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Extraction, characterization and evaluation of *Eruca sativa* against streptozotocin-induced diabetic nephropathy in rat

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Abstract

The present study was aimed to evaluate petroleum ether and hydroalcohol extracts of *Eruca sativa* seeds in the treatment of diabetic nephropathy in the rat model. GC-MS analysis of petroleum ether extract revealed the presence of fatty acids and erucic acid. Quercetin and kaempferol were isolated from the hydroalcohol extract. Diabetes was induced in rat by streptozotocin (65 mg/kg i.p.) 15 min after nicotinamide (230 mg/kg, i.p.) administration. Both the extracts at different doses produced a significant attenuation in the elevated serum glucose level. Elevated renal parameters, lipid and anti-oxidant enzyme levels were restored in a dose-dependent manner. Moreover, both extracts produced significant reduction in the formation of advanced glycation end products in the kidney. These findings suggest that *E. sativa* might inhibit the progression of diabetic nephropathy and could be a therapeutic agent for the management of diabetic nephropathy.

Introduction

Diabetic nephropathy is one of the major complications of diabetes mellitus. If left untreated, 20-40% of diabetic patients with microalbuminuria will progress to overt nephropathy and 20% of them will develop end-stage renal failure within 20 years. Diabetic nephropathy is characterized by initial oxidative stress, inflammatory response, thickening of basement membranes, expansion of mesangial matrix and interstitial fibrosis, podocytes and renal cell death, increased albuminuria, and renal dysfunction (Alpers and Hudkins, 2011). Hyperglycemia results in activation of multiple biochemical pathways like glucose flux through the polyol pathway, the hexosamine pathway, excess/inappropriate activation of protein kinase C isoforms and accumulation of advanced glycation end products (AGEs). These activated pathways are the major source of damage and are potential therapeutic targets in diabetic nephropathy (Edwards et al., 2008).

Herbs are the mine of medicinal agents and a large number of medicinal herbs are found to be efficacious, cheap and safe in preventing diabetes and diabetic complications. Moreover, use of herbal medicines for the treatment of diabetic complications is very important in developing countries where the cost of conventional medicines is a burden to the population. Plants like *Canscora decussate, Punica granatum Sesbania sesban* have the protective effect against diabetic nephro -pathy (Irshad et al., 2013; Mestry et al., 2016; Pandhare et la., 2011;).

Eruca sativa Millis (salad rocket) is a diploid annual herbaceous plant growing up to 80 cm (Hedrick, 1972). Leaves are increasingly eaten by humans either alone or as part of mixed salads, and are also used in herbal remedies (Mahran et al., 1991). There is sporadic information available about phytochemistry and bioactivity of this oily crop (Flanders and Abdulkarim, 1985). It is known as a diuretic, anti-inflammatory, antibacterial (Gulfraz et al., 2011) and also acts as an anti-cancer agent (Michael et al., 2011).

Eruca seeds have high oil contents, protein glucosi-

nolate and erucic acid contents and commonly used as animal feed in Asia, particularly in India and Pakistan. The major and structurally unique glucosinolate (GLS) in leaves of *E. sativa* was identified as 4-mercaptobutyl GLS (Kim et al., 2007). Three new quercetin glycosides isolated from leaves of *E. sativa* (Weckerle et al., 2001). Phytochemical investigations of the aqueous extract of *E. sativa* fresh leaves afforded the presence of nine natural flavonoid compounds (Michael et al., 2011). *E. sativa* is known to ameliorate hyperglycemia and also considering the potential flavanoidal moieties present in the plant gives the idea that it can potentially treat diabetes as well as the complications associated with diabetes (El-Missiry and El Gindy, 2000).

So, the present study was undertaken to assess the protective effects of petroleum ether and hydroalcohol (40%) extract of seeds of *E. sativa* in diabetic nephropathy in rats.

Materials and Methods

Animals

Adult male Wistar rats weighing 250-300 g were housed in standard environmental conditions maintained at 23 ± 2°C with 12 hours light-dark cycle. Animals were fed standard rodent diet and water.

Collection of plant material

Seeds of *E. sativa* were procured from a local supplier and identified by Dr. Sunita Garg, NISCAIR, New Delhi. A voucher specimen (NISCAIR/RHMD/Consult/2013/2296/76) was deposited in the herbarium of NISCAIR, New Delhi for future reference. Botanical name of the plant was verified from published literature and database (The Plant List, 2015).

Preparation of extract

The seeds were dried in the shade, powdered and then used for the extraction of potential anti-diabetic constituents into different solvents (petroleum ether, chloroform, ethanol and hydroalcohol). Seeds were sequentially extracted with solvents in order of increasing polarity i.e. petroleum ether 60-80°C, chloroform, ethanol and hydroalcohol (40%) by Soxhlet extraction method. The extraction procedure was continued until the extract gave no coloration. The extracts were distilled and concentrated under reduced pressure and finally freeze dried. Petroleum ether and hydroalcohol extracts were used for further studies.

Chemicals

Streptozotocin (STZ) was obtained from Sigma-Aldrich, Milwaukee, USA and nicotinamide from the Finar India Ltd. Diagnostic kits for the biochemical estimations were obtained from Reckon Diagnostics Pvt. Ltd., India. All the other chemicals used were of analytical grade.

Phytochemical screening

Both extracts were used to test the chemical compounds like alkaloids, carbohydrates, fixed oils and fats, terpenoids, phenols, tannins, glycosides, saponins, proteins, amino acids and flavonoids in accordance with the methods described elsewhere with a slight modification (Trease and Evans, 1989; Harbourne, 1998).

GC-MS analysis

Petroleum ether extract was subjected to GC-MS analysis. Samples were prepared by treating 10 mg of sample with 2 mL hexane followed by the addition of 0.2 mL of 2M methanolic KOH. The tube was vortexed for 2 min at room temperature and after a light centrifugation, aliquot of the hexane layer was collected for GC analysis. The column was held initially at 60°C for 5 min after injection, then the temperature was increased to 140°C with 10°C/min, heating ramp for 20 min and increased to 200°C with 5°C/min heating ramp for 20 min. The temperature was then increased to 220° C with 5°C/min heating ramp for 20 min. Injector temperature 250°C, detector temperature 275°C, carrier gas H_2: inlet pressure 45 psi linear, gas velocity 39 cm/sec, column flow rate 2.4 mL/min; split ratio, 40:1 and injector volume 1 µL.

Isolation of active compounds

Isolation of quercetin from hydroalcohol extract

Hydroalcohol extract (10 g) was subjected to column chromatography on silica gel (100-200 mesh; Merck) eluted with the mixtures of chloroform, ethyl acetate, ethanol and methanol of increasing polarity, to obtain fractions for yellow amorphous powder. Column fractions with ethyl acetate: ethanol (80:20) in the TLC mobile phase solvent ratio of chloroform: methanol (1:1) showed Rf value of 0.46 equal to that of standard quercetin. The fractions were then combined, crystallized and yielded quercetin.

Isolation of kaempferol from hydroalcohol extract

Hydroalcohol extract (10 g) was subjected to column chromatography on silica gel (100-200 mesh; Merck). Elution started with water, followed by increasing concentration of ethanol gradually to obtain 6 fractions. Kaempferol was isolated from the last fraction and was characterized using NMR, mass spectroscopy and literature (Michael et al., 2011).

Experimental design and animals

Adult male Wistar rats, procured from NIPER, Mohali, with initial weights of 280-300 g, were used in this study. Rats were housed under normal conditions with a 12 hours light/dark cycle at 23 ± 1°C and 40% humidity. Animals were fed standard rodent chow and water.

Diabetic nephropathy was induced by STZ i.p. at a dose of 65 mg/kg (dissolved in pH 4.5 citrate buffer imme-

diately before injection), 15 min after nicotinamide (230 mg/kg, i.p.). Induction of the diabetes was confirmed by measuring fasting blood glucose levels 72 hours after STZ injection. The rats with fasting blood glucose level ≥250 mg/dL were included in the study. Different doses of the extracts (100, 200 and 400 mg/kg) were selected on the basis of oral acute toxicity studies reported in the literature (Saleh et al., 2015). As diabetic nephropathy symptoms typically develop after 30 to 45 days so the levels of serum urea, uric acid, creatinine and blood urea nitrogen (BUN) were estimated on day 30. Elevated levels of serum urea, uric acid, creatinine and BUN suggested the development of diabetic nephropathy. After 30 days of STZ induction, treatment with extract and standard was continued for further 45 days.

Animals were divided into nine groups consisting of six rats each. Group I was normal control; Group II was diabetic nephropathy control; Group III-V consisted diabetic nephropathy rats treated with 100, 200, 400 mg/kg petroleum ether extract; Group VI-VIII consisted diabetic nephropathy rats receiving 100, 200, 400 mg/kg hydroalcohol extract; Group IX consisted diabetic nephropathy rats receiving 10 mg/kg glimepride.

Blood glucose level, body weight, lipid profile, renal function tests were estimated on 30th, 45th, 60th and 75th day of STZ induction. Biochemical estimation was carried out using commercially available kits of Reckon Diagnostics Pvt. Ltd. Kidney and pancreas were obtained and stored at -70°C until use.

Body weight and blood glucose estimation

Body weight of each animal was measured before induction of STZ and animals with similar weight were grouped together. Body weight of each group was measured periodically till the end of study. Blood glucose level was estimated after 72 hours of STZ-nicotinamide administration to confirm diabetes. Fasting blood glucose level was estimated at an interval of 15 days by using commercial enzymatic kits purchas-ed from Reckon Diagnostics Pvt. Ltd. (India) through-out the study.

Lipid profiles assay

Serum total cholesterol (TC), triglycerides (TG), low density lipoproteins (LDL), very low density lipoproteins (VLDL) and high density lipoproteins (HDL) were measured by the commercial enzymatic kits purchased from Reckon Diagnostics Pvt. Ltd. INDIA.

Renal function tests

Blood samples were collected on 30th, 45th, 60th, and 75th day from diabetic nephropathy control and treated rats for the estimation of serum creatinine, urea, uric acid and BUN by using commercial enzymatic kits purchased from Reckon Diagnostics Pvt. Ltd. India.

Estimation of anti-oxidant enzyme levels

Tissue (kidney and pancreas) homogenate was used to estimate the thiobarbituric acid reactive substances (TBARS) (Ohkawa et al., 1979) and level of anti-oxidant enzymes, *viz.* superoxide dismutase (SOD) and reduced glutathione (GSH) (Beutler et al., 1963).

Histopathology

Kidney and pancreatic tissues were harvested from the sacrificed animals and fixed in 10% neutral buffered formalin solution, dehydrated in ethanol and embedded in paraffin. Sections of 5 µm thickness were prepared using a rotary microtome and stained with hematoxylin and eosin dye for microscopic observations.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6. Values were expressed as mean ± SEM and one -way analysis of variance (ANOVA) was used for statistical analysis. ANOVA was followed by Tukey's as *post hoc* multiple comparison test. The results were considered significant if $p \leq 0.05$.

Results

Phytochemical screening

Preliminary phytochemical screening of petroleum ether extract showed the presence of terpenoids and fatty acids whereas carbohydrates, polyphenolic compounds, flavonoids and terpenoids were present in the hydroalcohol extract.

Gas chromatography mass spectroscopy analysis

GC-MS analysis was conducted on the crude petroleum ether extract of *E. sativa* seeds. The peaks in the chromatogram were integrated and compared with the database of spectra of known components (NIST-14) stored in the GC-MS library. Phytochemical by GC-MS analysis of the studied *E. sativa* seeds extract revealed the presence of different fatty acids. The flaxseed extract showed 7 major peaks in the GC-MS chromatogram indicating the presence of 7 phytochemical constituents (Table I).

Isolation of quercetin and kaempferol from hydro-alcoholic extract

Quercetin was obtained as a yellow amorphous powder with M.P. 315-17°C. Chemical tests of flavonoids were found positive and significant in hydroalcoholic extract of *E. sativa*. The UV λ_{max} (368 nm) and IR $_{max}\upsilon^{KBr}$ spectrum exhibited peaks (cm^{-1}) at 3291 (-OH), 1658 (C=O, aryl ketonic stretch), 1615 (C-C, aromatic ring stretch), 1349 (O-H bending of phenols), 1240, 1208, 1160, 991, 849, 584 have shown positive assignments for flavonoids. The structure of the quercetin was deter-

Table I

Phytochemicals identified in the petroleum ether extract by GC–MS

Peak	Retention time (min)	Area (%)	Database/NIST 14 Library
1	11.2	2.3	Myristic acid
2	14.0	4.0	Palmitic acid
3	18.4	3.3	Stearic acid
4	38.7	7.2	Linoleic acid
5	41.1	2.2	Linolenic acid
6	54.9	10.8	Erucic acid
7	57	5.9	Oleic acid

mined using ^{1}H NMR, ^{13}C NMR, MS (Table II).

Kaempferol was obtained as a yellow crystalline powder with M.P. 275-77°C. Chemical tests of flavonoids were found positive and significant in hydroalcoholic extract of *E. sativa*. The UV λ_{max} (265 and 280 nm) and IR $_{max}\upsilon^{KBr}$ spectrum exhibited peaks (cm^{-1}) at 32302 (-OH), 1650 (C=O, aryl ketonic stretch), 1617 (C-C, aromatic ring stretch), 1342 (O-H bending of phenols), 1241, 1210, 1165, 998, 846, 581 have shown positive assignments for flavonoids. The structure of the quercetin was determined using ^{1}H NMR, ^{13}C NMR and MS (Table I).

Effect of both extracts on body weight

Diabetic nephropathy control rats showed significant attenuation in body weight during the study (Figure 1). Administration of both extracts at a dose of 100, 200 and 400 mg/kg increased the body weight significantly in a dose-dependent manner. Glimepiride also increased the body weight in rats with diabetic nephropathy.

Effect of both extracts on blood glucose

Administration of different doses of extracts was started after 30 days of STZ administration and continued for up to 75th day. The serum blood glucose level of animals was measured on 30th, 45th, 60th and 75th day of study. The fasting blood glucose level of normal control rats ranged from 93.5 to 95.5 mg/dL in 75 days (Figure 2). Whereas, in diabetic rats fasting glucose level was found to be increased to 522.2 mg/dL from 324.8 mg/dL. Administration of different doses of both extracts (100, 200 and 400 mg/kg) for 45 days, produced significant attenuation in elevated blood glucose level and maximum attenuation was observed with 400 mg/kg of petroleum ether extract (173.8 ± 5.1 mg/dL) and hydroalcohol extract (127.8 ± 1.5 mg/dL) as compared to diabetic nephropathy control rats. Glimepiride treatment also resulted in significant attenuation (130.4 ± 2.9 mg/dL) of blood glucose level in rats.

Effect of both extracts on glycated hemoglobin (HbA1c)

Hb1Ac level was measured at the start of treatment and end of the study. Substantial elevation in Hb1Ac level was observed in untreated diabetic nephropathy control group (8.2 ± 0.1%) in comparison to normal control (4.0 ± 0.03%) on 30th day and the level of Hb1Ac was further increased in diabetic nephropathy rats at the end of study (75th day) to 11.0 ± 0.03% (Data not shown). Administration of 100, 200 and 400 mg/kg of both extracts for 45 days produced significant decreased in Hb1Ac level petroleum ether extract (6.9 ± 0.1, 6.2 ± 0.1, 5.1 ± 0.1% respectively); hydroalcohol extract (6.9 ± 0.1, 6.1 ± 0.1, 5.1 ± 0.03% respectively).

Effect of both extracts on fasting insulin level

A significant decrease in fasting insulin level (5.6 ± 0.2 µU/mL) was observed in STZ-treated diabetic nephropathy rats in comparison to normal control rats (14.8 ± 0.4 µU/mL)(Data not shown). Administration of 100, 200 and 400 mg/kg of both extracts for 45 days produced significant increase in serum insulin level (7.2 ± 0.2, 8.1 ± 0.3, 7.3 ± 0.2 µIU/mL respectively);

Table II

^{1}H and ^{13}C NMR of quercetin and kaempferol isolated from **hydroalcohol extract of *Eruca sativa***

^{1}H NMR of quercetin	^{1}H NMR of kaempferol	^{13}C NMR of quercetin	^{13}C NMR of kaempferol
6.2 (d, 1H, *Ar-H*)	6.2 (d, 1H, *Ar-H*)	93.2, 98.1, 103.0, 115.0,115.4 (*Ar-C*)	93.8, 98.9, 103.2, 115.0, 115.4 (*Ar-C*)
6.4 (d, 1H, *Ar-H*)	6.4 (d, 1H, *Ar-H*)	119.9, 122.0, 135.7 (Ar-C)	115.9, 123.7, 133.7 (Ar-C)
6.9 (d, 1H, *Ar-H*)	6.9 (d, 2H, *Ar-H*)	144.9 (Ar-C)	140.9 (Ar-C)
7.5 (q, 1H, *Ar-H*)	-----	146.6 (Ar-C)	141.6 (Ar-C)
7.7 (d, 1H, *Ar-H*)	8.1 (d, 2H, *Ar-H*)	147.5 (Ar-C)	144.5 (Ar-C)
9.4 (s, 2H, Ar-OH)	8.8 (s, 1H, Ar-OH)	156.1 (Ar-C)	156.4 (Ar-C)
9.6 (s, 1H, *Ar-OH*)	9.7 (s, 1H, *Ar-OH*)	160.7 (Ar-C)	161.3 (Ar-C)
10.9 (s, 1H, Ar-OH)	10.4 (s, 1H, Ar-OH)	163.8 (Ar-C)	163.4 (Ar-C)
12.5 (s, 1H, Ar-OH)	12.3 (s, 1H, Ar-OH)	175.7 (Ar-C=O)	176.6 (Ar-C=O)

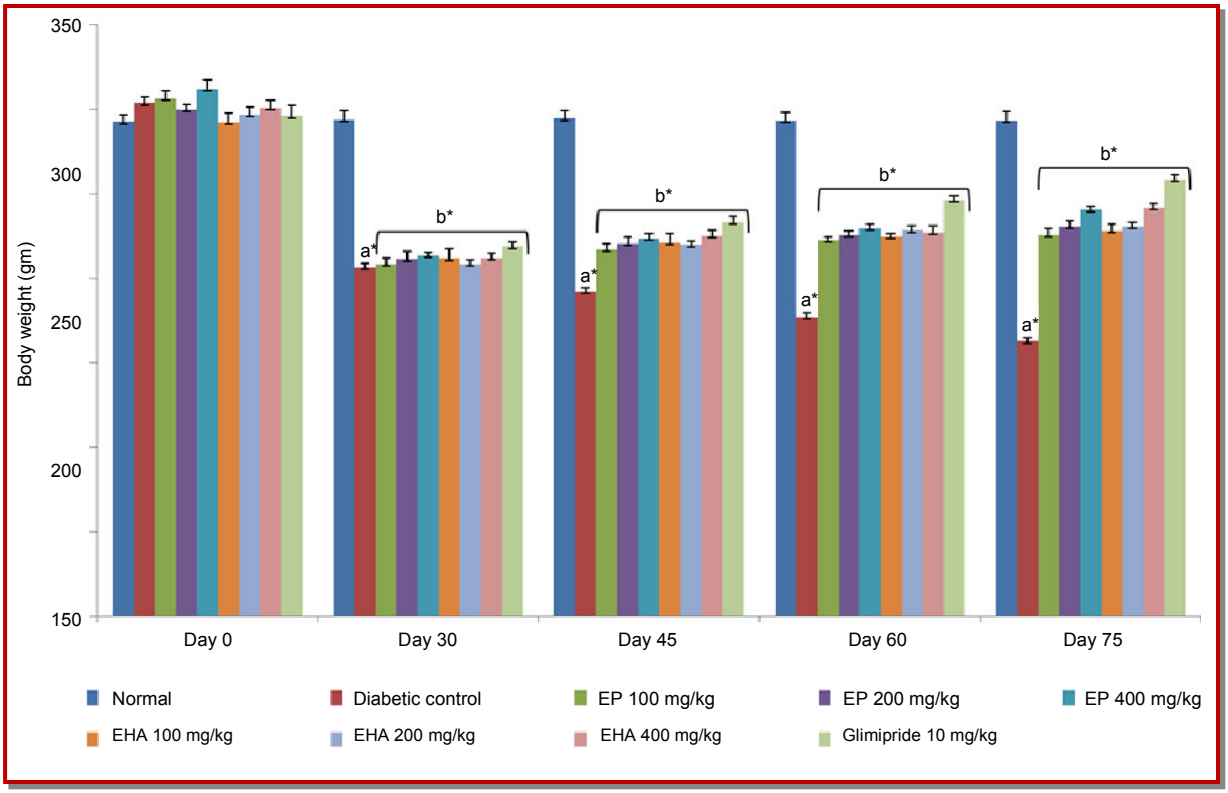

Figure 1: Effect of petroleum ether (EP) and hydroalcohol (EHA) extract on body weight. Data are mean ± SEM; n = 6; Data was analyzed by using one-way ANOVA followed by Tukey's multiple test; 'a' vs control, 'b' vs diabetic control; *p<0.001, #p<0.01, †p<0.05

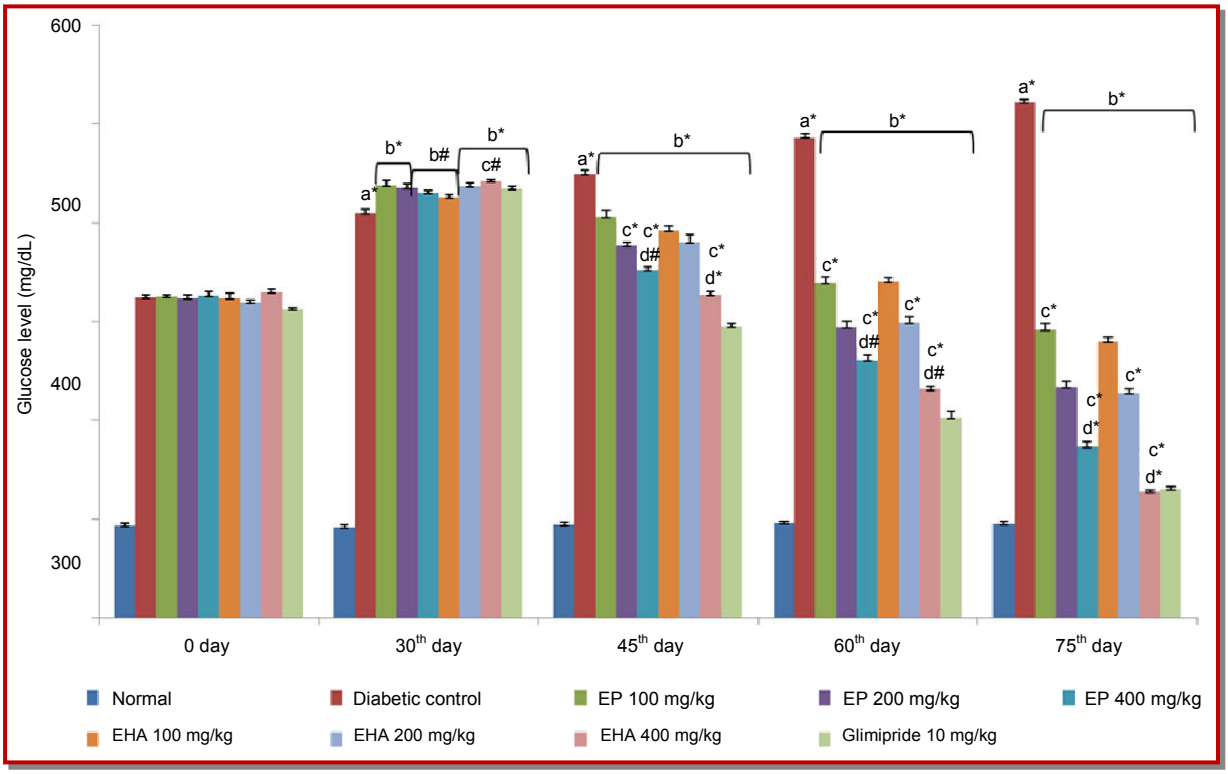

Figure 2: Effect of petroleum ether (EP) and hydroalcohol (EHA) extract on blood glucose level. Data are mean ± SEM; n = 6; Data was analyzed by using one-way ANOVA followed by Tukey's multiple test; 'a' vs control, 'b' vs diabetic control, 'c' vs *E. sativa* extract 100 mg/kg, 'd' vs *E. sativa* extract 200 mg/kg; *p<0.001, #p<0.01, †p<0.05

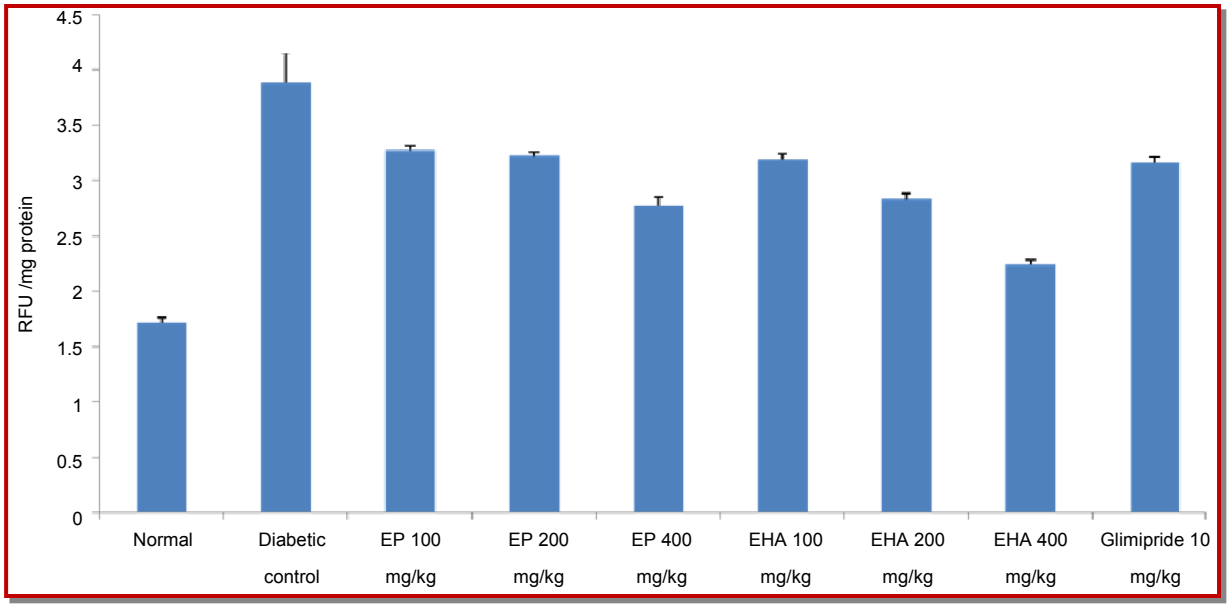

Figure 3: Effect of petroleum ether (EP) and hydroalcohol (EHA) extract on AGE's level (RFU/mg protein). Values are represented as mean ± SEM; (n = 6); Data was analyzed by using one-way ANOVA followed by Tukey's multiple test; 'a' vs control, 'b' vs diabetic control, 'c' vs *E. sativa* extract 100 mg/kg, 'd' vs *E. sativa* extract 200 mg/kg. *p<0.001, #p<0.01, †p<0.05

hydroalcohol extract (8.0 ± 0.3, 7.7 ± 0.2, 8.0 ± 0.3 µIU/mL respectively).

Effect of both extracts on AGEs in kidneys

Induction of diabetic nephropathy in rats led to a significant increase in kidney AGEs levels when compared to normal animals (Figure 3). Administration of 100, 200 and 400 mg/kg of petroleum ether extract and EHA for 45 days produced significant reduced AGEs level (3.3 ± 0.04, 3.2 ± 0.03, 2.8 ± 0.1 RFU/mg protein respectively); hydroalcohol extract (3.2 ± 0.04, 2.8 ± 0.1, 2.2 ± 0.04 RFU/mg protein respectively) compared to control group. However, administration of glimepiride (10 mg/kg) also produced significant change (3.2 ± 0.1 RFU/mg protein) in AGEs levels when compared to control group.

Effect of extracts on renal function

In the present study, there is increased microalbuminuria in diabetic control animals which are significantly attenuated by oral administration of both extracts. Uric acid was found to increase in diabetic nephropathy control rats to a significant extent in comparison to normal control. Administration of petroleum ether and ethanol extracts at 100, 200 and 400 mg/kg significantly reduced the uric acid at 75th day.

In diabetic nephropathy control rats, a significant increase in urea (98.0 mg/dL), uric acid (15.5 mg/dL), creatinine (4.1 mg/dL) and BUN (0.04 mg/dL) levels was observed in STZ-induced diabetic nephropathy rats over the period of study. Treatment of both extracts attenuated the increased renal parameters in a dose-dependent manner. Twelve hours urine volume and urinary creatinine significantly increased in diabetic control rats in comparison to normal control. Treatment with both extracts (100, 200 and 400 mg/kg) significantly reduced the level of urine volume and urine creatinine level. In addition, treatment with extracts significantly improved creatinine clearance (Table III).

Effect of both extracts on lipid profile

Serum total cholesterol (TC), triglyceride (TG), LDL and very low density lipoproteins (VLDL) were found to be significantly increased in diabetic nephropathy rats. Administration of extracts for 45 days resulted in significant decrease in serum TC at doses of 100, 200 and 400 mg/kg *i.e.* 185.6 ± 3.9, 151.1 ± 4.3, 136.3 ± 1.3 mg/dL and 177.3 ± 2.6, 156.9 ± 3.7, 119.7 ± 2.5 mg/dL respectively. The extracts significantly reduced the level of serum TG at doses of 100, 200 and 400 mg/kg, *i.e.* 122.5 ± 1.9, 104.7 ± 1.8, 97.9 ± 1.5, 105.1 ± 1.9, 96.6 ± 2.1, 77.5 ± 0.9 mg/dL respectively. Similarly, levels of LDL and VLDL were also reduced significantly by administration of both extracts and glimepiride in diabetic nephropathy rats. The serum HDL significantly increased i.e. 32.7 ± 0.6, 36.7 ± 1.0 and 39.2 ± 0.9 mg/dL respectively for the diabetic nephropathy rats treated with 100, 200 and 400 mg/kg of petroleum ether extract, whereas hydroalcohol extract (100, 200 and 400 mg/kg) increased HDL level to 33.5 ± 0.3, 37.6 ± 0.2 and 42.1 ± 0.2 mg/dL respectively.

The administration of 100, 200 and 400 mg/kg of both extracts increased the level of serum HDL-cholesterol and decreased the levels of total cholesterol, triglycerides and LDL-cholesterol.

Table III

Effect of pertroleum ether (EP) and hydroalcohol (EHA) extract on renal function and lipid profile on day 75 in diabetic-nephropathy rats

	Renal profile							Lipid profile				
Groups	Urea (mg/dL)	Uric acid (mg/dL)	BUN (mg/dL)	Creatinine (mg/dL)	Urine output (mL)	Urine creatinine (mg/dL)	Creatinine clearance (mL/min/kg)	TC (mg/dL)	TG (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)	HDL (mg/dL)
Normal	33.3 ± 0.6	5.3 ± 0.2	15.4 ± 0.6	0.8 ± 0.0	15.5 ± 0.3	0.1 ± 0.0	3.3 ± 0.2	103.7 ± 2.0	71.1 ± 1.6	33.4 ± 2.2	14.2 ± 0.3	56.1 ± 0.4
Diabetic control	98.0 ± 0.5^{a*}	15.6 ± 0.7^{a*}	45.8 ± 0.9^{a*}	4.1 ± 0.1^{a*}	45.8 ± 0.2^{a*}	0.0 ± 0.1^{a*}	4.79 ± 0.4$^{a\#}$	295.9 ± 2.5^{a*}	202.6 ± 0.8^{a*}	229.1 ± 2.6^{a*}	40.5 ± 0.2^{a*}	54.0 ± 0.4^{a*}
EP 100 mg/kg	65.5 ± 0.4^{b*}	9.5 ± 0.1^{b*}	30.6 ± 1.4^{b*}	2.5 ± 0.1 ^{b*}	32.6 ± 0.2^{b*}	0.1 ± 0.0^{b*}	3.5 ± 0.2	185.6 ± 4.0^{b*}	122.5 ± 1.9^{b*d*}	131.9 ± 4.2^{b*}	21.0 ± 0.4 ^{b*d*}	32.75 ± 0.59 ^{b*}
EP 200 mg/kg	63.5 ± 0.4^{b*}	8.1 ± 0.3 ^{b*}	29.7 ± 0.8^{b*}	2.0 ± 0.1^{b*c*}	28.7 ± 0.2^{b*}	0.1 ± 0.0^{b*}	3.6 ± 0.2	151.1 ± 4.3^{b*c*}	104.7 ± 2.0 ^{b*c*}	95.0 ± 5.0^{b*c*}	19.3 ± 0.4^{b*c*}	36.7 ± 1.0^{b*c*}
EP 400 mg/kg	54.4 ± 0.5^{b*c*d*}	7.9 ± 0.3 ^{b*}	25.4 ± 1.3^{b*c*d*}	1.4 ± 0.0^{b*c*d*}	24.4 ± 0.2 ^{b*c*d*}	0.1 ± 0.0^{b*c*}	4.0 ± 0.1^{b*c*}	136.4 ± 1.0^{b*c*d*}	97.9 ± 0.9 ^{b*c*d*}	73.6 ± 2.0^{b*c*d*}	15.2 ± 0.3^{b*c*d*}	39.2 ± 0.9^{b*c*}
EHA 100 mg/kg	63.1 ± 0.5^{b*}	8.1 ± 0.1^{b*}	29.5 ± 0.4^{b*}	2.3 ± 0.1^{b*}	23.9 ± 0.4^{b*}	0.1 ± 0.0^{b*}	3.1 ± 0.3^{b*}	177.3 ± 2.6 ^{b*}	105.1 ± 1.8 ^{b*d*}	121.3 ± 2.4^{b*}	22.5 ± 0.4^{b*d*}	33.6 ± 0.3^{b*}
EHA 200 mg/kg	53.3 ± 0.8^{b*c*}	7.7 ± 0.2^{b*}	24.9 ± 0.5^{b*c*}	1.8 ± 0.1 $^{b*c\#}$	20.1 ± 0.4^{b*c*}	0.1 ± 0.0$^{b*c\#}$	3.3 ± 0.1^{b*}	156.9 ± 2.7^{b*c*}	96.6 ± 1.8^{b*c*}	99.2 ± 2.5^{b*c*}	20.1 ± 0.4^{b*c*}	37.6 ± 0.2^{b*c*}
EHA 400 mg/kg	42.9 ± 0.8^{b*c*d*}	6.9 ± 0.2^{b*}	20.1 ± 0.3^{b*c*d*}	1.3 ± 0.0^{b*c*d*}	18.1 ± 0.4^{b*c*d*}	0.1 ± 0.0^{b*c*}	4.3 ± 0.3^{b*c*}	119.8 ± 3.2^{b*c*d*}	77.5 ± 1.0 ^{b*c*d*}	68.3 ± 2.6^{b*c*d*}	17.6 ± 0.2^{b*c*d*}	42.1 ± 0.2^{b*c*d*}
Glimepride 10 mg/kg	45.5 ± 0.8^{b*}	7.2 ± 0.1^{b*}	21.3 ± 0.4^{b*}	1.2 ± 0.0^{b*}	21.3 ± 0.4^{b*}	0.1 ± 0.0^{b*}	4.2 ± 0.3^{b*}	124.5 ± 1.4^{b*}	85.7 ± 1.1^{b*}	34.3 ± 1.4^{b*}	14.2 ± 0.3^{b*}	44.3 ± 0.4^{b*}

Each group (n = 6) represents Mean ± SEM. Data was analyzed by using one-way ANOVA followed by Tukey's multiple test; 'a' vs control, 'b' vs Diabetic control, 'c' vs *E. sativa* extract 100 mg/kg, 'd' vs *E. sativa* extract 200 mg/kg. $^{*}p<0.001$, $^{\#}p<0.01$, $^{\dagger}p<0.05$

Table IV

Effect of petroleum ether and hydro-alcohol extracts on the level of anti-oxidant enzymes and lipid peroxidation (TBARS) in diabetic-nephropathy Wistar rats

Parameters	SOD (U/mg protein)			GSH (μM/mg protein)			TBARS (nmol/mg protein)		
Groups	Kidney	Pancreas	Liver	Kidney	Pancreas	Liver	Kidney	Pancreas	Liver
Normal	4.9 (0.1)	4.2 (0.3)	3.7 (0.1)	74.9 (0.6)	69.4 (0.3)	66.2 (0.5)	0.5 (0.01)	0.5 (0.01)	0.4 (0.01)
Diabetic control	1.2 (0.05)^{a*}	1.1 (0.06)^{a*}	1.1 (0.04)^{a*}	38.6 (0.5)^{a*}	40.0 (0.1)^{a*}	41.4 (0.3)^{a*}	3.0 (0.02)^{a*}	2.7 (0.1)^{a*}	2.5 (0.1)^{a*}
EP 100 mg/kg	1.9 (0.02)^{b*}	1.2 (0.02)	1.1 (0.01)	40.7 (0.5)^{b*d*}	41.9 (0.3)	45.5 (0.5)^{b*}	2.6 (0.04)^{b*}	1.9 (0.01)^{b*}	1.6 (0.02)^{b*}
EP 200 mg/kg	2.5 (0.02)^{b*c*}	2.1 (0.04)^{b*c*}	1.7 (0.05)^{b*c*}	46.1 (0.6)^{b*c*}	46.5 (1.2)^{b*c*}	48.6 (0.7)$^{b*d\#}$	2.0 (0.03)^{b*c*}	1.4 (0.03)^{b*c*}	1.4 (0.02)^{b*c†}
EP 400 mg/kg	3.1 (0.02)^{b*c*d*}	2.8 (0.05)^{b*c*d†}	2.8 (0.07)^{b*c*d*}	55.4 (0.5)^{b*c*d*}	52.1 (0.4)^{b*c*d*}	54.8 (0.9)^{b*c*d*}	1.4 (0.02)^{b*c*d*}	1.2 (0.01)$^{b*c*d\#}$	1.2 (0.01)^{b*c*d*}
EHA 100 mg/kg	2.0 (0.00)^{b*}	1.4 (0.02)	1.3 (0.01)	42.7 (0.8)^{b*d*}	44.6 (1.0)	42.1 (0.2)^{b*}	2.3 (0.03)^{b*}	1.8 (0.03)^{b*}	1.5 (0.02)^{b*}
EHA 200 mg/kg	2.8 (0.05)^{b*c*}	2.7 (0.02)^{b*c*}	2.9 (0.05)^{b*c*}	52.9 (1.5)^{b*c*}	49.1 (0.6)^{b*c*}	47.5 (0.6)$^{b\dagger c*}$	1.9 (0.01)^{b*c*}	1.3 (0.03)^{b*c*}	1.3 (0.02)$^{b*c\#}$
EHA 400 mg/kg	3.9 (0.02)^{b*c*d*e†}	3.9 (0.01)$^{b*c*d\#}$	3.6 (0.1)^{b*c*d*}	67.8 (0.5)^{b*c*d*}	60.8 (0.4)^{b*c*d*e*}	61.3 (0.3)^{b*c*d*e*}	1.2 (0.03)^{c*e*d*}	1.0 (0.02)^{b*c*d*}	0.9 (0.03)^{b*c*d*}
Glimepiride 10 mg/kg	3.7 (0.02)^{b*}	3.8 (0.04)^{b*}	3.4 (0.1)^{b*}	58.7 (0.7)^{b*}	53.2 (0.4)^{b*}	52.0 (0.4)^{b*}	1.2 (0.03)^{b*}	1.1 (0.04)^{b*}	1.0 (0.02)^{b*}

Each group (n = 6) represents mean ± SEM (within parenthesis); EP means petroleum ether extract; EHA means hydro-alcohol extract; Data was analyzed by using one-way ANOVA followed by Tukey's multiple test; 'a' vs control, 'b' vs Diabetic control, 'c' vs *E. sativa* extract 100 mg/kg, 'd' vs *E. sativa* extract 200 mg/kg. *p<0.001, #p<0.01, †p<0.05

Effect of both extracts on anti-oxidant enzymes and lipid peroxidation

Reduced glutathione level (GSH) and superoxide dismutase (SOD) was significantly decreased in the kidney, liver and pancreas of diabetic nephropathy rats. Treatment with petroleum ether extract and hydro-alcohol extract for 45 days increased the level of this anti-oxidant enzyme in comparison to diabetic nephropathy control group. The level of TBARS was found to be high in case of diabetic nephropathy control rats in comparison to normal control group. Administration of *E. sativa* extracts reduced the formation of TBARS (Table IV).

Histopathology

Kidney of normal control animals showed normal renal parenchyma with renal glomeruli as a glomerulus and Bowman's capsule and surrounded by proximal and distal tubules. Kidney of diabetic nephropathy rats showed mesangial expansion and thickening of glomerular capillaries. Glomeruli infiltrated by inflammation cells along with infiltration seen in cortex and medulla area. Atrophy of glomeruli was seen in STZ-induced diabetic rats. In glimepiride treatment group, the necrotic condition was reduced in convoluted tubules with reduced infiltration of inflammatory cells in cortex and medulla. *E. sativa* extracts treated group also showed protection *viz.*, reduction in mesangial expansion, membrane thickness and atrophy (Figure 4). Oxidative stress coupled with chronic hyperglycemia may have an important role in the pathogenesis of glomerular and tubular functional and structural abnormalities, even before the onset of microalbuminuria. These changes include extracellular deposition of the matrix in the mesangium, promotion of a hypoxic environment by early microvascular damage, induction of cellular oxidant injury and apoptosis and, finally, promotion of tubulointerstitial fibrosis by activation of TGF-β, which stimulates several pathways of fibrosis.

Pancreatic cells of the normal control group showed normal architecture with normal acini and islets cells with no signs of edema and inflammation. In diabetic nephropathy rats, inflammation, disorganization of the islets and steatosis were observed. Cell infiltration was

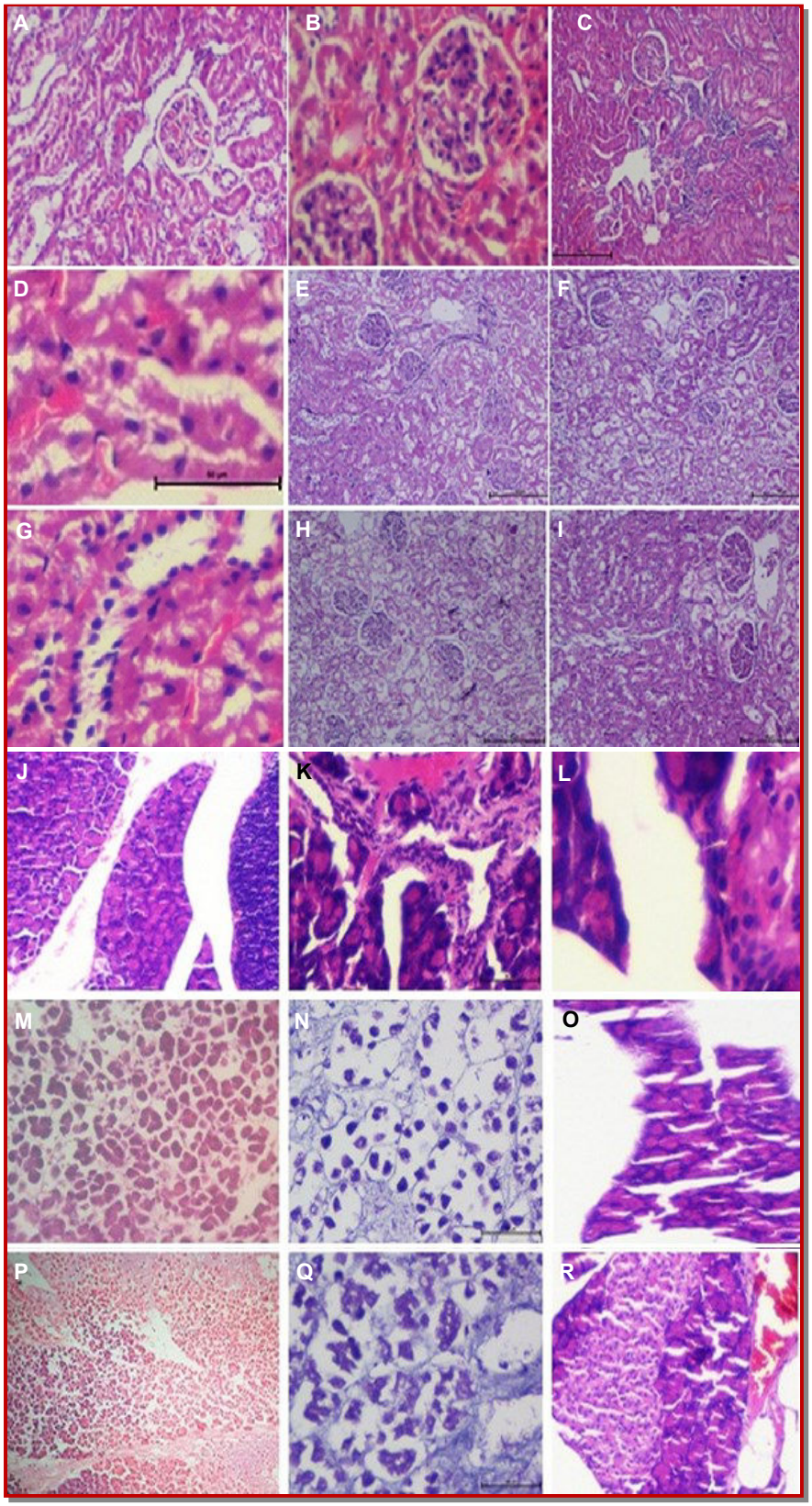

Figure 4: Histopathological changes in kidney and pancreas of normal and treated rats (hematoxylin and eosin 10×); "a" shows the structure of glomerulus. (A, J) normal, (B, K) diabetic nephropathy control, (C, L) standard, (D, M) EP 100 mg/kg treated, (E, N) EP 200 mg/kg, (F, O) EP 400 mg/kg treated, (G, P), EHA 100 mg/kg treated, (H, Q) EHA 200 mg/kg treated and (I, R) EHA 400 mg/kg treated

seen in the acinar cells along with necrosis and shrinkage of islet cells. Treatment with *E. sativa* extracts and glimepiride, showed a protective effect on islets of Langerhans and acinar cells as compared to diabetic rats and further reduction in edema, inflammation and shrinkage of islets (Figure 4).

Discussion

The current study was performed to determine the therapeutic potential of *E. sativa* in the treatment of STZ -induced diabetic nephropathy in rodents. STZ treatment resulted in an increase in blood glucose level of experimental animals. Extracts and glimepride treatment restored the blood glucose level to a significant extent. Elevated renal parameters (UAE, creatinine, BUN, urea and uric acid levels) were used to assess the development of diabetic nephropathy. *E. sativa* restored elevated renal parameters, lipid levels and level of anti-oxidant enzymes in a dose-dependent manner.

A previous study by El-missry et al., 2000 on amelioration of alloxan-induced diabetes mellitus and oxidative stress in rats by oil of *E. sativa* seeds gave supporting evidence to our study. NAD is an anti-oxidant which exerts a protective effect on the cytotoxic action of STZ by scavenging free radicals and causes only minor damage to pancreatic beta cell mass producing type II diabetes. STZ and NAD provide good opportunity to investigate diabetes in a much closely similar pathophysiological situation as in humans (Srinivasan and Ramarao, 2007). The earliest clinical manifestation for incipient diabetic nephropathy is the development of the persistent microalbuminuria (Urinary albumin excretion rate; 20-200 µg/min). In type 2 diabetes, if no treatment is initiated, up to 20-40% of patients will progress to overt albuminuria and 20% of those with overt albuminuria will develop ESRD over the next 20 years (Yamagishi et al., 2005). In a 10-year study of type 2 diabetics, 38% developed micro-albuminuria as a symptom of nephropathy (Torffvit and Agardh, 2001). In the present study, there is increased microalbuminuria in diabetic control animals which is significantly attenuated by oral administration of either extract.

The earliest clinical manifestation for incipient diabetic nephropathy is the development of the persistent microalbuminuria (urinary albumin excretion rate; 20-200 µg/min). In type 2 diabetes, if no treatment is initiated, up to 20-40% of patients will progress to overt albuminuria and 20% of those with overt albuminuria will develop ESRD over the next 20 years. In a 10-year study of type 2 diabetics, 38% developed microalbuminuria as a symptom of nephropathy (Torffvit and Agardh, 2001). Chronic hyperglycemia increased the concentration of metabolic waste like urea, uric acid, BUN and creatinine due to which renal function is compromised. After 30 days there was increased level of serum urea, uric acid, BUN and creatinine levels which suggested the development of diabetic nephropathy. Continue administration of 100, 200 and 400 mg/kg of petroleum ether extract and EHA for 45 days decreased the level of all these parameters. Abnormalities in the lipoprotein metabolism are associated with patients with diabetic nephropathy due to the development of microalbuminuria (Kishore et al., 2016). The lipoprotein abnormalities include a higher level of VLDL-C, LDL-C and a suppressed level of HDL -C. Lipid control appears to be important in the prevention and treatment of diabetic nephropathy (Srivastava et al., 2014) The administration of 100, 200 and 400 mg/kg of petroleum ether extract and EHA increased the level of serum HDL-C and decreased the levels of total cholesterol, triglycerides and LDL-C.

Glycosylated hemoglobin (Hb1Ac) can be used as an excellent marker of overall glycemic control. Since it is formed slowly and does not dissociate easily, it reflects the real blood glucose level (Guoyan, 1992). HbA1c had been found to increase in patients with diabetes mellitus (Baskaran et al., 1990). Oral administration of either extract decreased the level of HbA1c, thereby significantly attenuating hyperglycemia. Rajkumar et al., 1997 have reported that increased catabolic reactions leading to muscle wasting might also be the cause for the reduced weight gain by the diabetic rats. An increase in the body weight of diabetic rats by extract might be due to an improvement in insulin secretion and glycemic control (Genet et al., 1999).

Literature review shows the existence of kaempferol and quercetin in *E. sativa*. (Weckerle et al., 2001; Michael et al., 2011). The results of HPLC analysis also showed that there was a high concentration of kaempferol and quercetin in EHA and these compounds also isolated by column chromatography. Previous study reported that quercetin, a flavonoid which produces an increase in the number of pancreatic islets, probably increases insulin release in STZ-diabetic rats and also have plasma glucose lowering property (Vessal et al., 2003; Coskun et al., 2005) A recent study by Zang et al., 2015 also suggested anti-diabetic potential of kaempferol glycoside fractions by decreasing fasting blood glucose, serum HbA1c levels and improved insulin resistance.

GC-MS analysis revealed the presence of different fatty acids and a glucosinolate i.e. erucin in abundant form. Previous literature showed Trans fatty acids as an effective dietary strategy for the decrease in postprandial glucose responses (Cunnane et al., 1993). The major glucosinolate in *eruca* seeds is erucin which is potentially capable of protecting cells against oxidative stress via three mechanisms: a) induction of phase II enzymes, b) scavenging hydrogen peroxide and alkyl hydroperoxides accumulated in cells and peripheral blood, and

c) acting as a precursor of sulforaphene, a potent inducers detoxifying electrophiles and increase cellular anti-oxidant defenses. As oxidative stress is the important factor for the development of diabetic nephropathy, therefore, erucin might be responsible for attenuating effect of *E. sativa* against diabetic nephropathy.

Oxidative stress plays an important part in the pathogenesis of endothelial cell dysfunction and microalbuminuria. Chronic hyperglycemia is the single most important factor in the generation of early and sustained oxidative stress. Other complications of chronic hyperglycemia that promote oxidative stress include enhanced production of AGEs, decreased nitric oxide production, increased cytokine activation and levels of inflammatory markers (Kishore et al., 2016). There is accumulating evidence that AGE-RAGE axis is involved in the pathogenesis of diabetic nephropathy. Among various types of AGE receptors, RAGE is a signal transducing receptor for AGEs that could mediate the inflammatory reactions evoked by AGEs (Yamagishi et al., 2005). Engagement of RAGE with AGEs elicits oxidative stress generation, thus participating in diabetic nephropathy. The kidney plays an important role in the clearance and metabolism of AGEs and serum AGE concentrations increase in chronic renal insufficiency, partly by an increase in oxidative stress. It has been reported that STZ-induced diabetic mice develop renal changes seen in human diabetic nephropathy such as glomerular hypertrophy, glomerular basement membrane thickening, mesangial matrix expansion, connective tissue growth factor (CTGF) overexpression, and NFκB activation, all of which are blocked by the administration of neutralizing antibody raised against RAGE (Alam et al., 2007). ROS are cytotoxic to renal cells and promote inflammatory and fibrogenic reactions in diabetic kidney. AGEs are associated with structural renal changes leading to CKD-progression followed by a further increase in AGE concentration (Flyvbjerg et al., 2004). In diabetes, there is a pathogenic relationship between AGE-formation, deposition and nephropathy. It can be concluded that petroleum ether or hydroalcohol extract showed a protective effect against diabetic nephropathy, since they exhibited beneficial effects on the blood glucose level and associated biomarkers of diabetic nephropathy.

GSH and SOD were depleted in diabetic rats as compared to non-diabetic rats. The increase in thiobarbituric acid-reactive substances (TBARS), an index of lipid peroxidation in the diabetic rats might be due to increased levels of oxygen free radicals. In animal studies, 100, 200 and 400 mg/kg of petroleum ether or hydroalcohol extract administration was shown to decrease serum TBARS level and increase SOD and GSH due to its potential anti-oxidant activity. The effectiveness of *E. sativa* against oxidative stress is widely recognized and has been discussed in other studies (Barillari et al., 2005; Heimler et al., 2007; Alam et al., 2007), which supported our present study. Histopathological observations showed glomeruli with mesangiocapillary proliferation in the kidney of diabetic rats along with amelioration of hemodynamic parameters of the kidney due to increased formation of AGEs. The ultra-structure of the diabetic pancreas showed a considerable reduction in the islet of Langerhans and depleted islets (Kumar and Padhy, 2011).

Conclusion

The supplementation with *E. sativa* might be beneficial in chronic diabetics and thus may find application in diabetic nephropathy via reducing the formation of AGEs and amelioration of oxidative stress.

Ethical Issue

The experimental protocol was approved by Institutional Animal Ethical Committee (MMCP/IAEC/13/09) and the experiments were performed according to the CPCSEA guidelines.

Conflict of Interest

All authors have completed the ICMJE uniform disclosure form and declare no support from any organization for the submitted work.

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