

Therapeutic effect of *Linum usitatissimum* L. in STZ-nicotinamide induced diabetic nephropathy via inhibition of AGE's and oxidative stress

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Abstract The present study was aimed to evaluate the potential of petroleum ether and hydro-alcoholic extract of *Linum usitatissimum* (FPE and FHE) in STZ-nicotinamide induced diabetic nephropathy. GC–MS analysis of FPE revealed the presence of different fatty acids, heterocyclic compounds etc. Moreover, chromatography of FHE isolated Secoisolariciresinol diglycoside. After 30 days of STZ-administration, 100, 200 and 400 mg/kg of FPE and FHE were administered for 45 days. FPE and FHE produced significant attenuation in the glycemic status, renal parameter, lipid profile and level of antioxidant enzymes proving efficacy in diabetic nephropathy. Moreover, FPE and FHE produced significant reduction in the formation of AGEs in kidney. The results indicated that via amelioration oxidative stress and formation of AGEs, FPE and FHE produced significant nephroprotective effect in STZ-induced diabetic nephropathy in rats.

Keywords Advanced glycation end products · Hyperglycemia · Oxidative stress · Secoisolariciresinol diglycoside

Abbreviations

ANOVA	Analysis of variance
BUN	Blood urea nitrogen
FHE	Flaxseed hydroalcoholic extract
FPE	Flaxseed petroleum ether extract
GSH	Glutathione

HDL	High density lipoproteins
LDL	Low density lipoproteins
VLDL	Very low density lipoproteins
<i>i.p.</i>	Intraperitoneal administration
LPO	Lipid peroxidation
MAPKs	Mitogen activated protein kinases
SOD	Superoxide dismutase
SDG	Secoisolariciresinol diglycoside
STZ	Streptozotocin
TBARS	Thiobarbituric acid reactive substances
TNF- α	Tumor necrosis factor- α

Introduction

Chronic hyperglycemia is the main culprit for the development of diabetic complications like diabetic nephropathy, diabetic neuropathy, diabetic retinopathy etc. Among these, diabetic nephropathy is the major cause of morbidity and mortality due to end stage renal disease (Pan et al. 2014). Diabetic nephropathy is a multistage condition characterized by decreased glomerular filtration rate, hypertrophy and increased renal blood flow (Thomas and Karalliedde 2015). Various metabolic hypotheses have been proposed for the development of diabetic complication among which polyol pathway is considered as a potential target for management of diabetic nephropathy. In polyol pathway, aldose reductase utilizes nicotinamide adenine dinucleotide phosphate (NADPH) to reduce glucose to sorbitol (Brownlee et al. 2008). In addition, two key mechanisms implicated in the development of diabetic nephropathy include advanced glycation and oxidative stress. Glycation of proteins leads to partial loss of protein activity and is one serious consequences of chronic hyperglycemia (Yamagishi and Matsui 2010; Tupe et al.

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2015). Advanced glycation is the irreversible attachment of reducing sugars to amino groups of proteins to form advanced glycation end products (AGEs). The glycation reaction is subdivided into three main stages: early, intermediate and late (Baker et al. 1985). In the early stage, excessive glucose present in blood reacts with active amino groups of circulating proteins to yield chemically reversible glycosylation products (Schiff bases), fructosamines and Amadori products. In the intermediate stage, Amadori products degrade into a variety of carbonyl compounds (glyoxal, methylglyoxal, and deoxyglucosones). In the late stage, yellow–brown, often fluorescent, insoluble, irreversible advanced glycation end-products (AGEs) are formed. Furthermore, there is a growing body of evidence to indicate that glycated protein, AGEs and their signal-transducing receptor interactions induce the formation of reactive oxygen species, leading to concomitant oxidative stress and vascular inflammation, thereby playing a central role in the pathogenesis of various vascular complications in diabetes (Goh and Cooper 2008). Intracellular formation of AGEs can also cause generalized cellular dysfunction. Oxidative stress occurs as a result of the imbalance between ROS production and antioxidant defenses. Sources of ROS include the mitochondria, auto-oxidation of glucose, and enzymatic pathways including nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase (Singh et al. 2013).

Normalization of glycemia, inhibition of aldose reductase, AGE formation inhibition and amelioration of oxidative stress reverses early changes of diabetic nephropathy (UK Prospective Diabetes Study (UKPDS) Group 1998). So, the management of hyperglycemia may pave a way to attenuation of diabetic nephropathy. But allopathic drugs used for the management of diabetes mellitus have plethora of side effects. So, the focus is shifted to herbal drug. But, herbal drugs which are considered safer than allopathic drugs are not purely devoid of side effects. So, food or food supplements have an upper edge in the management of diabetes and diabetic complications. Moreover, dietary measures are integral part of antidiabetic regimen. If dietary intervention could reduce the ensuing diabetic complications it can be a powerful adjunct to conventional antidiabetic treatment.

Linum usitatissimum L. (Linaceae), commonly known as Flaxseeds are used in daily food, preparation in many Asian countries. Various studies on the potential health benefits associated with consumption of flaxseed have increased in the past decade. These potential health benefits include anticancer effects, antiviral and bactericidal activity, anti-inflammatory activity, laxative and reduction of atherogenic risks (Cunnane et al. 1995; Thompson et al. 1996). Flaxseeds are a part of food in many parts of the

world due to their dietary fiber content and other secondary plant metabolites viz, fixed oils, triterpenoids and polyphenolic compounds, lignans, α -linoleic acid etc. (Cunnane et al. 1995; Liggins et al. 2000). Flaxseeds inhibit glucosidase (Bhat et al. 2011) and porcine pancreatic α -amylase leading to the reduction in starch hydrolysis and eventually reduced glucose levels (Sudha et al. 2011). Moreover, the flaxseeds are reported to have renoprotective activity in ischemic reperfusion injury (Ghule et al. 2011). Flaxseed contains ω -3 fatty acids, lignans, and fiber that together may provide benefits to patients with cardiovascular disease. Circulating α -linolenic acid levels correlated with systolic and diastolic blood pressure, and lignan levels correlated with changes in diastolic blood pressure. Flaxseed showed one of the most potent antihypertensive effects achieved by a dietary intervention (Rodríguez-Leyva et al. 2013). Gok et al. 2016 investigated the effects of flaxseed intake on general metabolism, pentose phosphate pathway and glutathione-dependent enzymes in diabetic rats. The study concluded that Flaxseed has beneficial effects against diabetes-induced glucotoxicity by modulating glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and glutathione reductase activities in tissues. Keeping these view points, present study was designed to evaluate the antihyperglycemic and renoprotective activity of petroleum ether and hydro-alcoholic (40%) extract of flaxseeds in diabetic nephropathy.

Materials and methods

Preparation of extract

Flaxseeds were procured from local supplier and authenticated by Dr. Sunita Garg, NISCAIR New Delhi. A voucher specimen (NISCAIR/RHMD/Consult/2013/2295/75) was deposited in the herbarium of NISCAIR, New Delhi for future reference. Seeds were powdered and extracted with Petroleum Ether 60–80 °C and hydro-alcoholic (40%) sequentially using soxhlet extraction method and extracts were stored at 4–8 °C until further use. The petroleum ether extract (FPE) was prepared in 0.25% w/v Tween-80 and hydroalcoholic extract (FHE) was redissolved in water.

Chemicals

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

Quantitative analysis

Determination of total flavonoids

10 g of seed powder was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter Paper No. 42. The filtrate was later transferred into a crucible and evaporated into dryness and weighed to a constant weight (Boham and Kocipal-Abyazan 1974).

Determination of total saponins

20 g of powdered seeds were taken in conical flask and 100 ml of 20% aq. ethanol was added. The mixture was heated over a hot water bath for 4 h with continuous stirring at about 55 °C. The mixture was filtered and the residue re-extracted with another 200 ml of 20% aq. ethanol. The combined extracts were reduced to 40 ml over water bath at about 90 °C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. Then 60 ml n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the sample was dried in the oven to a constant weight, the saponins content was calculated as percentage (Obadoni and Ochuko 2001).

Terpenoid extraction and TLC analysis

50 g of the powdered seeds were extracted with solvent combination of methanol and water (4:1) at room temperature for 24 h. The solution was filtered using Whatman filter Paper No. 1 and the filtrate was then evaporated to 1/10 volume at 40 °C. The remaining filtrate was acidified with 2 M sulphuric acid (pH 0.89) followed by chloroform extraction (three times the volume), stirred and allowed to stand in a separating funnel. Out of the two layers formed, the non-aqueous layer was taken and evaporated till dryness. The dried extract contained components like terpenoids which were further used for thin layer chromatography analysis. Glass plates (20 × 20 cm) were coated (0.5 mm) with silica gel (Qualigen fine chemicals) and samples (approximately 5 mg/ml) of each extract dissolved in methanol were applied. Chromatography was performed in 100% chloroform according to the method described by Harborne. The spots were visualized using concentrated sulphuric acid as a spray reagent followed by heating of plates at 100 °C for 10 min. The spots were

identified based on the color, produced on reacting with a spray reagent (Harborne 1998).

Gas chromatography–mass spectrometry (GC–MS) analysis

FPE 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 2 M 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 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. Then 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 H₂: inlet pressure 45 psi linear, gas velocity 39 cm/s, column flow rate 2.4 mL/min; split ratio, 40:1 and injector volume 1 µL.

Isolation of active compounds

The dried hydroalcoholic extract (10 g) was then subjected to column chromatography (silica gel packed column, Molychem 100–200 mesh, 160 g) by pre-adsorbing with silica gel. 5 g extract was eluted using chloroform (100%) and the mixture of chloroform and methanol up to 25%. The fractions (200 ml each) obtained from the column were collected and combined on monitoring TLC. At 20% (Fraction 80) yielded the compound.

In-vitro anti-glycation activity

In vitro antiglycation activity of FPE and FHE was evaluated by measuring their ability to inhibit the fluorescence of BSA (Matsuda et al. 2003). The reaction mixture of BSA (10 mg/ml), 1.1 M fructose in 0.1 M phosphate buffered-saline (PBS), pH 7.4 containing 0.02% sodium azide with or without extract (FPE and FHE; dissolved in PBS; 50–500 µg/ml) was incubated in darkness at 37 °C for 1, 2, 3, and 4 weeks. AGE formation was measured using Spectrofluorometer at an excitation wavelength 355 nm and emission wavelength 460 nm. Aminoguanidine (AG) was used as a positive control for this study. The percentage inhibition of AGE formation was determined by following formula:

$$\% \text{ Inhibition} = \left[\frac{\text{Fluorescence of control} - \text{Fluorescence of test}}{\text{Fluorescence of control}} \right]$$

Animals

Adult male Wistar rats weighing 250–300 g were housed in standard environmental conditions maintained at 23 ± 2 °C with 12 h light–dark cycle. Animals were fed with standard rodent diet and water ad libitum. Experimental protocol was approved by Institutional Animal Ethical Committee and the experiments were performed according to the guidelines of CPCSEA (MMCP/IAEC/13/11).

Induction of diabetic nephropathy

Diabetes was induced by a single *i.p.* of STZ (65 mg/kg) after 15 min of nicotinamide injection (230 mg/kg, *i.p.*). STZ was freshly prepared in 0.1 M citrate buffer (pH 4.5) and nicotinamide was prepared in normal saline. Diabetes mellitus was confirmed after 72 h of STZ injection and blood samples were collected via retro-orbital plexus. Rats with fasting blood glucose level more than 200 mg/dl were selected for the study. Doses of FPE and FHE (100, 200 and 400 mg/kg) were selected on the basis of oral acute toxicity studies reported in literature (Zanwar et al. 2011). After 30 days of STZ administration, different doses of FPE, FHE and Glimepride were continued for 45 days.

Rats were divided into nine groups and each group consists of six rats.

Group 1: normal control; Group 2: diabetic nephropathy (DN) control; Group 3: DN + 100 mg/kg FPE; Group 4: DN + 200 mg/kg FPE; Group 5: DN + 400 mg/kg FPE; Group 6: DN + 100 mg/kg FHE; Group 7: DN + 200 mg/kg FHE; Group 8: DN + 400 mg/kg FHE; Group 9: DN + 10 mg/kg Glimepride.

Rats were fasted overnight and blood samples were collected from retro-orbital plexus under light anesthesia. Biochemical estimation was done by measuring blood glucose level, serum insulin level, lipid profile (total cholesterol, triglyceride, HDL, LDL, VLDL levels), uric acid, urea, BUN and creatinine levels by using commercially available kits of Reckon Diagnostics Pvt. Ltd. Body weight was measured before the induction of diabetes and during the experimental period. Animals were sacrificed at the end of the study and tissues (liver, kidney and pancreas) were harvested and stored at -70 °C for histopathological studies.

Lipid peroxidation and activities of antioxidant enzymes in different tissues

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

AGEs estimation in kidneys

AGEs level in kidneys were determined by a method as previously described by Sensi et al. 1996. Briefly, perfused kidneys were homogenized in 2 ml of 0.25 M sucrose followed by centrifugation at 900 g at 5 °C and the supernatant was separated. The pellet was resuspended in 2 ml sucrose solution and centrifuged and the supernatant obtained was mixed with the previous one. The proteins present were precipitated by adding equal volume of trichloroacetic acid (TCA). After centrifugation at 4 °C with 900 g, the protein pellet obtained was mixed with 1 ml methanol twice to remove the lipid fraction. The insoluble protein, after washing with 10% cooled TCA was centrifuged and the residue was solubilized in 1 ml of 1 N NaOH and the protein concentration was estimated by measuring the absorbance at 280 nm against BSA standard curve. The AGEs content was then measured fluorometrically with an emission at 440 nm and excitation at 370 nm, and the results were expressed as relative fluorescence units (RFU)/mg protein.

Histopathology

Liver, kidney and pancreas were harvested from the animals and fixed in 10% neutral buffered formalin solution, dehydrated in ethanol and embedded in paraffin. Sections of 5 μ m thickness were prepared using a rotary microtome and stained with hematoxylin and eosin (H & E) dye for histopathological examination.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 6. Values were expressed as mean \pm 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 statistically significant if $p \leq 0.05$.

Results

Quantitative analysis

The quantity of flavonoids, saponins and terpenoids was found in the following order $4.3\% > 1.3\% > 0.03\%$ respectively. The terpenoidal extract when further subjected to TLC analysis using 100% chloroform showed 2 spots out of which spot 1 was brown and spot 2 was yellowish in color suggesting that spot 1 was limonene or α -pinene, and spot 2 of Pulgeone (Harborne 1984).

Gas chromatography mass spectroscopy analysis

GC–MS analysis was conducted on the crude petroleum ether extract of Flaxseeds. 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. Detailed tabulations of the GC–MS analysis of the extract is given in Table 1. Flaxseed extract revealed the presence of different fatty acids, heterocyclic compounds etc. The flaxseed extract showed 10 peaks in the GC–MS chromatogram indicating the presence of 10 phytochemical constituents.

Secoisolariciresinol diglycoside from *L. usitatissimum* (Flaxseed)

Chemical tests of phenolics were found positive and significant in hydroalcoholic extract of *Linum usitatissimum*. The hydroalcoholic extract was subjected to column chromatography and its 20% (chloroform: methanol; 20: 80) fraction showed single spot on TLC and also have positive tests for phenolics. The UV λ_{max} (265 & 358) and IR peaks (cm^{-1}) [3391 (-OH), 2939 (C-H), 1637 (C = C), 1428 (C-C), 1346, 1279, 1240, 1208, 1130, 1067, 1052, 991, 941, 921, 867, 849, 682, 584, 472] showed presence of phenolics. The LC–MS Q-TOF micromass fragmentation pattern [704 ($\text{M}^+ + \text{NH}_4$); 687 ($\text{M}^+ + 1$), 524.3 (Secoisolariciresinol glycoside; 381.1, 441), 362.1 (Secoisolariciresinol; 325, 319, 281, 273, 247, 199, 183, 133, 73; Ricci and Piccolella 2012; Ghose et al. 2014] provides the confirmation of Secoisolariciresinol diglycoside (Fig. 1).

AGEs inhibition activity

In the present study, the formation of AGEs was monitored weekly by measuring fluorescence intensity of the BSA-fructose solutions for 4 weeks. A significant inhibition of AGEs formation (93.37%) was observed in fructose-induced glycated BSA plus aminoguanidine (500 $\mu\text{g}/\text{ml}$). In the 1st week, % inhibition of AGEs formation using FPE (50–500 $\mu\text{g}/\text{ml}$) was 23.54% whereas in the 4th week, the percentage inhibition was found to be 83.97%. Similarly, FHE % inhibition in the 1st week found to be 28.44% and in 4th week it was 91.74%.

Effect of FPE and FHE on body weight

Significant increase in reduced body weight was observed in DN control rats as compared to control animals. Whereas, administration of FPE and FHE (100, 200 and 400 mg/kg) in rats produced significantly ($p < 0.001$) reduced the weight loss as compared to DN control rats. Different doses of FHE (100, 200 and 400 mg/kg) also produced significant

elevation in BW (6.55, 9.5 and 14.61% respectively) in comparison to DN control rats (Fig. 2a).

Effect of FPE and FHE on fasting blood glucose level

Administration of different doses of extracts was started after 30 days of STZ administration and continued for up to 75th day. Serum blood glucose level of animals was measured on 30th, 45th, 60th and 75th day of study. Fasting blood glucose level of control rats ranged from 93.68 to 96.03 mg/dl in 75 days. Whereas, in diabetic rats fasting glucose level was increased to 521.73 mg/dl from 325.82 mg/dl . Administration of different doses of FPE and FHE (100, 200 and 400 mg/kg) for 45 days, produced significant attenuation in blood glucose level and maximum attenuation was observed with 400 mg/kg of FHE as compared to DN control rats (Fig. 2b). Glimepride treatment also resulted in significant attenuation of blood glucose level in rats.

Effect of FPE and FHE on fasting insulin level

Administration of STZ produced a significant decrease in fasting insulin level ($7.05 \pm 0.209 \mu\text{U}/\text{ml}$). Whereas administration of 100, 200 and 400 mg/kg of FPE and FHE (Fig. 2c) for 45 days produced significant increase in serum insulin level [FPE (8.20 ± 0.108 , 9.45 ± 0.068 , $9.45 \pm 0.082 \mu\text{U}/\text{ml}$ respectively); FHE (9.23 ± 0.111 , 10.33 ± 0.059 , $12.92 \pm 0.081 \mu\text{U}/\text{ml}$ respectively)].

Effect of FPE and FHE on serum urea, uric acid, creatinine and BUN

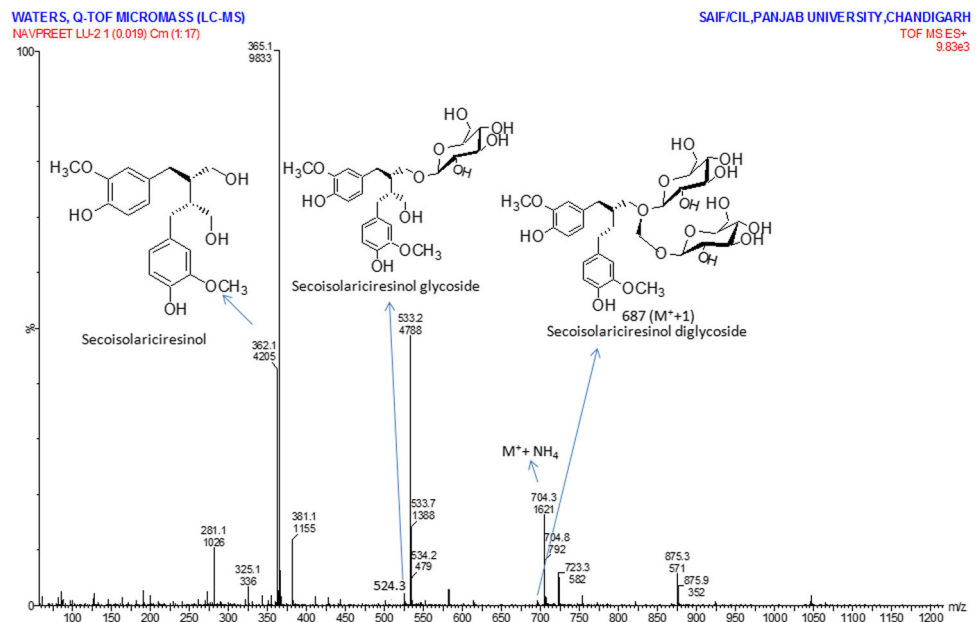
Administration of STZ resulted in induction of diabetic nephropathy which can be assessed by the elevated level of serum urea, uric acid, creatinine and BUN as compared to normal control rats (Table 2). Whereas, administration of different doses of FPE, FHE (100, 200 and 400 mg/kg) significantly decreased the elevated level of serum urea, uric acid, creatinine and BUN. Moreover, attenuating effect was found to be dose dependent and 400 mg/kg of FPE and FHE produced maximum decrease in the level of markers of diabetic nephropathy. Glimepride also significantly attenuated the elevated level of these parameters.

Effect of FPE and FHE on serum lipid profile

Administration of STZ also produced dyslipidemia depicted in the form of elevated serum TC, TG, LDL, VLDL and reduced HDL level. 100, 200 and 400 mg/kg of FPE and FHE produced dose dependent attenuation in TC. Moreover, 400 mg/kg of FPE and FHE produced 56.94 and 64.40% respectively decrease in TC as compared to DN

Table 1 Phytochemicals identified in FPE by GC–MS

Peak	Retention time	Area %	Database/NIST 14 Library	CAS
1	12.17	2.30	cis-4-Isopropyl-5-methylhexa-2,4-dien-1-ol	19898-86-3 00000-00-0
2	14.01	2.00	Cyclohexasiloxane, dodecamethyl-Fluoren-9-ol, 3,6-dimethoxy-9-(2-phenylethynyl)- Dihydrofuro-[2,3-e] phenalene, 6-methoxy-8-oxo-2,3,3,10-tetramethyl-4,5,7-trihydroxy	00540-97-6 00000-00-0 00000-00-0
3	17.79	2.25	4,5diepiaristolochene	01137-12-8
4	18.03	6.25	1,2,4-Methenoazulene, decahydro1,5,5,8atetramethyl, [1S-(1à,2à,3aá,4à,8aá,9R*)]-à-ylangene	
5	18.37	57.99	Caryophyllene	00087-44-5
6	18.66	6.84	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a, 8-dimethyl-2-(1-methylethenyl), [2R-(2α,4α,8αβ)]-	17066-67-0
7	19.89	14.38	Decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-, [4aR-(4aà,7à,8aá)]	
8	21.53	2.54	Epiglobulol	00473-13-2
9	41.06	4.40	Stearic acid	00000-00-0
10	56.98	3.59	Linolenic acid, ethyl ester (Ethyl 9,12,15-octadecatrienoate)	1191-41-9
			Phthalic acid, di-(2-propylpentyl) ester	00000-00-0
			Phthalic acid, di(oct3yl) ester	00000-00-0
			Diisooctyl phthalate	00131-20-4

Fig. 1 Mass spectra of Secoisolariciresinol diglycoside

rats. Similarly, TG level was also found to be attenuated (120.08, 127.78, 107.24 mg/dl and 103.94, 90.86 and 79.70 mg/dl) by administration of different doses of (100, 200 and 400 mg/kg) of FPE and FHE respectively. Attenuation was also observed in level of LDL and VLDL at 100, 200 and 400 mg/kg of FPE, FHE. Serum HDL-c level was significantly reduced in diabetic nephropathy control rats whereas the level of HDL-c was found to be

significantly elevated in FPE, FHE (100, 200 and 400 mg/kg) treated animals (Table 3).

Effect of FPE and FHE on antioxidant enzymes and TBARS

Oxidative stress ensues with the reduction of antioxidant enzymes and increased lipid peroxidation. Level of

Fig. 2 Effect of FPE and FHE on **a** body weight (g) in diabetic nephropathy wistar rats, **b** blood glucose level (mg/dl) in diabetic nephropathy wistar rats, **c** serum insulin level (μ IU/ml) in diabetic nephropathy wistar rats

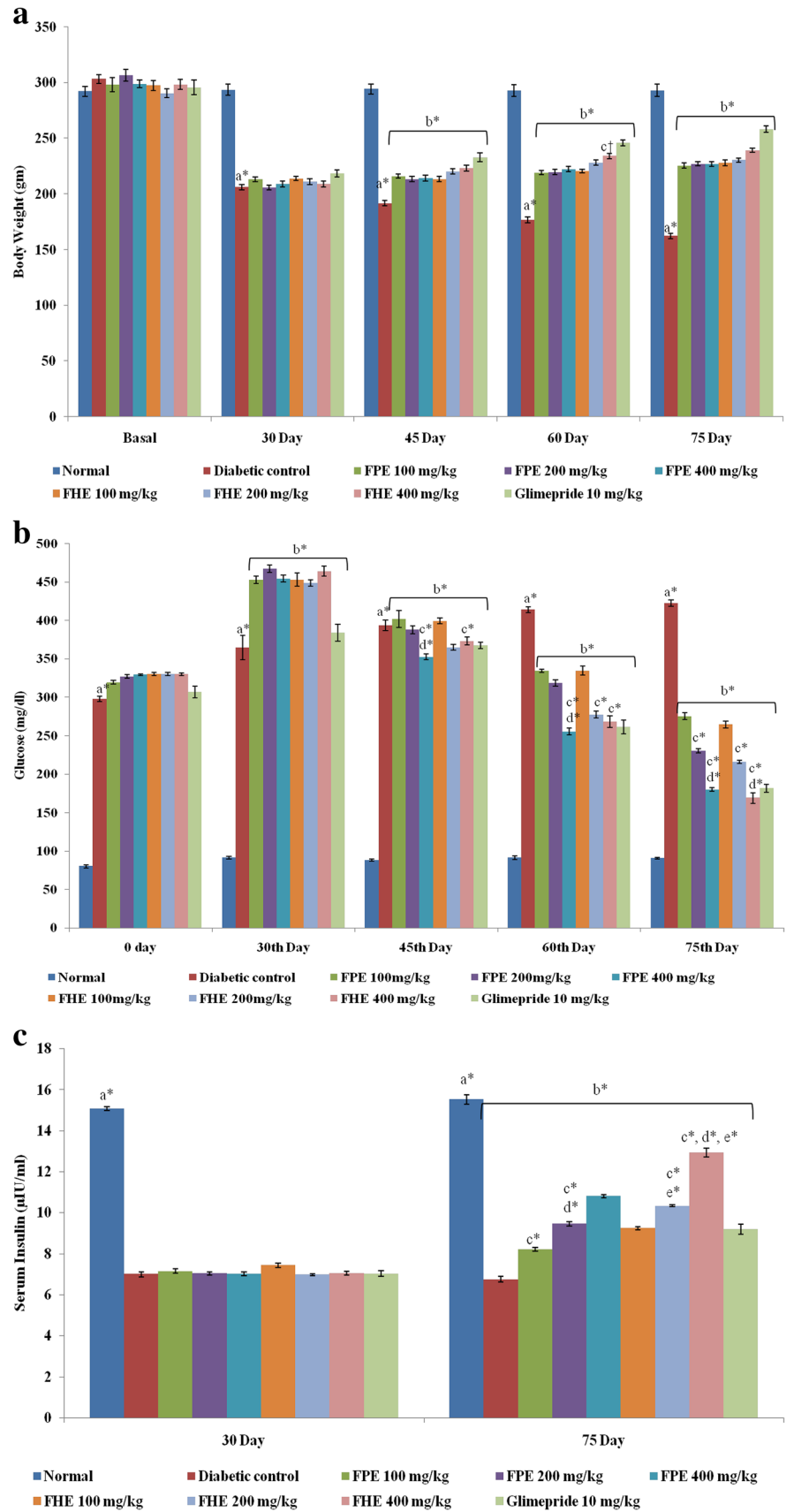


Table 2 Effect of FPE and FHE on renal function estimation in diabetic-nephropathy wistar rats

Parameters	Urea (mg/dl)		Uric Acid (mg/dl)		Creatinine (mg/dl)		BUN (mg/dl)	
	30th day	75th day	30th day	75th day	30th day	75th day	30th day	75th day
Normal	33.15 ± 0.789	33.28 ± 0.629	5.22 ± 0.216	5.27 ± 0.198	0.72 ± 0.009	0.81 ± 0.009	15.48 ± 0.368	15.54 ± 0.294
Diabetic control	82.13 ± 1.64 ^{a*}	97.96 ± 0.518 ^{a*}	12.59 ± 0.854 ^{a*}	16.04 ± 0.612 ^{a*}	2.83 ± 0.046 ^{a*}	4.22 ± 0.092 ^{a*}	38.35 ± 0.769 ^{a*}	45.74 ± 0.242 ^{a*}
FPE 100 mg/kg	86.53 ± 1.62	56.56 ± 0.726 ^{b*}	12.46 ± 0.900	8.59 ± 0.153 ^{b*}	4.06 ± 0.237 ^{b*}	2.22 ± 0.149 ^{b*}	40.40 ± 0.439	26.41 ± 0.172 ^{b*}
FPE 200 mg/kg	83.57 ± 1.029	50.52 ± 0.714 ^{b*c*}	12.26 ± 0.635	8.52 ± 0.151 ^{b*}	4.03 ± 0.277 ^{b*}	2.11 ± 0.159 ^{b*}	39.02 ± 0.504	23.59 ± 0.407 ^{b*c*}
FPE 400 mg/kg	83.33 ± 1.677	41.03 ± 0.768 ^{b*c*d*}	11.65 ± 0.355	7.53 ± 0.159 ^{b*}	4.18 ± 0.223 ^{b*}	1.44 ± 0.117 ^{b*c*d*}	38.91 ± 1.026	19.16 ± 0.322 ^{b*c*d*}
FHE 100 mg/kg	83.91 ± 1.054 [*]	60.42 ± 0.295 ^{b*}	12.64 ± 0.539	8.28 ± 0.118 ^{b*}	3.38 ± 0.265 ^{b*}	1.82 ± 0.109 ^{b*}	39.18 ± 0.539	28.21 ± 0.151 ^{b*}
FHE 200 mg/kg	83.33 ± 1.397 [*]	49.06 ± 0.869 ^{b*c*}	13.26 ± 0.414	7.17 ± 0.094 ^{b*}	3.91 ± 0.176 ^{b*}	1.88 ± 0.078 ^{b*}	38.91 ± 0.714	22.91 ± 0.444 ^{b*c*}
FHE 400 mg/kg	83.62 ± 1.397	42.87 ± 0.669 ^{b*c*d*}	12.32 ± 0.392	6.15 ± 0.132 ^{b*}	4.38 ± 0.104 ^{b*}	1.24 ± 0.064 ^{b*c*d*}	39.05 ± 1.056	20.02 ± 0.342 ^{b*c*d*}
Glimepride 10 mg/kg	83.87 ± 1.098	34.35 ± 0.933 ^{b*}	12.29 ± 0.634	6.01 ± 0.167 ^{b*}	3.00 ± 0.223	0.96 ± 0.052 ^{b*}	39.16 ± 0.562	16.04 ± 0.477 ^{b*}

Each group (=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 Flaxseed extract 100 mg/kg, ^d vs Flaxseed extract 200 mg/kg. * $p < 0.001$, # $p < 0.01$, [†] $p < 0.05$

antioxidant enzymes (SOD and GSH) reduced significantly in pancreas, kidney and liver of diabetic nephropathy rats. Treatment with 100, 200 and 400 mg/kg of FPE and FHE significantly increased the level of antioxidant enzymes viz. SOD and GSH compared to DN rats. Level of TBARS was found to be elevated in DN rats as compared to normal control group whereas administration of FPE and FHE significantly reduced the level of TBARS in comparison to DN rats (Table 4).

Effect of FPE and FHE on AGEs in kidneys

Administration of STZ led to a significant increase in formation of AGEs in kidney of diabetic rats. FPE, FHE (100, 200 and 400 mg/kg) and Glimepride significantly inhibited the formation of AGEs when compared to diabetic control group (Table 4).

Histopathological studies of liver, kidney and pancreas

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 DN 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 Glimepride treatment group, necrotic condition was reduced in convoluted tubules with reduced infiltration of inflammation cells in cortex and medulla. Flaxseed extracts treated group also showed protection viz. reduction in mesangial expansion, membrane thickness and atrophy (Fig. 3a).

Normal central vein with radiating sinusoid cords were present in Liver of normal rats. There was no sinusoid congestion; swelling and necrotic cells. DN rats demonstrated perivenular inflammatory collection and hyperplasia of kupffer cell with condensed nuclei and fatty infiltration. These pathological changes were reversed by FPE and FHE extracts administered DN rats (Fig. 3b).

Pancreatic cells of normal control group showed normal architecture with normal acini and islets cells with no signs of edema and inflammation. In DN rats, inflammation, disorganization of the islets and steatosis were observed. Cell infiltration was seen in the acinar cells along with necrosis and shrinkage of islet cells. Treatment with FPE and FHE extracts and Glimepride, showed protective effect on islets of Langerhans and acinar cells as compared to diabetic rats and further reduction in edema, inflammation and shrinkage of islets (Fig. 3c).

Table 3 Effect of FPE and FHE on Lipid Profile in diabetic-nephropathy wistar rats

Parameters	TC (mg/dl)		TG (mg/dl)		LDL (mg/dl)		VLDL (mg/dl)		HDL (mg/dl)	
	30th day	75th day	30th day	75th day	30th day	75th day	30th day	75th day	30th day	75th day
Normal	103.5 ± 2.75	103.6 ± 2.02	72.44 ± 2.06	71.38 ± 1.46	33.00 ± 2.207	32.96 ± 2.47	33.07 ± 0.94	32.59 ± 0.66	56.09 ± 0.63	56.44 ± 0.54
Diabetic control	250.4 ± 2.54*	295.8 ± 2.49*	161.72 ± 3.27*	203.26 ± 1.12*	187.26 ± 2.31*	176.97 ± 2.72*	73.84 ± 1.49*	92.81 ± 0.51*	30.86 ± 0.42*	26.07 ± 0.329*
FPE 100 mg/kg	271.0 ± 2.56*	161.5 ± 3.23*	155.29 ± 1.36	120.08 ± 0.98*	213.09 ± 2.52	100.82 ± 3.25*	70.90 ± 0.62	57.05 ± 0.62*	26.85 ± 0.784*	35.72 ± 0.663*
FPE 200 mg/kg	241.5 ± 2.31*	135.9 ± 1.79*	152.66 ± 1.13*	127.78 ± 8.41*	183.30 ± 2.69	72.22 ± 1.54*	69.71 ± 0.51*	54.54 ± 0.404*	27.66 ± 0.579*	39.81 ± 0.617*
FPE 400 mg/kg	238.5 ± 2.62*	127.9 ± 2.53*	155.15 ± 1.33	107.24 ± 1.03*	181.67 ± 2.96	63.45 ± 2.46*	70.84 ± 0.61	48.97 ± 0.47*	25.79 ± 1.033*	43.00 ± 0.964*
FHE 100 mg/kg	258.2 ± 5.06	143.5 ± 6.98*	151.78 ± 1.54*	103.94 ± 1.84*	201.41 ± 5.32*	88.17 ± 7.01*	69.30 ± 0.70	47.70 ± 0.76*	26.51 ± 0.753*	34.54 ± 0.599*
FHE 200 mg/kg	245.9 ± 2.88	115.2 ± 2.42*	153.52 ± 1.73	90.86 ± 1.22*	189.07 ± 3.01	59.43 ± 2.74*	70.10 ± 0.79*	41.48 ± 0.55*	26.19 ± 0.403*	37.60 ± 0.691*
FHE 400 mg/kg	240.6 ± 3.12*	105.3 ± 2.63*	153.11 ± 1.71	79.70 ± 1.45*	182.41 ± 3.16*	44.68 ± 2.84*	69.91 ± 0.78	36.39 ± 0.66*	27.63 ± 0.481*	44.72 ± 0.552*
Glimepride 10 mg/kg	252.2 ± 2.91	102.5 ± 1.33*	158.89 ± 2.24	71.68 ± 2.02*	189.74 ± 3.19	34.39 ± 1.57*	72.55 ± 1.02	32.73 ± 0.92*	30.68 ± 0.703	53.81 ± 0.295*

Each group (=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 Flaxseed extract 100 mg/kg, ^d vs Flaxseed extract 200 mg/kg. * $p < 0.001$, # $p < 0.01$, † $p < 0.05$

Table 4 Effect of FPE and FHE on level of antioxidant enzymes, lipid peroxidation (TBARS) and AGE's formation in diabetic-nephropathy wistar rats

Parameters	SOD (U/mg protein)			GSH (μM/mg protein)			TBARS (nmol/mg protein)			AGE's (RFU/mg protein)	
	Kidney	Pancreas	Liver	Kidney	Pancreas	Liver	Kidney	Pancreas	Liver	Kidney	Liver
Normal	4.87 ± 0.083	4.17 ± 0.298	3.67 ± 0.096	74.93 ± 0.621	69.45 ± 0.289	66.21 ± 0.476	0.55 ± 0.01	0.46 ± 0.011	0.41 ± 0.005	1.71 ± 0.051	1.71 ± 0.051
Diabetic control	1.23 ± 0.049*	1.15 ± 0.058*	1.06 ± 0.045*	38.60 ± 0.481*	41.90 ± 0.347*	45.53 ± 0.482*	2.98 ± 0.017*	2.71 ± 0.008*	2.48 ± 0.066*	3.88 ± 0.271*	3.88 ± 0.271*
FPE 100 mg/kg	1.93 ± 0.023*	1.27 ± 0.016	1.16 ± 0.015	40.57 ± 0.218*	39.55 ± 0.572	41.36 ± 0.374*	2.71 ± 0.017*	2.01 ± 0.021*	1.69 ± 0.021*	3.26 ± 0.063*	3.26 ± 0.063*
FPE 200 mg/kg	2.82 ± 0.025*	2.38 ± 0.023*	2.16 ± 0.029*	54.75 ± 0.601*	49.31 ± 1.216*	48.53 ± 0.798*	2.12 ± 0.024*	1.68 ± 0.016*	1.46 ± 0.042*	2.96 ± 0.070*	2.96 ± 0.070*
FPE 400 mg/kg	3.93 ± 0.020*	3.43 ± 0.032*	3.13 ± 0.030*	60.83 ± 0.532*	59.36 ± 0.742*	56.29 ± 0.327*	1.50 ± 0.006*	1.33 ± 0.009*	1.22 ± 0.059*	2.84 ± 0.042*	2.84 ± 0.042*
FHE 100 mg/kg	2.13 ± 0.012*	1.25 ± 0.017	1.28 ± 0.095	45.40 ± 0.838*	44.22 ± 0.263*	47.19 ± 0.299	2.52 ± 0.021*	1.94 ± 0.020*	1.56 ± 0.033*	2.81 ± 0.035*	2.81 ± 0.035*
FHE 200 mg/kg	3.13 ± 0.006*	2.23 ± 0.026*	2.31 ± 0.095*	58.58 ± 0.300*	51.81 ± 0.553*	53.69 ± 0.512*	1.97 ± 0.035*	1.50 ± 0.013*	1.37 ± 0.006*	2.24 ± 0.034*	2.24 ± 0.034*
FHE 400 mg/kg	4.09 ± 0.025*	3.49 ± 0.100*	3.20 ± 0.049*	65.05 ± 0.43*	61.53 ± 0.406*	56.95 ± 0.406*	1.30 ± 0.006*	1.13 ± 0.009*	1.10 ± 0.072*	1.86 ± 0.044*	1.86 ± 0.044*
Glimepride 10 mg/kg	3.86 ± 0.018*	4.03 ± 0.148*	3.63 ± 0.110*	67.83 ± 0.535*	60.77 ± 0.375*	61.26 ± 0.350*	1.19 ± 0.03*	0.98 ± 0.020*	0.92 ± 0.031*	3.16 ± 0.053*	3.16 ± 0.053*

Each group (=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 Flaxseed extract 100 mg/kg, ^d vs Flaxseed extract 200 mg/kg. * $p < 0.001$, # $p < 0.01$, † $p < 0.05$

Discussion

Hyperglycemia is the vital initiator of diabetic complications and instigates the activation of various metabolic pathways including polyol pathway and formation of AGEs. Excessive formation of AGEs under hyperglycemic condition plays a major role in the pathogenesis of diabetic nephropathy. Hyperglycemia leads to increased glucose level in tissues viz., kidneys which in turn leads to accumulation of AGEs. Accumulation of AGEs has been implicated in structural alterations in extracellular matrix leading to decreased glomerular filtration rate. Advanced glycation is the only pathway by which renal injury can be induced in diabetes (Forbes et al. 2003). The present study demonstrates that FPE and FHE attenuated the formation of AGEs in vitro which prompted us to evaluate the effect of FPE and FHE on diabetic nephropathy.

STZ has been widely used to induce diabetes in the experimental animals. It produces diabetes via its cytotoxic effects on pancreatic β -cells (Kim et al. 2003). The cytotoxic action of STZ in rats produces hyperglycemia, oxidative stress and renal injuries which closely reflect the natural progression and metabolic characteristics of diabetic nephropathy. Moreover, excessive production of reactive oxygen species ensues oxidative stress which plays a key role in the development of diabetic nephropathy (Singh et al. 2013). Along with hyperglycemia and oxidative stress, induction of diabetic nephropathy with STZ is associated with characteristic weight loss due to muscle wasting and catabolism of tissue proteins (Swanston-Fiatt et al. 1990).

The increased levels of fasting blood glucose in STZ-induced DN rats were lowered by the administration of FPE and FHE in dose and time dependent manner. The reduced glucose levels might be suggested by insulin like effect on peripheral tissues by either promoting glucose uptake metabolism by inhibiting hepatic gluconeogenesis, or by absorption of glucose into the muscle and adipose tissues, through the stimulation of a regeneration process and revitalization of the remaining beta cells (Kaur et al. 2016). Moreover, oral administration of FPE and FHE effectively reversed body weight loss by reversing muscle wasting and protein loss and also significantly attenuated hyperglycemia as compared to DN rats.

Hyperglycemia is responsible for diminution of antioxidant enzymes viz. SOD and GSH. LPO is another process which involves a source of secondary free radical; thus, it acts as a second messenger or can directly react with other biomolecules, further enhancing the biochemical lesions. Moreover, Administration of different doses of FPE and FHE ameliorated the oxidative stress via increasing level of antioxidant enzymes.

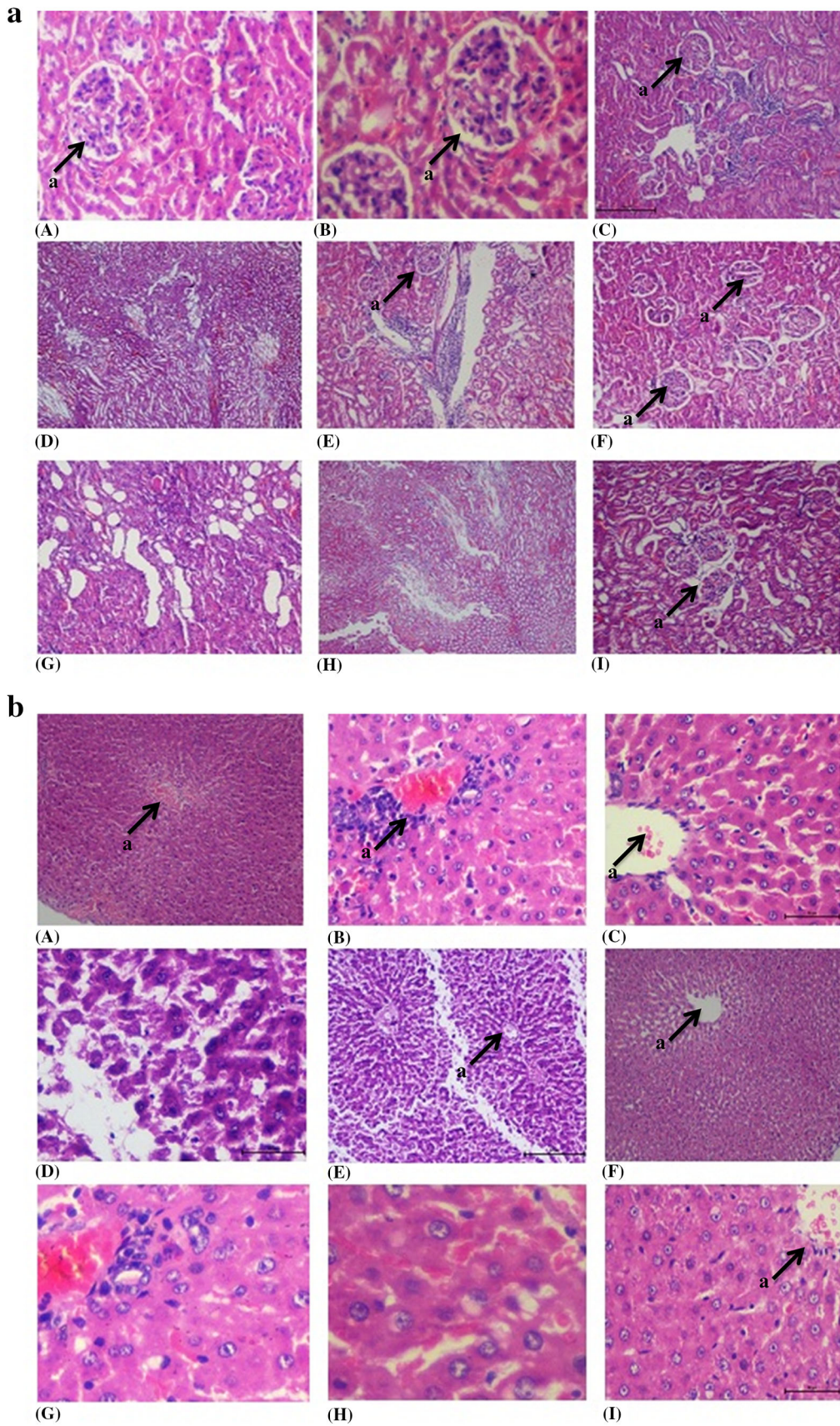
Phytochemical screening revealed that fatty acids, triterpenoid, phenolic compounds and saponins are present

Fig. 3 Histopathological changes in **a** Kidney; **b** Liver; **c** Pancreatic islet of normal and treated rats (H&E \times 100); “**a**” shows the structure of β -cells. (A) Normal, (B) diabetic nephropathy control, (C) standard, (D) FPE 100 mg/kg treated, (E) FPE 200 mg/kg, (F) FPE 400 mg/kg treated, (G) FHE 100 mg/kg treated, (H) FHE 200 mg/kg treated and (I) FHE 400 mg/kg treated

in FPE and FHE. Flaxseeds are grouped as “functional food and/or endocrine active food” by virtue of the presence of physiologically active food components that may provide health benefits beyond basic nutrition (Hasler et al. 2000). Trans fatty acids present in flaxseed oil offers an effective dietary strategy for the prevention of atherosclerotic cardiovascular disease (Bassett et al. 2011) and decrease postprandial glucose responses. Flaxseeds are richest source of plant lignans. The lignan SDG is present in greater quantity in the seed coat. In humans, ingested SDG is converted by bacteria in colon to biologically active lignans enterodiol and enterolactone (Hano et al. 2006). These SDG metabolites possess antioxidant activity and have been shown to effectively inhibit the development of type 1 and 2 diabetes (Prasad 2000). Effect of FHE on amelioration of DN can thus be attributed to the presence of SDG in the extract.

Diabetic Nephropathy is known by not only dysfunctional glucose metabolism but also dyslipidemia, especially in type 2 diabetes. Lipid abnormalities including increased level of TC, TG, LDL, VLDL and decreased HDL-c level predisposes diabetic patient to atherosclerosis and other cardiovascular complications like coronary heart disease (Thomas and Karalliedde 2015). Hyperglycemia, atherosclerosis and dyslipidemia are major causes of cardiovascular diseases and are linked to have interrelation with free radical generation (Singh et al. 2015). Similarly, in the present study, a positive correlation between hyperglycemia and dyslipidemia was observed. Serum TC, TG LDL and VLDL levels were found to be significantly elevated whereas HDL levels decreased. This dyslipidemia occurs due to increased breakdown of lipid and free fatty acids from peripheral stores. Moreover, hyperlipidemia with elevated levels of triglycerides, and free fatty acids results in oxidative stress and inflammation and may independently potentiate the adverse effects of hyperglycemia (O’Keefe and Bell 2007). Administration of FPE and FHE significantly reduced TC, TG, LDL and VLDL levels. Moreover, FPE and FHE effectively increased the level of serum HDL-c. Dyslipidemic effect of flaxseeds can be attributed to the presence of SDG (Prasad 2008).

DN is one of the most serious complications of diabetes and it is characterized by elevated the level of serum creatinine, blood urea nitrogen, creatinine clearance as well as kidney hypertrophy (Cowie et al. 1989). Hyperglycemia could enhance glomerulosclerosis and accelerate the



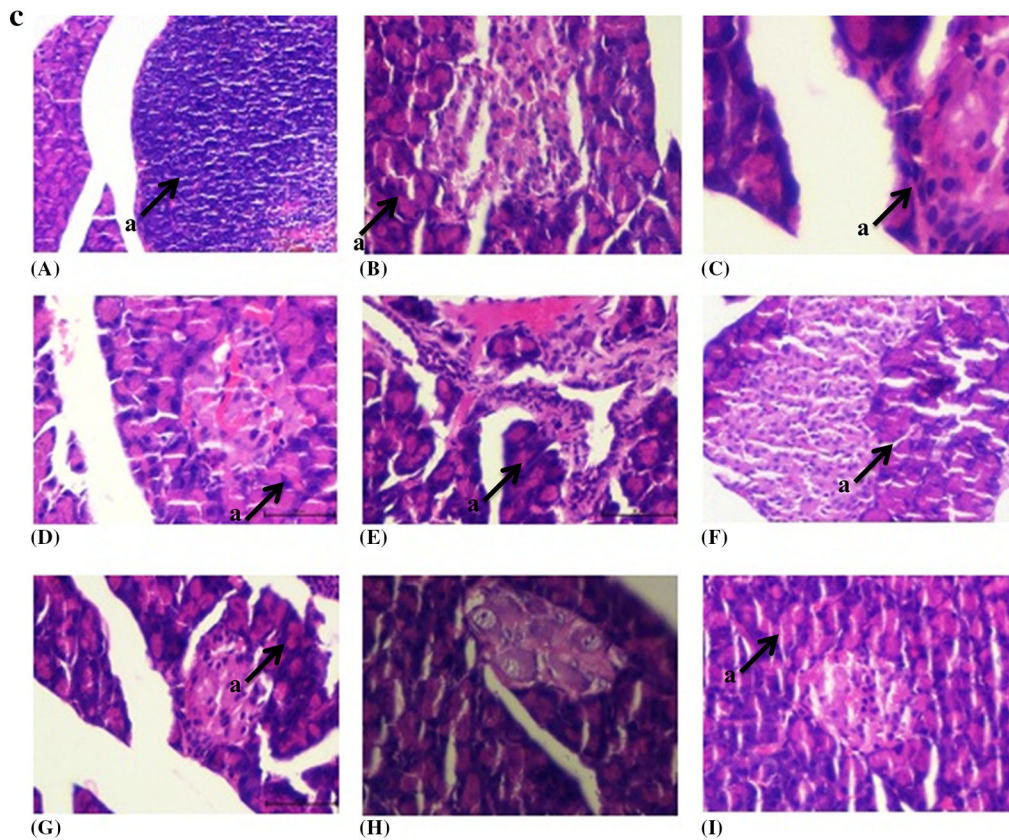


Fig. 3 continued

progress of DN. These changes are a result of abnormal glucose regulation, hemodynamic changes within the kidney and increased oxidative stress (Aurell and Björck 1992). Free radicals attack important macromolecules leading to cell damage and homeostatic disruption, evidently leading to the degenerative diseases that afflict humanity (Tomás-Zapico and Coto-Montes 2007). DN is characterized by a series of renal structure abnormality including basement membrane thickening, mesangial expansion, glomerulosclerosis and tubulointerstitial fibrosis (Abe et al. 2011). These changes are attributed to persistent hyperglycemia and increased levels of BUN, creatinine, urea and uric acid. In the present study, histopathological observations of kidney showed glomeruli with mesangio-capillary proliferation along with amelioration of hemodynamic parameters of kidney. Liver showed perivenular inflammatory collection. Increased oxidative stress due to excess generation of free radicals, lipid peroxidation and decreased level of antioxidant enzymes causes deleterious structural changes in the liver of diabetic nephropathy rats (Polavarapu et al. 1998). The ultra structure of diabetic pancreas showed considerable reduction in the islet langerhans and depleted islets. Induction of hyperglycemia by STZ also produced similar structural alterations in liver, kidney and pancreas of experimental animals in the present

study. Interaction between metabolic and hemodynamic factors adds to the deleterious effects of diabetic nephropathy (Cooper 2001). Good glycemic control ameliorates progression of diabetic nephropathy on both type 1 and 2 diabetes aided by slow development of kidney lesions (Thomas and Karalliedde 2015). Saponins isolated from medicinal plants are found to be renoprotective as they reduce fasting blood glucose and albuminuria, reverses the glomerular hyper-filtration state and ameliorates proliferative glomerular pathological changes during the early stages of diabetic nephropathy in rat models (Zhang et al. 2009). Treatment of DN rats with FPE and FHE effectively reduced the levels of urea, uric acid, creatinine and BUN indicating their increased clearance from kidney. Administration of FPE and FHE reversed these structural alterations in liver, kidney and pancreas of experimental animals. Moreover, administration of FPE and FHE also led to significant reduction in the level of AGEs in kidneys.

Conclusion

It can be concluded that FPE and FHE possess potential renoprotective activity. FPE and FHE ameliorated diabetic nephropathy by attenuating hyperglycemia and oxidative

stress. Moreover, markers of diabetic nephropathy (uric acid, urea, creatinine and BUN level) and lipid profile was also improved after the administration of FPE and FHE in diabetic animals.

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