal GPx, Gred, GST, CAT and SOD enzymes level were significantly increased by 50%, 51%, 49%, 47%, 68% and 13% , 27%, 74%, 35%, 66%, respectively, after treatment with CT-1 in STZ-induced diabetic rats followed by CT-2, PET-1 and PET-2 groups, respectively. These results revealed that CT-1 exhibited a better free radical scavenging activity and prevent pathological alteration caused by hydroxyl radicals and are in concordant with previously published report from same species and other plants (Shabeer *et al.*, 2009; Garg and Bansal, 2000). These findings concluded that among all the treated groups CT-1 significantly ameliorate the enzymatic and non enzymatic antioxidant levels.

Long term damage, dysfunction and kidney failure are the major secondary complications of DM that results in end stage renal diseases (Onozato *et al.*, 2002). Our study was carried out in STZ induced diabetic rats that are seen to have similar early stage human diabetic–associated disorders of kidney (Kiran *et al.*, 2012). However the histological evaluation of present study shows significant messengial cell proliferation, reduction in bowman capsule spacing and thinning of membrane of bowman capsule in DC group as compared to NC group. The primary function of the glomeruli is to help in ultra filteration of the plasma and thus helping in maintenance of fluid and electrolyte homeostasis. These results are in concordant with the report of Kiran *et al*. (2012) that exhibited similar finding. Administration of *P. virgatus* methanolic extract and its partially purified fraction improves to architecture of kidney and helps restoring its functionality. The group administered with CT-1 at a dose of 0.5mg/rat/day demonstrated a distinct regenerative capacity over the other treated groups.

Angiogenesis is the key player in vision dysfunction during the process of diabetes retinopathy (Yoshida *et al.*, 2012). Moreover, corneal sections of diabetic rats revealed intense degenerative changes e.g. epithelial cells, stromal spacing, degradation of its collagen fibers and increased corneal thickness. Our data demonstrated that the stromal cells of the diabetic cornea are having darkly stained nuclei and vacuolated cytoplasm which is in agreement with the previous record of other scientists who declared that in diabetes, formation of advanced glycation end products lead to proteins cross linking that caused destruction of intracellular organelles. Along with it, stromal vascularization was also detected in diabetic

animals. Corneal neovascularization in diabetes was recorded by some investigators who suggested that the presence of stromal edema might allow blood vessels into compact stroma (Chang *et al.*, 2001). Treatment for over 28 days with *P. virgatus* methanolic extract and its partially isolated fraction (CT-1) showed significant restoration of the histological features of the cornea and there was a decrease in the thickness of the corneal layer in the entire treated group. Although glibenclamide, a known antidiabetic agent, which is identified to exert a host of side effects (Moller, 2001) also showed some protection against oxidative stress induced by STZ but are not comparable to protection exhibited by *P. virgatus* methanolic extracts and its bioactive compound.

CONCLUSION

The present results are in agreement and continuation of our previously published data that showed *in vitro* antioxidant and *in vivo* hypoglycemic activity (Hashim *et al.*, 2013; Hashim *et al.*, 2014) of *P. virgatus* methanolic extract and its partially purified fraction. The combined results demonstrated significant protection against STZ-induced oxidative stress, nephropathy and retinopathy condition by *P. virgatus* methanolic extract and its bioactive compound which can be due to its profound antioxidant activity. However, in view of several toxic effects exhibited by glibenclamide and its inadequacy to exhibit pleiotropic therapeutic effects, use of *P. virgatus* methanolic extract or its purified fraction as an effective hypoglycemic, hypolipidemic, antioxidant and antidiabetic agent could be preferred that will be both efficacious and cost effective.

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