

## Unveiling the pharmacological potential of *Murraya koenigii* against diabetic nephropathy

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This study aimed to explore the efficacy of various extracts derived from *Murraya koenigii* (L.) in managing diabetic nephropathy (DN). DN was induced via intraperitoneal administration of streptozotocin (65 mg/kg), following a 15-minute pre-treatment with NAD (230 mg/kg), to develop diabetes. Evaluation of DN involved monitoring level of fasting blood glucose, glycated haemoglobin, serum insulin, albuminuria, serum urea, uric acid, creatinine, BUN, and alterations in lipid profile (TC, TG, LDL, and HDL). The oral administration of hydroalcoholic extract derived from *Murraya koenigii* to diabetic rats showed a notable improvement in renal dysfunction, as demonstrated by a marked decrease in inflammatory markers. Moreover, the treated groups experienced significant increase in body weight and notable reductions in fasting blood glucose and glycated haemoglobin levels. The administration of the extracts also effectively alleviated hyperglycemia-induced oxidative stress, evidenced by a noteworthy rise in superoxide dismutase (SOD) and reduced glutathione (GSH) levels, coupled with a considerable decrease in malondialdehyde (TBARS) levels. Additionally, the treatment led to the reversal of histopathological abnormalities observed in the kidneys, pancreas, and liver of diabetic rats. These findings indicate that treatment with *M. koenigii* extracts alleviated DN.

**Keywords:** Diabetic nephropathy, Hyperglycaemia, *Murraya koenigii*, Oxidative stress, Streptozotocin

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### Introduction

Diabetic nephropathy (Kimmelstiel-Wilson syndrome) is defined as a partial loss of function of the kidney associated with nephrotic syndrome, glomerulosclerosis, persistent albuminuria, declining glomerular filtration rate (GFR), elevated arterial blood pressure and fluid retention<sup>1</sup>. It is also called nodular diabetic glomerulosclerosis or intercapillary glomerulonephritis. Albuminuria more than 300 mg/day or 200 mcg/min tested at least two times in 3-6 months characterises diabetic nephropathy (DN)<sup>2</sup>. DN is one of the main reasons for end stage renal diseases (ESRD) in the United States. Characteristic histological changes like glomerular basement membrane (GBM) thickening and mesangial expansion develop along with diabetic albuminuria in humans. As albuminuria advances and renal insufficiency proceeds, glomerulosclerosis, arteriolar hyalinosis, and tubulointerstitial fibrosis

develop<sup>3</sup>. Diabetic nephropathy is a chronic complication of both type 1 DM and type 2 DM. Chronic hyperglycaemia results in oxidative stress that leads to increased polyol pathway flux, hexosamine flux, activation of protein kinase C (PKC) isoforms, and advanced glycation end products (AGEs) formation, which alters the protein functioning and initiates pathological cellular responses. The activation of various signal transduction cascades, which release pro-inflammatory and pro-fibrotic mediators, alters the intrarenal haemodynamic. Together, these result in the structural and functional alterations of kidney<sup>4,6</sup>.

Hyperglycaemia and oxidative stress together are responsible for the development and progression of microvascular complications of diabetes like diabetic neuropathy, diabetic nephropathy, etc<sup>7,8</sup>. Thus, there remains an urgent need to find new compounds that not only produce antidiabetic effects but also ameliorate the complications associated with diabetes. The progression of diabetic complications can be lessened by the

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debilitation of hyperglycaemia and oxidative stress<sup>8</sup>. Pharmacotherapy of diabetes is very intricate. The synthetic medicines available for the pharmacotherapy of diabetes mellitus have a large number of side effects. However, herbal drugs that are considered safer than synthetic drugs are not completely without side effects. So, food or food supplements having medicinal value can have an upper edge in the management of diabetes and the complications of diabetes. More than 75% of the world population mainly depends on extracts of plants for health, and 30% of plants find their use as medicine<sup>8</sup>.

*Murraya koenigii* (L.) Sprengel (Rutaceae) is commonly called 'Curry patta' or karipatta in Indian dialects<sup>9</sup>. *M. koenigii* originated in Uttar Pradesh and is now found all over India. It is also found in Australia, China, and Pacific islands<sup>10</sup>. Various phytoconstituents have been isolated from the leaves e.g., carotenoids, carbazole alkaloids<sup>11,12</sup> and coumarin glycoside<sup>13</sup>. It also contains calcium, phosphorus, iron, thiamine etc. Essential oil is also obtained from leaves<sup>14</sup>. *M. koenigii* possess antioxidant<sup>9-13</sup>, antifungal<sup>15</sup>, antibacterial<sup>15</sup>, mosquitocidal, anti-carcinogenic<sup>16</sup>, hepatoprotective<sup>17</sup>, anti-caries<sup>18</sup>, anti-hyperlipidaemic<sup>19</sup>, anti-diabetic<sup>20</sup>, vasodilating activity<sup>21</sup>, anti-lipidperoxidative<sup>22</sup>. The oil is used externally for eruptions and bruises. Various parts of *M. koenigii* have been used in traditional and folk medicine to treat several traumatic injuries, snake bites, and rheumatism. It has been reported to possess antioxidant, anti-dysenteric and antidiabetic activities. Curry leaves are used as stimulants and in managing diabetes mellitus in traditional medicine<sup>23</sup>.

Therefore, the present study evaluated the protective role of hydroalcoholic extract of *M. koenigii* leaves in diabetic nephropathy in rats.

## Materials and Methods

### Animals

The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of M. M. College of Pharmacy, Maharishi Markandeshwar (Deemed to be) University, Mullana, Ambala, Haryana, India, (MMCP/21/IAEC/24). Animals were kept as per the guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of Environment and Forest, Government of India. Adult male Wistar rats weighing 250-300 gm were housed in standard environmental conditions maintained at 23±2°C with 12 hours light-dark cycle. They were fed with a standard rodent diet

(Ashirwad Industries, Chandigarh, India) and tap water *ad libitum*.

### Collection of plant material

Fresh leaves of *M. koenigii* were purchased from the local market of Ambala on April 2020, Haryana, India and were authenticated by Dr. K. Madhava Chetty, Department of Botany, Sri Vankateshwara University, Tirupati, India. The plant specimen (voucher no. 0384) is available in the herbarium of the University for future reference.

### Preparation of extract

Fresh leaves of *M. koenigii* were shade-dried and crushed to powder. Powdered leaves were extracted sequentially with hydro-alcohol (40%) by Soxhlet extraction. The extract was then filtered and concentrated using a rotary evaporator (40°C) under reduced pressure. The crude extracts were used for further studies.

### Chemicals

Streptozotocin (STZ) was obtained from MP Biomedicals, LLC and nicotinamide (NAD) from Sigma-Aldrich, Milwaukee, USA. Diagnostic kits for different biochemical estimations were obtained from Erba Mannheim by Transasia Bio-Medical Ltd. All other chemicals used were of analytical grade and were obtained from Molychem, India.

### Phytochemical screening

Phytochemical analysis was carried out to determine the chemical constituents like alkaloids, fixed oils, steroids, phenolic compounds, fats, terpenoids, tannins, saponins, glycosides, and flavonoids present in hydroalcoholic extract of *M. koenigii* leaves according to the methods given by Trease, Evans, and Harborne<sup>24,25</sup>.

### Liquid chromatography-mass spectroscopy (LC-MS) analysis

LC-MS analysis was performed on an XEVO TQ-S triple quadrupole mass spectrometer (Waters, USA) controlled by Mass Lynx software (version 4.1). The instrument was linked to an Acquity I-Class LC system (Waters, USA). Chromatography was performed using a gradient combining solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). Separation was carried out using Acquity UPLC BEH Shield C18 column, 2.1 mm × 50 mm, 1.7 µm (Waters, USA). The gradient elution was carried out using the method by commencing 0% solvent B for 2 minutes, followed by a linear increase to 60% solvent B at 2 minutes, which was maintained for 15 minutes. The percentage of this

solvent was then gradually decreased to 0% over a 0.5-minute period, which was maintained until the chromatographic run. The column temperature was maintained at 40°C. The injection volume was kept at 5 µL, and the run time was 6.5 minutes. MS data were acquired in positive mode with an ion spray voltage of 3000 V, a source and a desolvation temperature set to 450°C. The entrance potential was kept at 12 eV, and cone voltage was set at 40 V.

#### FTIR

Dried powder of hydroalcoholic extract of plant material was used for FTIR analysis. 10 mg of dried extract powder was encapsulated in 100 mg of KBr pellet to prepare a translucent sample disc. The powdered sample of plant specimen was loaded in FTIR Spectroscopy (Shimadzu, IR Affinity1, Japan), with a scan range from 400-4000 cm<sup>-1</sup> with a resolution of 4 cm<sup>-1</sup>.

#### Isolation of the compound from *M. koenigii*

The hydroalcoholic extract of *M. koenigii* (50 g) was subjected to column chromatography to isolate phytoconstituents. The silica gel with 100-200 mesh was used as a stationary phase. The elution was carried out using mobile phase in an increasing polarity manner using solvents mixtures of hexane, chloroform, ethyl acetate, ethanol and methanol in different ratios, i.e., 90:10, 80:20, 70:30 and 50:50, respectively. Fraction containing ethyl acetate and ethanol (80:20) yielded an amorphous yellow powder.

#### Thin layer chromatography (TLC)

The TLC chamber for the isolated compound was set, and several solvents in different ratios (9.5:0.5, 9:1, 7:3, 1:1) were used. F254 pre-coated aluminium plates were used to run the compound. The vanillin-sulphuric acid reagent was used as a visualisation agent. The retention factor (Rf) value was calculated as

$$R_f = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent}}$$

#### UHPLC/MS analysis

HPLC-MS/MS analysis was performed on an XEVO TQ-S triple quadrupole mass spectrometer (Waters, USA) controlled by Mass Lynx software (version 4.1). The instrument was linked to an Acquity I-Class LC system (Waters, USA). Chromatography was performed using a gradient combining solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). Separation was achieved using a gradient elution solvent system in the ratio of 30:70 for 5 minutes initially, which was subsequently changed to 40:60 for 3 minutes and 50:50 for 6 minutes, at a flow rate of 0.5 mL/min and a temperature of 40°C. MS data were acquired in negative mode with an ion spray voltage of 3000 V, a source and a desolvation temperature set to 450°C. The entrance potential was kept at 12 eV, and cone voltage was set at 40 V.

#### Characterisation of quercetin

The isolated quercetin from ethyl alcohol and ethanol (80:20) fraction was analysed for identification using XEVO TQ-S triple quadrupole mass spectrometer (Waters, USA) controlled by Mass Lynx software (version 4.1). The isolated sample was diluted in methanol along with silica gel G. The methanol was evaporated, and then the extract containing isolated quercetin was reconstituted in 2 mL acetonitrile. The sample was vortexed, and then 0.1% formic acid was added to the sample before filtering it in a 0.22-micron propylene syringe filter. The reconstituted sample was then injected into the LC-MS for analysis.

#### Induction of diabetic nephropathy

##### Experimental protocol and design

The experimental protocol for diabetic nephropathy is outlined in Fig. 1.

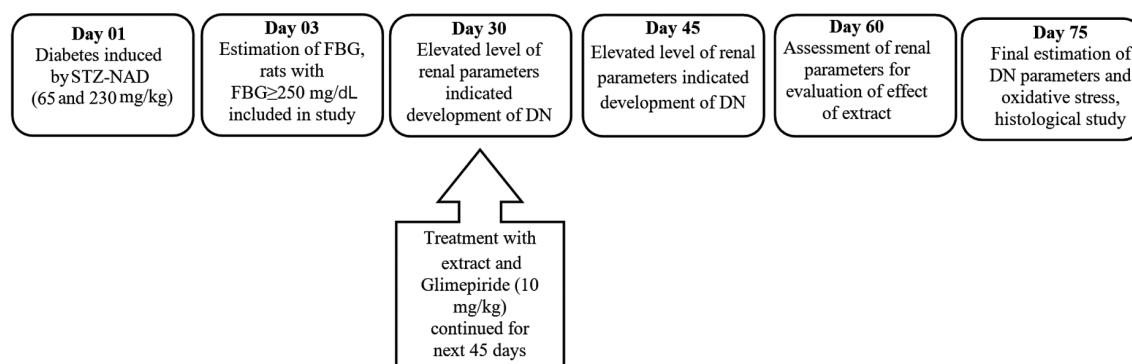


Fig. 1 — Experimental protocol for diabetic nephropathy.

#### **Estimation of parameters**

Body weight, food intake, water intake, and urine output were measured throughout the study and biochemical parameters like fasting blood glucose, insulin, renal function, lipid profile and liver function were estimated by using diagnostic kits on 30<sup>th</sup>, 45<sup>th</sup>, 60<sup>th</sup>, and 75<sup>th</sup> day after the injection of streptozotocin<sup>25-32</sup>.

#### **Estimation of urine output**

For measuring urine output, animals were kept in metabolic cages, and urine was collected for 24 hours at a specified time. The collected urine was measured and stored at 4°C for further analysis.

#### **Estimation of antioxidant enzyme levels**

To estimate the levels of antioxidant enzymes, homogenates of kidney, pancreas, and liver tissues were prepared. Tissues from both control and experimental groups were collected, rinsed with ice-cold saline, and then homogenised in 0.1 M Tris-HCl buffer at pH 7.4 at 4°C. The homogenates were subsequently centrifuged at 3000 g for 10 minutes at 4°C. The resulting supernatant was collected and utilised for biochemical estimations. The protein levels in the tissue homogenate were determined using the BSA method<sup>33</sup>.

#### **Estimation of reduced glutathione**

Reduced glutathione (GSH) was estimated according to the method described by Beutler *et al.*<sup>34</sup>. Equal volumes of the supernatant of tissue homogenate and 10% w/v TCA were mixed well and centrifuged (1000 rpm at 4°C for 10 minutes) and supernatant collected. 2 mL of 0.3 M disodium hydrogen phosphate and 0.25 mL of 0.001 M DTNB was added to supernatant and absorbance was measured at 412 nm and reduced glutathione was expressed as  $\mu\text{M}/\text{mg}$  protein.

#### **Estimation of superoxide dismutase**

To 50  $\mu\text{L}$  of homogenate, 1.15 mL of distilled water, 1.2 mL of sodium pyrophosphate buffer of pH 8.3 (0.052 M), 100  $\mu\text{L}$  post mitochondrial supernatant (186  $\mu\text{M}$ ) + 300  $\mu\text{L}$  of NBT (300  $\mu\text{M}$ ) + 200  $\mu\text{L}$  of NADPH (780  $\mu\text{M}$ ), 50  $\mu\text{L}$  of potassium phosphate buffer of pH 7.5 (0.1 M) were added for control reading replacing enzyme source and Optical density was measured in both control and test samples. The activity was expressed as unit enzyme/mg protein<sup>35-37</sup>.

#### **Estimation of lipid peroxidation**

Quantitative measurement of thiobarbituric acid reactive species (TBARS) (an index of lipid peroxidation) in the kidney, pancreas, and liver were

performed according to the method of Ohkawa *et al.*<sup>38</sup>. Comparison was carried out between absorbance of extracts treated groups and MDA standard; Final concentration was expressed as nanomoles per mg of protein.

#### **AGEs estimation in kidney**

Briefly, the kidney was homogenised in 0.25 M sucrose and centrifuged at 900 rpm at 5°C. The pellets were again centrifuged at 900 rpm at 5°C. The supernatant was collected and mixed with previously obtained supernatant, and equal volumes of TCA were added to the supernatant to precipitate proteins precipitated and centrifuged at 4°C at 900 rpm. 1 mL methanol was added twice to remove the lipid fraction and solubilised in 1 mL of 1 N NaOH. Absorbance was measured at 280 nm against the BSA standard curve. AGEs were estimated by Fluorometric estimation at an emission of 440 nm and excitation at 370 nm. Results were expressed as relative fluorescence units (RFU)/mg protein<sup>39</sup>.

#### **Histopathology**

Animals were sacrificed, and kidney, pancreas, and liver were harvested and collected in 10% neutral buffered formalin solution, dehydrated in ethanol and embedded in paraffin and sections of 5  $\mu\text{m}$  thickness were prepared using a rotary microtome and stained with hematoxylin and eosin (H & E) dye for microscopic observations<sup>39</sup>.

#### **Statistical analysis**

GraphPad Prism 6 software and one-way analysis of variance (ANOVA) followed by Tukey's multiple tests were used for statistical analysis. Values were expressed as mean $\pm$ SEM.

## **Results**

#### **Phytochemical screening**

The phytochemical screening of hydroalcoholic extract of leaves of *M. koenigii* revealed the presence of chemical constituents such as alkaloids, carbohydrates, glycosides, saponins, terpenoids, phenolic compounds, proteins and flavonoids. The percentage yield of hydroalcoholic extract was found to be 24.45%.

#### **LC-MS analysis**

The LC-MS analysis of a hydroalcoholic extract of leaves of *M. koenigii* was carried out, and the interpretation of peaks was carried out by using a database of National Institute Standard and Technology (NIST-14) stored in the LC-MS library.

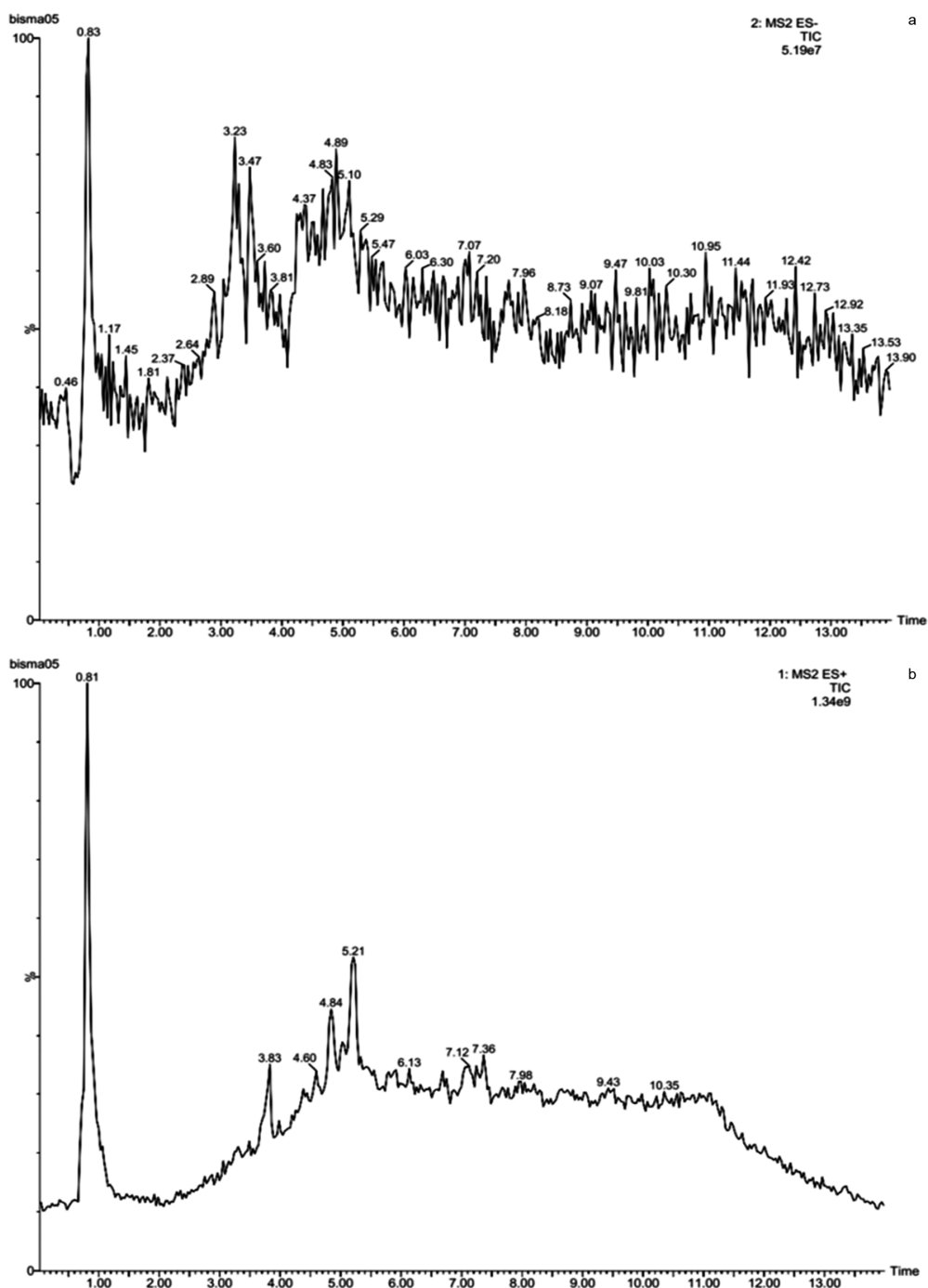


Fig. 2 — Positive and Negative LC-MS chromatograms of *M. koenigii* extracts, a) Negative chromatogram of MKHA; and b) Positive chromatogram of MKHA.

LC-MS result analysis includes the active principles with their formula, molecular mass, exact mass retention time (in minutes) and mode.

#### LC-MS analysis of *M. koenigii* (L.) hydroalcoholic extract

The LC-MS analysis of the hydroalcoholic extract of leaves of *M. koenigii* revealed the presence of several

flavonoids, terpenoids and phenolic acids such as quercetin, apigenin, myricetin, rutin, kaempferol, naringin, blumenol,  $\beta$ -sitosterol etc. (Fig. 2a, b and Table 1).

#### FT-IR analysis

The hydroalcoholic extract of leaves of *M. koenigii* was subjected to FT-IR analysis to identify different

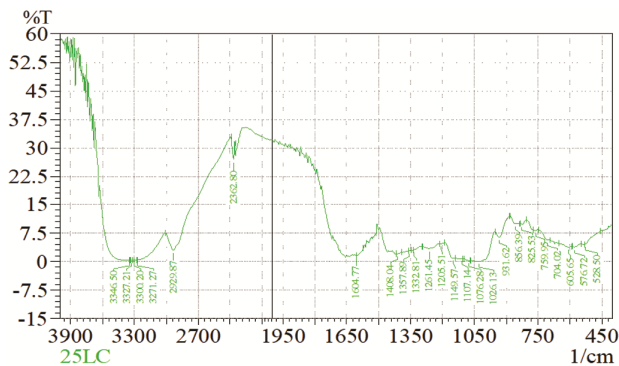
Table 1 — The LC-MS analysis of hydroalcoholic extract of leaves of *M. koenigii* (L.)

S. No.	Name	Formula	Mol. mass	Extract mass	RT	Mode
Flavonoids						
1	Quercetin	C15H10O7	302.23	301.2214	5.2	-
				302.0667	5.2	-
				302.3267	5.2	-
2	Apigenin	C15H10O5	270.24	269.1693	4.5	-
3	Kaempferol	C15H10O6	286.24	286.2031	3.5	-
4	Rutin	C27H30O16	610.1533	610.2256	0.7	+
				609.9656	4.3	-
				610.8757	4.8	+
5	Catechin	C15H14O6	290.27	289.8440	0.7	-
				289.0638	4.3	-
6	Myricetin	C15H10O8	318.23	317.1499	5.2	-
				318.0601	4.8	-
				318.1901	4.8	-
7	4-O- $\beta$ -d-Rutinosyl-3-methoxyphenylpropanone	C22H32O12	488.5	487.1587	3.2	-
				489.1740	0.8	+
8	1-O- $\beta$ -d-Rutinosyl-2 9(R)-ethyl-1-pentanol	C19H36O10	424.5	425.7871	1.1	+
9	8-Phenylethyl-O- $\beta$ -d-rutinoside	C20H30O10	430.4	431.8332	0.7	+
10	Epicatechin	C15H30O10	290.27	289.8440	0.7	-
				289.0638	4.3	-
11	Naringin	C27H32O14	580.5	581.0355	3.7	+
				580.2554	5.2	-
				579.3453	5.2	-
				580.5154	5.2	-
Terpenoids						
1	Blumenol A	C13H20O3	224.30	223.9187	6.1	-
2	IcarisideB1	C19H30O8	386.4	386.9094	0.7	+
3	Loliolide/ (-)-Epiloliolide	C11H16O3	196.24	195.7019	1.1	-
				197.3272	4.5	+
				195.1167	4.8	-
				195.2467	4.8	-
4	$\beta$ -sitosterol	C29H50O	414.7	415.3200	7.3	+
				415.1900	4.8	+
				415.3200	4.8	+
5	$\alpha$ -copaene/ $\alpha$ -selinene	C15H24	204.35	203.7638	3.5	-
				203.3737	5.07	-
				205.0642	5.2	+
				205.0642	4.3	+
				204.9991	5.2	+
6	Juniper camphor	C15H26O	222.37	223.9187	6.1	+
				221.1881	4.3	-
				222.2283	0.8	-
				221.2531	4.8	-
Phenolics						
1	Selin-11-en-4-ol	C15H26O	222.37	223.9187	6.1	+
				221.1881	4.3	-
				222.2283	0.8	-
				221.25.31	4.8	-
2	Gallic acid	C7H6O5	170.12	---	---	---
3	Cinnamic	C9H8O2	148.16	149.0849	5.2	-
				149.1499	6.1	+
				148.9549	4.4	+
				148.8899	5.2	+
				149.1499	5.2	+

(Contd.)

Table 1 — The LC-MS analysis of hydroalcoholic extract of leaves of *M. koenigii* (L.)

S. No.	Name	Formula	Mol. mass	Extract mass	RT	Mode
Phenolics						
4	Ferulic acid	C <sub>8</sub> H <sub>8</sub> O <sub>4</sub>	194.18	195.1167	4.8	+
5	Vanillic acid	C <sub>8</sub> H <sub>8</sub> O <sub>4</sub>	168.15	168.8501	0.7	+
				168.1999	0.8	+
				168.1999	0.7	+
6	Caffeic acid	C <sub>9</sub> H <sub>5</sub> O <sub>4</sub>	180.16	180.6181	1.1	+
7	Chlorogenic acid	C <sub>16</sub> H <sub>28</sub> O <sub>9</sub>	354.31	354.4028	7.3	+

Fig. 3 — FTIR of extracts of leaves of *M. Koenigii*.

functional groups. The results showed several major functional groups, like the hydroxyl group and aromatic –CH stretch, which are the predominant groups for phenolic compounds. The results of the FT-IR analysis of *M. koenigii* are given in Fig. 3.

#### Isolation of active constituent from hydroalcoholic extract of leaves of *M. koenigii*

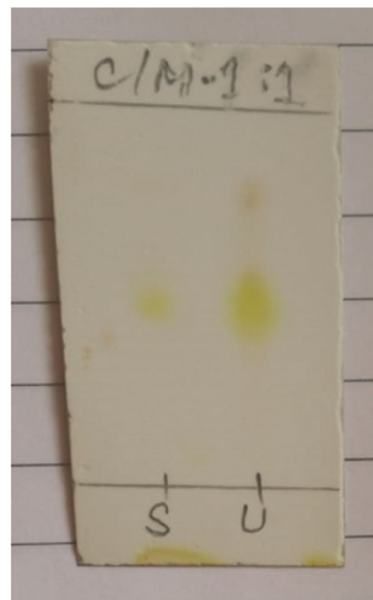
Quercetin was isolated from ethyl acetate and ethanol (80:20) fraction as an amorphous yellow powder. The melting point of the isolated compound was found to be 316–18°C. Besides this, the hydroalcoholic extract of leaves of *M. koenigii* showed positive chemical tests for flavonoids and LC-MS, as well as FTIR results, revealed the presence of quercetin in the hydroalcoholic extract of leaves of *M. koenigii*.

#### Thin layer chromatography (TLC)

This fraction in TLC mobile phase solvent chloroform: methanol (1:1) showed an R<sub>f</sub> value of 0.46, which was equal to that of standard quercetin (Fig. 4).

#### UHPLC/MS Analysis

The quercetin has a molecular weight of 302, which is confirmed by the mass spectra (M+H) of Quercetin in Fig. 5. The isotopic peak of 304 confirms the elemental C-13 carbon in the quercetin. The mass-based fingerprint of the Quercetin was confirmed by fragmenting the compound, resulting in the formation of daughter ions indicated in Fig. 6. The

Fig. 4 — TLC of isolated quercetin from hydroalcoholic extract of leaves of *M. koenigii* (L.) and standard quercetin.

Compound showed that [M+H]<sup>+</sup> molecular ion was at m/z 303.04 and other abundant fragment ions, such as ions at m/z 257.17, 229.13, 201.16, 165.11, 153.01, 137.01 and 95.04. Among them, fragments were more commonly formed by cracking 1, 3 bonds. Meanwhile, it was compared with the standard product information in Fig. 7 and confirmed as quercetin.

#### Quantitative estimation of phytoconstituents in hydroalcoholic extract of leaves of *M. koenigii*

The phytoconstituents were quantitatively estimated for total phenolic content (TPC) and total flavonoid content (TFC) in hydroalcoholic extract of leaves of *M. Koenigii*. The results are summarized as TAOA (AAE) = 458.78 μM/g, TPC (GAE) = 172.30 mg/g.

#### Diabetic nephropathy

The assessment of diabetic nephropathy (DN) was evaluated by assessing different morphological parameters such as body weight, water intake, food intake, urine output (Table 2), and biochemical

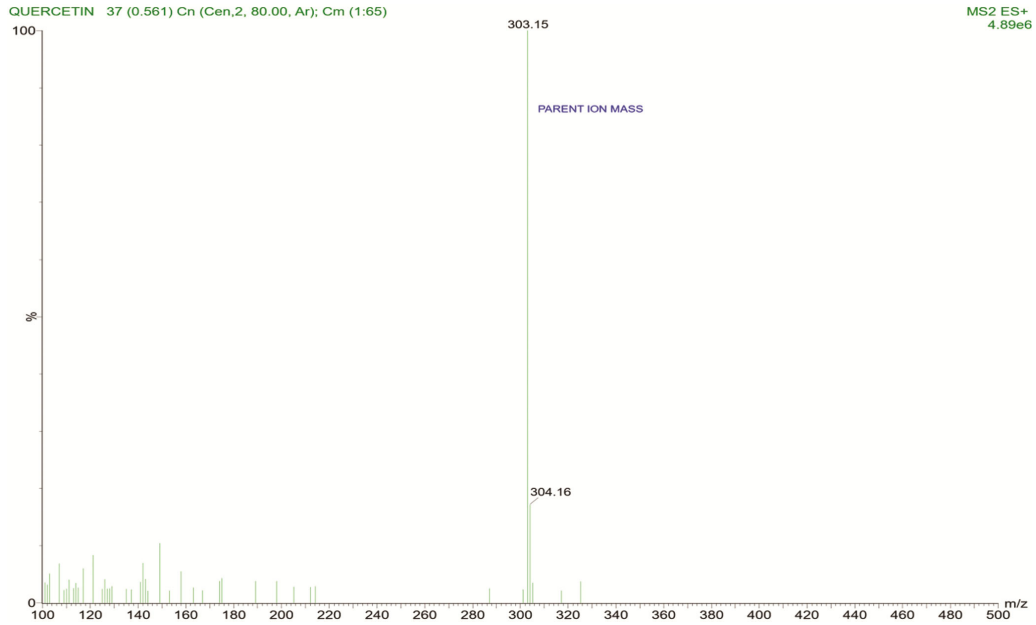


Fig. 5 — Molecular ion peak of quercetin.

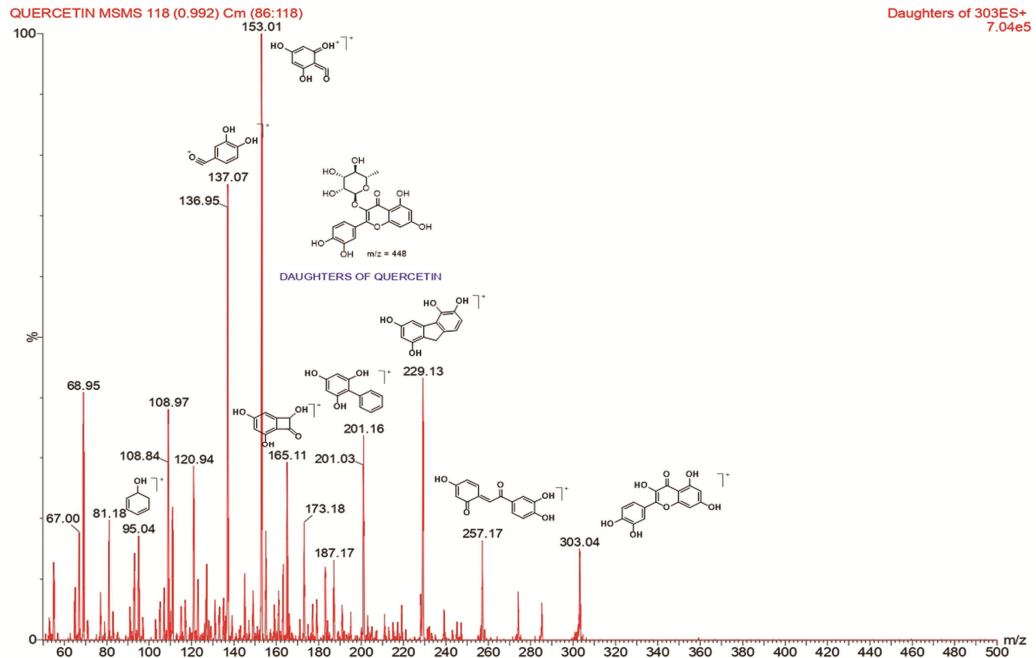


Fig. 6 — The LC-MS of isolated quercetin; Mass range- 100-1000; Injection volume-10  $\mu$ L; Run time- 1 min; Flow rate-0.3 mL/min; Detector-MS; Ion source-ESI (-VE); Ion spray voltage-3000; Nebulizer gas curtain gas-12; Temperature-450°C; Entrance potential-12.

parameters such as fasting blood glucose level, insulin level, HbA1c level, renal function parameters like serum urea level, uric acid level, blood urea nitrogen level (BUN), urine albumin level, creatinine level and kidney index as well as lipid profile like triglycerides, cholesterol level, high density lipoprotein level (HDL), and low density lipoprotein level(LDL). AST/

SGOT and ALT/SGPT levels were also assessed. Development of DN was suggested by the significant changes in renal function parameters after 30 days of STZ-NAD administration. The morphological and structural changes in the kidney, liver, and pancreas of experimental animals were studied by carrying out the histology of these organs.

An *in-vivo* evaluation of DN was carried out using *M. Koenigii* hydroalcoholic extract (MKHA) from leaves of *M. koenigii*. Different doses of MKHA (125, 250, 500 mg/kg body weight) were administered orally, after 30 days of induction of STZ-NAD, i.e., after the DN onset till the 75<sup>th</sup> day.

#### Effect on body weight

No considerable difference was observed in the body weight of normal control rats  $254 \pm 3.02$  g and diabetic control rats  $256 \pm 3.56$  g on basal day. However, a significant decrease in body weight was observed in diabetic control rats at the end of the study compared to normal control rats. Oral administration of different doses of MKHA reduced loss in body weight from the 30<sup>th</sup> to the 75<sup>th</sup> day (Fig. 8).

#### Effect on food and water intake

Food intake and water intake were significantly increased in diabetic control rats as compared to normal control rats. Administration of MKHA125,

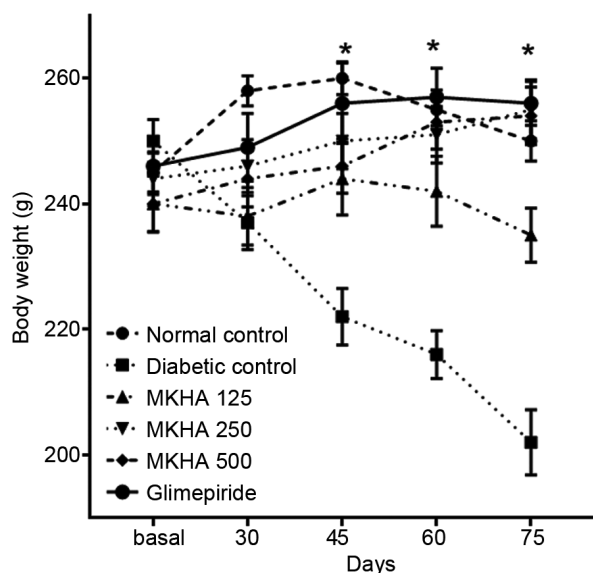


Fig. 7 — The MS/MS of standard quercetin (The m/z ratio of peaks correspond to the individual daughter ions along with the structures).

250 & 500 mg/kg extract attenuated food and water intake in a dose-dependent manner (Table 2).

#### Effect on fasting blood glucose level

Glucose level was measured at different intervals during the experimentation period, i.e., 0<sup>th</sup> (basal day), 30<sup>th</sup>, 45<sup>th</sup>, 60<sup>th</sup> and 75<sup>th</sup> day. Administration of STZ-NAD led to a marked elevation in fasting blood glucose levels in experimental animals.

Oral administration of different doses (125, 250, 500 mg/kg) of MKHA and glimepiride (10 mg/kg) attenuated glucose levels in experimental animals as compared to diabetic control rats ( $393.21 \pm 1.59$  mg/dL) ( $P < 0.001$ ). Moreover, the attenuating effect was found to be dose dependent also (Fig. 9).

#### Effect on serum insulin level

Serum insulin was measured at the start and end of the study. A marked reduction in serum insulin level was observed in the diabetic control group compared to the normal control at the end of the study (75<sup>th</sup> day). Administration of 125, 250, and 500 mg/kg

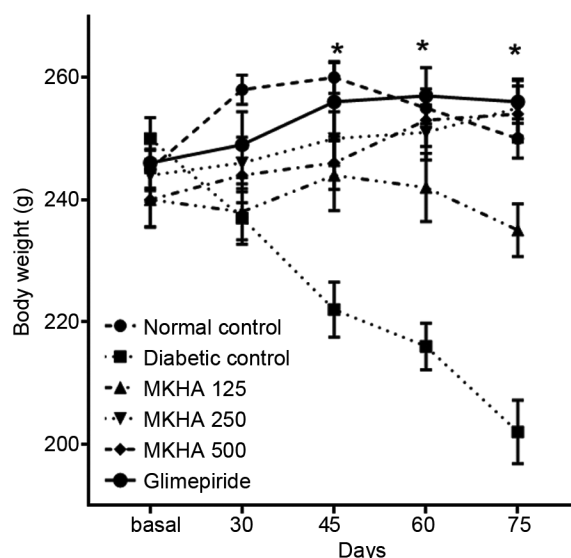


Fig. 8 — Effect of *M. koenigii* (L.) leaf extracts (MKHA) on body weight (g) in diabetic nephropathy Wistar rats.

Table 2 — Effect of *M. koenigii* (L.) leaf extract (MKHA) on water intake, food intake and urine output

Parameters	Water intake (mL/rat/day)		Food intake (mg/rat/day)		Urine output (mL/rat/day)	
	30th day	75th day	30th day	75th day	30th day	75th day
Normal	48.97 $\pm$ 1.457	49.77 $\pm$ 1.070	26.14 $\pm$ 0.872	25.22 $\pm$ 0.307	15.25 $\pm$ 0.849	15.73 $\pm$ 0.538
Diabetic	142.5 $\pm$ 0.846 <sup>###</sup>	146.4 $\pm$ 0.230 <sup>###</sup>	75.05 $\pm$ 1.297 <sup>###</sup>	74.95 $\pm$ 1.132 <sup>###</sup>	84.86 $\pm$ 1.538 <sup>###</sup>	86.60 $\pm$ 0.870 <sup>###</sup>
MKHA 125 mg/kg	143.2 $\pm$ 0.894	115.5 $\pm$ 1.207*	74.27 $\pm$ 1.272	61.61 $\pm$ 0.683*	84.45 $\pm$ 0.738	59.75 $\pm$ 0.683***
MKHA 250 mg/kg	142.4 $\pm$ 0.588	101.0 $\pm$ 1.019**	70.17 $\pm$ 1.207	54.26 $\pm$ 0.620**	84.51 $\pm$ 0.498	53.88 $\pm$ 0.385***
MKHA 500 mg/kg	142.6 $\pm$ 0.908	85.01 $\pm$ 0.971***	74.12 $\pm$ 1.901	46.29 $\pm$ 0.733***	85.04 $\pm$ 0.992	42.07 $\pm$ 1.941***
Glimperide 10 mg/kg	141.9 $\pm$ 0.854	69.78 $\pm$ 0.774***	68.53 $\pm$ 0.934	51.73 $\pm$ 1.313**	85.03 $\pm$ 1.708	45.23 $\pm$ 1.012***

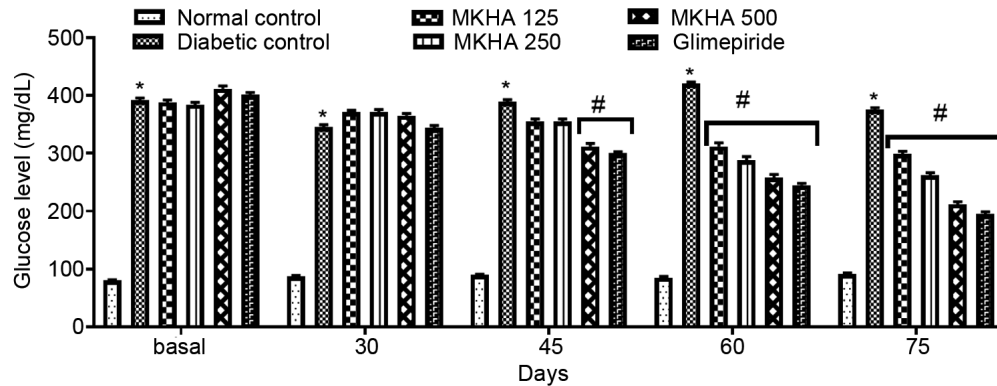


Fig. 9 — Effect of *M. koenigii* (L.) leaf extracts (MKHA) on fasting glucose level (mg/dL) in diabetic nephropathy Wistar rats.

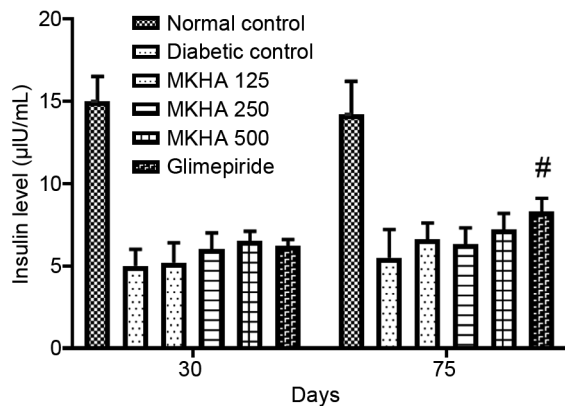


Fig. 10 — Effect of *M. koenigii* (L.) leaf extracts (MKHA and MKAQ) on serum insulin level (µIU/mL) in diabetic nephropathy Wistar rats.

doses of MKHA for 45 days exhibited a significant increase in serum insulin level (Fig. 10).

#### Effect on renal function

Serum urea, uric acid, creatinine, BUN and kidney index are imperative indicators of renal function. These renal functional markers were found to be elevated in diabetic rats in comparison to the normal control group. MKHA (125, 250, and 500 mg/kg doses) reduced urine output, serum urea level, serum uric acid level, serum creatinine level and serum BUN level (Table 3).

#### Effect on kidney index

Kidney index (kidney weight/body weight ratio) increased in diabetic control rats ( $0.99 \pm 0.02\%$ ) compared to normal control. Oral administration with MKHA 125, 250, and 500 mg/kg doses significantly alleviated the kidney index in comparison to diabetic control rats. Glimepiride at a dose of 10 mg/kg also reduced kidney index (Table 3).

#### Effect on lipid profile

A marked increase in serum triglyceride, total cholesterol, and LDL and a decrease in HDL was evident after the administration of STZ-NAD in experimental rats, and the administration of MKHA (125, 250, and 500 mg/kg) significantly attenuated elevated triglycerides and serum cholesterol levels. Whereas the level of serum HDL was found to be elevated after administration of MKHA (125, 250, 500 mg/kg) in experimental animals (Table 4).

#### Effect on liver enzymes

A significant elevation in AST level and ALT level (was observed in diabetic control rats in comparison to the normal control group. Oral administration of 125, 250, and 500 mg/kg doses of MKHA significantly reduced the elevated level of serum AST and ALT in diabetic rats in comparison to diabetic control rats at the end of the study (Table 5).

#### Effect on antioxidant enzymes (GSH, SOD and TBARS)

The level of antioxidant enzymes (SOD and GSH) reduced significantly in the kidney, pancreas and liver of diabetic rats in comparison to the normal control group and treatment with 125, 250 & 500 mg/kg doses of MKHA increased the level of GSH and SOD in a dose-dependent manner in experimental animals. Whereas elevated level of TBARS was found to be attenuated significantly in the kidney, pancreas and liver of diabetic rats after administration of MKHA (125, 250, and 500 mg/kg) in experimental animals (Table 5).

#### Effect on AGEs in kidney

Induction of diabetes in rats resulted in a substantial increase in the level of AGEs in the kidney in comparison to the normal control group. Administration of MKHA significantly alleviated

Table 3 — Effect of *M. koenigii* (L.) leaf extract (MKHA) on renal function tests

Parameters	Serum urea (mg/dL)	Uric acid (mg/dL)	Creatinine (mg/dL)	BUN (mg/dL)	Kidney Index (%)	Urine output (mL/rat/day)
Groups	75 <sup>th</sup> day	75 <sup>th</sup> day	75 <sup>th</sup> day	75 <sup>th</sup> day	75 <sup>th</sup> day	75 <sup>th</sup> day
Normal	35.74±0.46	5.28±0.19	0.76±0.15	16.70±0.21	0.48±0.04	15.73±0.54
Diabetic	93.03±0.43 <sup>####</sup>	15.56±0.66 <sup>####</sup>	3.58±0.09 <sup>####</sup>	43.47±0.20 <sup>####</sup>	0.99±0.02 <sup>####</sup>	86.60±0.87 <sup>####</sup>
MKHA 125 mg/kg	68.73±0.90 <sup>**</sup>	10.64±0.29 <sup>*</sup>	2.41±0.15	34.47±0.42 <sup>*</sup>	0.71±0.05 <sup>*</sup>	59.75±0.68 <sup>*</sup>
MKHA 250 mg/kg	61.32±0.63 <sup>***</sup>	9.85±0.45 <sup>*</sup>	1.70±0.16 <sup>*</sup>	27.68±0.29 <sup>**</sup>	0.62±0.09 <sup>*</sup>	53.88±0.39 <sup>**</sup>
MKHA 500 mg/kg	50.65±0.56 <sup>***</sup>	8.29±0.34 <sup>*</sup>	1.17±0.24 <sup>*</sup>	22.67±0.26 <sup>***</sup>	0.56±0.07 <sup>**</sup>	42.07±1.94 <sup>**</sup>
Glimperide 10 mg/kg	52.3±1.72 <sup>***</sup>	7.63±0.38 <sup>**</sup>	1.47±0.30 <sup>*</sup>	21.39±0.81 <sup>***</sup>	0.54±0.01 <sup>**</sup>	40.39±2.30 <sup>**</sup>

Here, Data represents Mean±SEM, n=6. \**P*<0.05, \*\**P*<0.01, and \*\*\**P*<0.001 vs diabetic control. ####*P*<0.001 vs normal control

Table 4 — Effect of *M. koenigii* (L.) leaf extract (MKHA) on lipid profile

Parameters	AST (IU/L)		ALT (IU/L)		LDL (mg/dL)		HDL (mg/dL)		TG (mg/dL)		Cholesterol (mg/dL)	
	30 <sup>th</sup> day	75 <sup>th</sup> day	30 <sup>th</sup> day	75 <sup>th</sup> day	30 <sup>th</sup> day	75 <sup>th</sup> day	30 <sup>th</sup> day	75 <sup>th</sup> day	30 <sup>th</sup> day	75 <sup>th</sup> day	30 <sup>th</sup> day	75 <sup>th</sup> day
Normal	37.38±0.381	37.88±0.578	29.01±0.383	53.58±0.795	54.29±0.589	71.04±1.038	71.77±0.707	80.21±3.735	78.08±1.751	29.39±0.350	96.29±1.346	96.26±1.114
Diabetic	70.66±0.576 <sup>####</sup>	84.26±0.731 <sup>####</sup>	59.60±0.322 <sup>####</sup>	31.36±0.787 <sup>####</sup>	26.02±0.180 <sup>####</sup>	162.2±1.325 <sup>####</sup>	202.1±0.180 <sup>####</sup>	136.3±3.595 <sup>####</sup>	170.4±1.344 <sup>####</sup>	72.48±0.463 <sup>####</sup>	164.2±0.870 <sup>####</sup>	275.5±0.954 <sup>####</sup>
MKHA 125 mg/kg	70.98±0.395	57.30±0.563 <sup>*</sup>	59.13±0.284	32.71±0.175	41.71±0.425 <sup>*</sup>	161.4±1.318	117.6±2.878 <sup>**</sup>	141.5±2.217	112.0±1.628 <sup>***</sup>	55.68±0.394 <sup>**</sup>	164.1±1.187	126.1±2.028 <sup>***</sup>
MKHA 250 mg/kg	70.73±0.566	52.20±0.177 <sup>**</sup>	59.64±0.522	32.07±0.173	46.77±0.436 <sup>*</sup>	160.6±1.274	106.5±1.386 <sup>***</sup>	139.3±3.240	100.9±1.017 <sup>**</sup>	50.24±0.430 <sup>**</sup>	165.1±1.387	95.81±1.808 <sup>***</sup>
MKHA 500 mg/kg	70.72±0.655	47.15±0.286 <sup>***</sup>	59.10±0.404	31.99±0.326	51.48±0.542 <sup>*</sup>	161.7±0.923	84.78±1.216 <sup>***</sup>	140.9±2.132	92.52±0.761 <sup>***</sup>	43.88±0.474 <sup>***</sup>	165.0±1.529	84.20±1.984 <sup>***</sup>
Glimperide 10 mg/kg	71.54±0.460	45.61±0.857 <sup>***</sup>	59.40±0.322	32.31±0.369	40.74±0.382 <sup>*</sup>	162.8±1.201	101.2±0.01 <sup>***</sup>	138.1±2.549	96.47±1.398 <sup>***</sup>	42.87±1.022 <sup>***</sup>	165.8±0.581	89.87±2.202 <sup>***</sup>

Here, Data represents Mean±SEM, n=6. \**P*<0.05, \*\**P*<0.01, and \*\*\**P*<0.001 vs diabetic control. ####*P*<0.001 vs normal control

Table 5 — Effect of *M. koenigii* (L.) leaf extract (MKHA) on liver enzymes

Parameters	SOD (U /mg protein)			GSH (µM/mg protein)			TBARS (nmoles /mg protein)			AGEs (RFU/mg protein)
	Kidneys	Pancreas	Liver	Kidneys	Pancreas	Liver	Kidneys	pancreas	Liver	Kidneys
Normal	4.91±0.099	4.22±0.157	3.61±0.102	75.2±0.314	69.4±0.182	66.2±0.336	0.58±0.024	0.47±0.009	0.41±0.015	1.71±0.045
Diabetic	1.11±0.021 <sup>####</sup>	1.12±0.039 <sup>####</sup>	1.08±0.046 <sup>####</sup>	39.1±0.371 <sup>####</sup>	41.4±0.193 <sup>####</sup>	44.7±0.155 <sup>####</sup>	2.96±0.053 <sup>####</sup>	2.72±0.020 <sup>####</sup>	2.48±0.030 <sup>####</sup>	3.84±0.037 <sup>####</sup>
MKHA 125 mg/kg	1.37±0.059	1.31±0.027	1.20±0.025	48.9±0.247 <sup>*</sup>	45.4±0.247	49.3±0.163	1.88±0.125	1.80±0.044 <sup>*</sup>	2.31±0.024 <sup>*</sup>	3.35±0.019
MKHA 250 mg/kg	1.84±0.025	1.67±0.030	1.55±0.030	56.7±0.345 <sup>**</sup>	51.3±0.182 <sup>*</sup>	53.1±0.188 <sup>*</sup>	1.46±0.010	1.48±0.033 <sup>*</sup>	1.85±0.024 <sup>**</sup>	2.59±0.085 <sup>*</sup>
MKHA 500 mg/kg	2.16±0.031	2.09±0.026	1.88±0.043	66.1±0.290 <sup>**</sup>	58.1±0.179 <sup>**</sup>	59.5±0.188 <sup>**</sup>	1.03±0.012 <sup>*</sup>	0.94±0.039 <sup>**</sup>	1.23±0.050 <sup>**</sup>	1.95±0.064 <sup>*</sup>
Glimperide 10 mg/kg	3.37±0.041	2.76±0.066	3.35±0.039	69.1±0.088 <sup>**</sup>	60.6±0.156 <sup>***</sup>	61.5±0.325 <sup>***</sup>	1.25±0.032 <sup>*</sup>	1.01±0.027 <sup>*</sup>	1.16±0.013 <sup>***</sup>	3.15±0.030 <sup>*</sup>

Here, Data represents Mean±SEM, n=6. \**P*<0.05, \*\**P*<0.01, and \*\*\**P*<0.001 vs diabetic control. ####*P*<0.001 vs normal control

AGEs levels in the kidney in comparison to diabetic control rats (3.85±0.04 RFU/mg protein). MKHA at 125, 250, and 500 mg/kg doses reduced AGEs to 3.36±0.02, 2.597±0.09 and 1.96±0.06 RFU/mg protein, respectively (Table 5).

### Histopathology studies

#### Histopathology study of kidney

*M. koenigii* extracts were evaluated by assessing the pathological changes in the kidneys. The diabetic group had signs of different pathological biomarkers

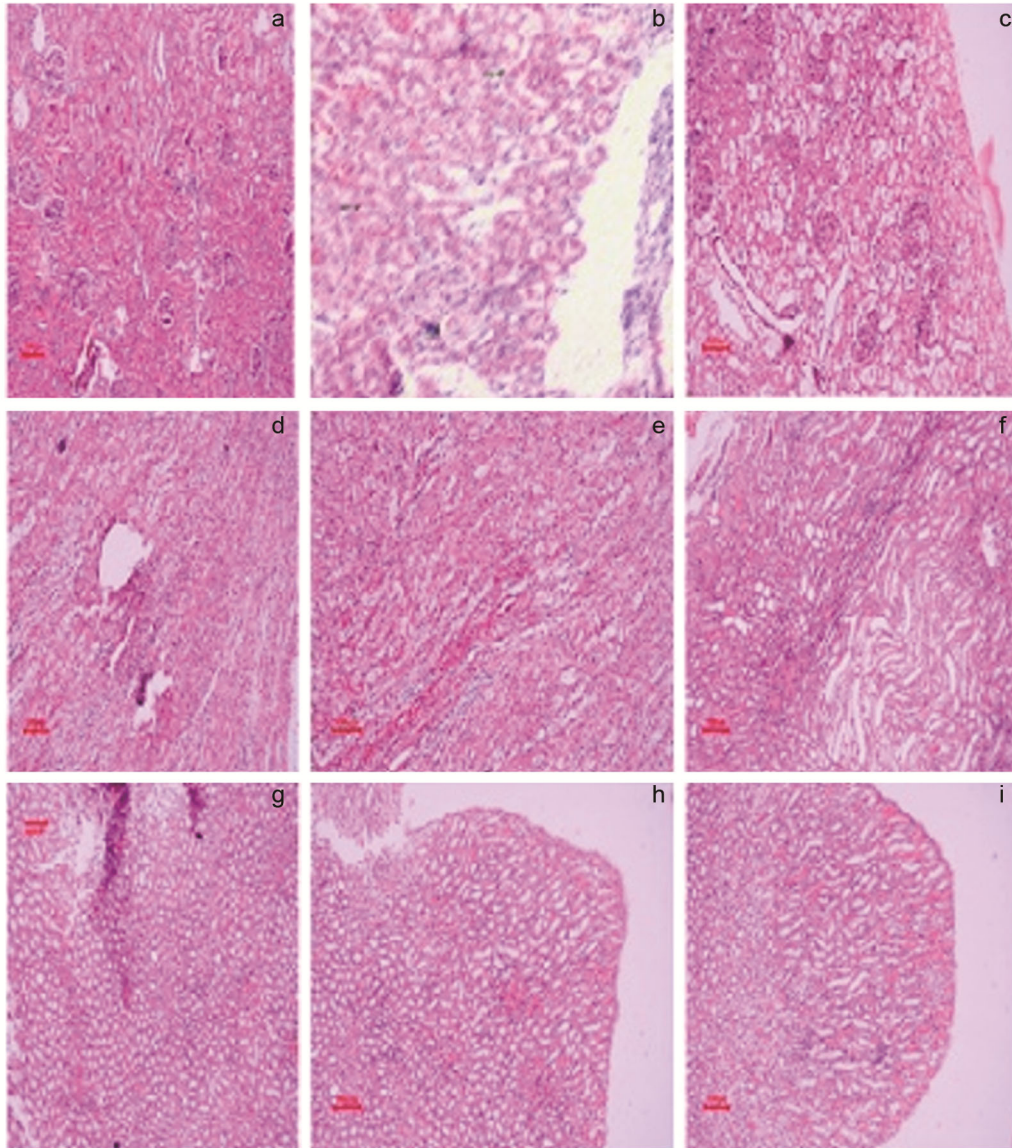


Fig. 11 — Effect of *M. koenigii* (L.) leaf extracts (MKHA and MKAQ) on histology of kidneys in diabetic nephropathy Wistar rats. a) Normal; b) Diabetic; c) Glibenclamide 10 mg/kg; d) MKHA 125; e) MKHA 250; f) MKHA 500; g) MEAQ 125; h) MEAQ 250; and i) MEAQ 500.

of nephropathy, such as thickening of glomerular capillaries, atrophy of glomeruli, increased glomerular space, and thickness of basement membrane as well as mesangial expansion as compared to the normal group, which showed normal renal parenchyma, normal shape and size of glomeruli as well as basement membrane and mesangial thickness. The cortex, medulla and glomeruli were found to be infiltrated with inflammation cells. However, the standard control group was seen to have diminished necrotic condition in convoluted tubules as well as diminished infiltration of inflammation cells in the

cortex and medulla. Moreover, different doses of *M. koenigii* leaves also improved the architecture of the glomerulus by reducing membrane thickness, atrophy of glomeruli and mesangial cell expansion (Fig. 11).

#### *Histopathology study of liver*

The liver of diabetic rats showed hyperplasia of kupffer cells with condensed nuclei, perivenular inflammatory collection and fatty infiltration, while that of the normal rats was seen with normal architecture where the central vein with radiating sinusoid cords was present without sinusoid

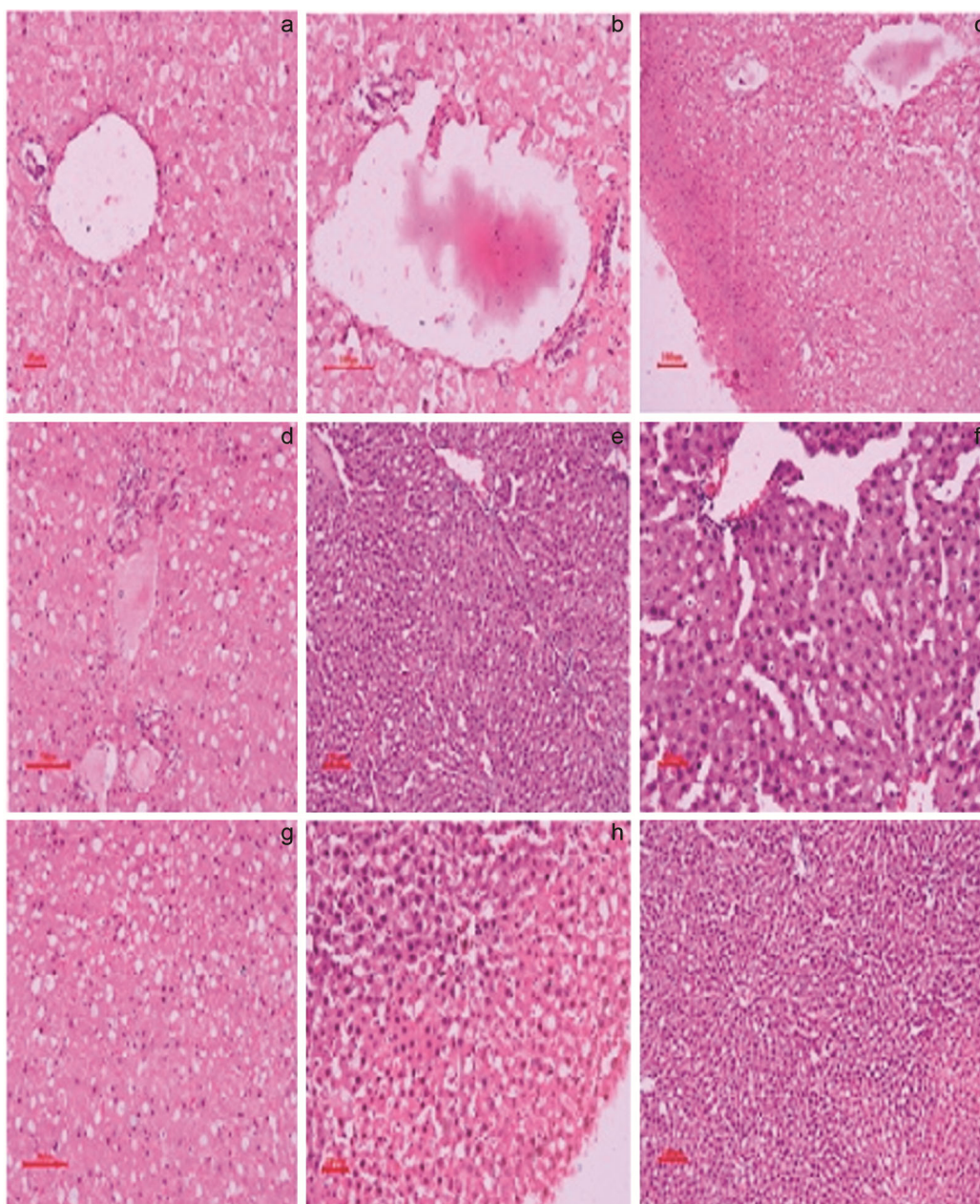


Fig. 12 — Effect of *M. koenigii* (L.) leaf extracts (MKHA and MKAQ) on histology of liver in diabetic nephropathy Wistar rats. a) Normal; b) Diabetic; c) Glimepiride 10 mg/kg; d) MKHA 125; e) MKHA 250; f) MKHA 500; g) MEAQ 125; h) MEAQ 250; and i) MEAQ 500.

congestion, swelling and necrotic cells. Moreover, different doses of *M. koenigii* extracts alleviated these pathological conditions in diabetic rats (Fig. 12).

#### ***Histopathology study of pancreas***

For studying the histopathology of the pancreas, the arrangement of islets cells and acini cells was considered. Normal control group rats were found to have normal shape, normal acini and

islets cells without any signs of inflammation, while as in the case of the diabetic control group, necrosis and shrinkage of islets cells, inflammation and disorganisation of acini cells were observed. Different doses of oral administration of *M. koenigii* extracts showed a protective effect on islets of Langerhans and acini cells and reduced oedema, inflammation and shrinkage of islets (Fig. 13).

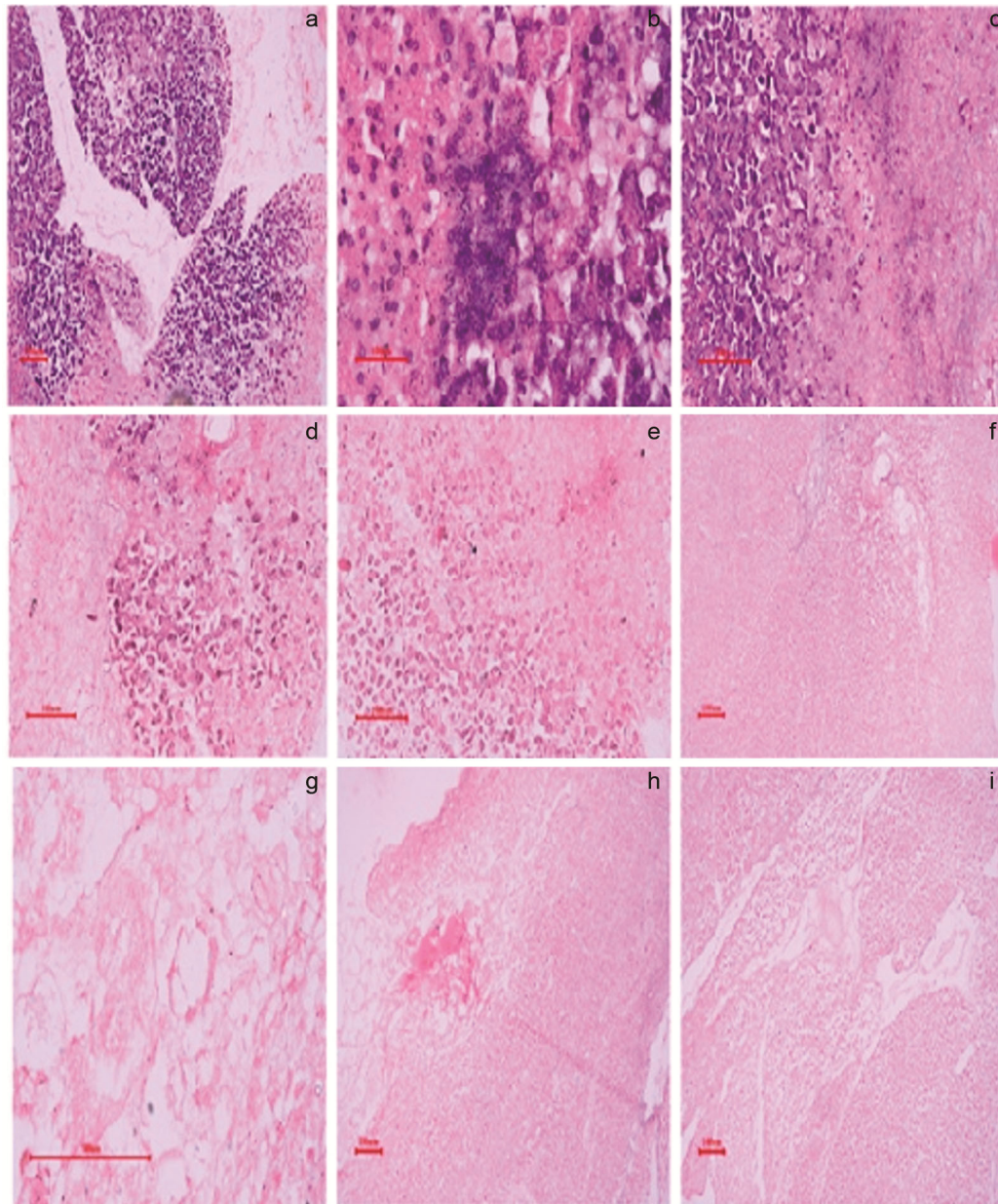


Fig. 13 — Effect of *M. koenigii* (L.) leaf extracts (MKHA and MKAQ) on histology of pancreas in diabetic nephropathy Wistar rats. a) Normal; b) Diabetic; c) Glimpiride 10 mg/kg; d) MKHA 125; e) MKHA 250; f) MKHA 500; g) MEAQ 125; h) MEAQ 250; and i) MEAQ 500.

### Discussion

The qualitative phytochemical analysis of the hydroalcoholic extract of leaves of *M. koenigii* revealed the presence of alkaloids, carbohydrates, glycosides, saponins, terpenoids, phenolic compounds, proteins and flavonoids<sup>40</sup>. FT-IR of the extracts of leaves of *M. koenigii* has revealed main functional groups like aromatic –CH stretch, –OH stretch, –CO stretch and hydroxyl groups, thereby confirming the presence of phenolic compounds. The

results of the LC-MS analysis confirmed the presence of quercetin, rutin, kaempferol, catechin, naringin, and various terpenoids and phenolic acids in the MKHA extracts. The antioxidants present in the food, such as phenolic acids, flavonoids, isoflavones, tocopherols, carotenoids, etc, are comparatively higher, possess important pharmacological activities and have been reported for their beneficial effects in several diseases, etc<sup>41-46</sup>. Various studies have confirmed the antioxidant properties of plants with

respect to different flavonoids, saponins and terpenoids, and these studies emphasized that the flavonoids, saponins and terpenoids can be used as potential drugs to prevent oxidative stress<sup>47,48</sup>. The present study revealed that hydroalcoholic extract of leaves of *M. koenigii* attenuated fasting blood glucose levels in diabetic rats. The diabetic rats showed a characteristic weight loss because of the insufficient level of insulin, which prevented glucose transportation to the cells. Hence, the body starts to utilize fats and muscles for energy, leading to body weight loss<sup>49,50</sup>. The significant decrease in the level of glucose and improvement in body weight may be elucidated by the stimulation of the residual pancreatic  $\beta$ -cells or their regeneration, partially destroyed by STZ. Increased levels of insulin secretion from protected  $\beta$ -cells and an increase in the peripheral utilization of glucose may also contribute to the antihyperglycemic action of extracts. Some previous studies have also demonstrated that improved levels of insulin may influence weight loss in diabetics, which supports our study<sup>49,50</sup>. The biochemical estimations were supported by histopathological examination of the pancreas, which showed disorganized acini cells and necrosis as well as shrinkage of islet cells in the pancreas of diabetic control rats. Moreover, oral administration of extracts with different doses improved the arrangement of acini cells, and the size of islet cells was also regained compared to that of diabetic control rats. It is known that STZ causes hyperglycemia and insulin deficiency, leading to various derangements in metabolic and regulatory processes. Insulin deficiency suppresses glycogenesis, and excess of glucose is converted to lipids via fatty acid synthesis pathways. Subsequently, lipolysis increases with consecutive increases in the synthesis of triglycerides, LDL synthesis and quick breakdown of HDL<sup>51-54</sup>. Moreover, hyperlipidemia with enhanced levels of triglycerides results in oxidative/nitrosative stress, which may independently potentiate the adverse effects of hyperglycemia<sup>55</sup>. In the present study, increased levels of total cholesterol, triglycerides, LDL, and decreased HDL levels were found, and these altered levels were alleviated by different doses of extracts of leaves of *M. Koenigii*, therefore demonstrating their potential to improve lipid metabolism. The possible reason for amelioration of dyslipidemia could be its anti-hyperglycemic activity as well as increased production of insulin from pancreatic  $\beta$ - cells<sup>56</sup>.

The magnitude of hyperglycemia and hyperglycemia-induced oxidative stress are principal culprits for the onset of diabetic complications<sup>57-59</sup>. Free radicals are generated during regular cellular metabolism and functional activities in biological systems and play certain roles such as cell signalling, apoptosis, etc<sup>59,60</sup>. However, the production of free radicals during a hyperglycemic state has devastating effects on DNA, RNA, lipids and proteins, which are consequent to various pathological conditions, including diabetes and its complications. Extracts of leaves of *M. koenigii* significantly counteracted AGE formation in both *in vitro* and *in vivo* studies. The present study demonstrated that the administration of hydroalcoholic and aqueous extracts of leaves of *M. koenigii* extracts suggestively reduced the kidney weight/body weight ratio (kidney index) in comparison to diabetic control rats. Histopathological studies also suggested mesangial expansion and thickening of glomerular capillaries along with increased glomerular space in diabetic control rats. Atrophy of glomeruli and inflammation of renal cells was also seen in diabetic rats. Moreover, administration extracts of leaves of *M. koenigii* improved the structural aberrations in comparison to the diabetic control rats in the kidneys. Administration of MKHA extract significantly reduced the elevated levels of liver enzymes<sup>61,62</sup>.

### Conclusion

In conclusion, the *in-vivo* study of this extract revealed *M. koenigii*'s potential to alleviate hyperglycaemia and oxidative stress, which are the main factors responsible for the development of diabetic complications. Hence, it can be a promising candidate for diabetic nephropathy. However, the clinical efficacy of MKHA should be investigated further.

### Conflict of interest

All the authors declare no conflict of interest.

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