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Litsea cubeba fruit attenuates diabetes-associated metabolic complications in mice

Waqar Ahmed Goldar¹, Sandipan Jana¹, Reshma Kumari¹, Sanjib Bhattacharya² and Pallab Kanti Haldar^{1*}

Abstract

Background: *Litsea cubeba* (Lour) Pers. (Lauraceae) fruit has traditionally been used in treatment of diabetes in Sikkim, India. The purpose of the present study is to investigate the antidiabetic activity of methanol extract of *Litsea cubeba* fruit (MELCF) against streptozotocin (STZ)-induced diabetes in male albino mice. MELCF was assessed for in vitro α -amylase, α -glucosidase inhibitory activity and in vitro antioxidant activity against 2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide radicals. STZ was given intraperitoneally at 60 mg/kg for consecutive 3 days to mice. MELCF was administered orally in three doses 100 mg/kg, 200 mg/kg, 300 mg/kg body weight to STZ-induced mice. Metformin (200 mg/kg body weight) served as reference. Blood glucose and body weight were checked in seven days interval. After 28 days of treatment, the glycosylated hemoglobin (HbA1c), cholesterol (TC), triglyceride (TG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, urea were measured. Hepatic and renal antioxidant parameters of viz. lipid peroxidation (LPO), reduced glutathione (GSH) and superoxide dismutase (SOD) were estimated. The histopathological evaluation of pancreas, liver and kidney was performed.

Results: MELCF demonstrated significant α -amylase, DPPH and nitric oxide inhibitory effects. It significantly reduced blood glucose in a dose dependent manner. It has normalizing effect on HbA1c, AST, ALT, TC, TG, urea and creatinine. It has a modulator effect on tissue antioxidant status i.e., LPO, GSH and SOD. Histopathological findings revealed regenerative effect in pancreatic islets, liver and kidney.

Conclusion: It can hence be concluded that, *Litsea cubeba* fruit has significant attenuative effect against diabetic complications in mice.

Keywords: *Litsea cubeba*, Streptozotocin, Mice, Dyslipidemia, Antioxidant

Background

Diabetes mellitus is a metabolic disorder which is considered as a main threat in twenty-first century to the quality of human lives. It is characterized by high blood glucose level and lipid metabolic disorders. It is a group of symptoms where there are defects in secretion of insulin, action of insulin or both as a result hyperglycemia happens. Diabetic patient is increasing in the world day

by day. There are around 415 million people found with diabetes, their age from 20 to 79 years. Around 352.1 million people are estimated with impaired glucose tolerance with age from 20 to 79 years. They have a high risk of development of diabetes. It is estimated that by the year 2045, around 629 million people will suffer by this disease. Due to chronic hyperglycemia, damage or malfunction of tissue can be happen which lead to increase the risk of macrovascular and microvascular disease of diabetic retinal disease, kidney disease (nephropathy), cardiovascular diseases and sclerosis of arteries (Huisman 2007; Anonymous 2019; Kumari et al. 2021).

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There are many therapies in diabetes to decrease the blood glucose level and two of them are alpha-amylase and alpha glucosidase inhibitors which inhibit the digestion of starch from small intestine to decrease the postprandial hyperglycemia. There are several phytochemicals which have this type of activity they are alkaloids, terpenoids, glycosides, steroids etc. Inhibition of these enzymes helps to reduce the postprandial blood glucose (Bhat et al. 2011; Patra et al. 2020). As the diabetes develops, there is also aggravation of oxidative stress due to increased production of reactive oxygen species (ROS) as a result decreased endogenous antioxidant defense. The new antidiabetic drug (oral hypoglycemic) development has been shifted to the natural products to decrease the adverse effects. The World Health Organization (WHO) recommended the effectiveness of evaluation of medicinal plants in pursuit of newer therapeutic entities (Bhattacharya 2011; Biswas et al. 2018).

The new plants which play the major role for the new therapeutic purpose in diabetes having hypoglycemic, antioxidant and hypolipidemic agents. *Litsea cubeba* (Lour) Pers. commonly known as mountain pepper or Chinese pepper is a plant belonging to the family Lauraceae. It is a small evergreen tree and it has aromatic leaves with branches and flowers. It is distributed in tropical and subtropical regions. It is found in southeastern Asian countries. In India, it is found in Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim, Uttarakhand, Uttar Pradesh and West Bengal. It is traditionally used for the treatment of stomachache, dyspepsia, diabetes, edema, cold, asthma, arthritis, pain, traumatic injury etc. (Bhuinya et al. 2010; Kamle et al. 2019). The essential oil extracted from the fruit has antibacterial, antifungal, antimicrobial, anti-anxiety, antiasthma, anticancer activity (Thielmann and Muranyi 2019).

It has been found that, the *Litsea cubeba* fruit is traditionally used by the Nepali people of Sikkim, India for decreasing the high blood sugar level (Chhetri et al. 2005). *Litsea cubeba* fruit is reported to possess in vitro antidiabetic and antioxidant activity (Chakraborty and Mandal 2018). There is no scientific data on in vivo antidiabetic activity of this plant till date, despite relevant traditional usage. Therefore, the present study was aimed to evaluate the possible antidiabetic activity of *Litsea cubeba* fruit on streptozotocin (STZ)-induced diabetic complications in mice.

Methods

Plant material and extraction

The mature fruits from *Litsea cubeba* (Lour) Pers. (Lauraceae) were collected from Gangtok, East Sikkim, India in the month of August 2018. The plant specimen was authenticated at the Central National Herbarium, Botanical Survey of India, Shibpur, Howrah, West Bengal, India and a voucher specimen has been kept in the Department of Pharmaceutical Technology, Jadavpur University, Kolkata, West Bengal, India.

The air-dried fruits of *Litsea cubeba* were ground mechanically, weighed (500 g) and then it was immersed in petroleum ether and kept it in a mechanical shaker for 48 h, after that the solvent was evaporated in air and the air-dried powder was again extracted with methanol for 48 h using cold maceration process. The solvent was evaporated under reduced pressure by rotary vacuum evaporator and the dry extract (MELCF) was obtained. Then it was stored in a refrigerator at 40 °C for further use. To determine the chemical constituents present in the sample of MELCF, the phytochemical screening was performed by following the qualitative methods (Bhattacharya and Zaman 2009).

Drugs and chemicals

The chemicals and drugs used in the experiments were of analytical grade and procured from Hi-Media, Mumbai, India. All the test kits were purchased from Arkray Health Care Private Limited (Autospan), Gujarat, India.

In vitro assessments

Alpha amylase inhibitory activity

Alpha amylase inhibitory assay in vitro was performed by the 3,5-dinitrosalicylic acid (DNSA) method (Patra et al. 2020). MELCF was dissolved in 10% dimethyl sulphoxide (DMSO) and again dissolved in phosphate buffered saline (PBS) of pH 6.9. Various concentrations (10 to 500 µg/ml) of extract was prepared. α-amylase solution 200 µl (2U/ml) was mixed with it and incubated at 30 °C for 10 min. Afterwards, 200 µl (1% w/v) starch solution was added to it again incubate for 3 min. Reaction was stopped by addition of 200 µl DNSA (3,5-dinitro salicylic acid) reagent and boiled it for 10 min in hot water bath. Afterwards, it was cooled in running tap water and absorbance was taken at 540 nm using a UV-visible spectrophotometer. In a same manner, a positive control test was performed by using acarbose. The alpha amylase inhibitory activity was expressed by using percentage of inhibition which is calculated by using the following equation:

$$\text{Percentage of inhibition} = (\text{Absorbance of control} - \text{Absorbance of sample}) / \text{Absorbance of control} \times 100$$

Alpha glucosidase inhibitory activity

Alpha-glucosidase inhibitory activity of MELCF *in vitro* was carried out according to the standard method with minor modifications (Kumari et al. 2021). 50 μ l of phosphate buffer taken (100 mM, pH = 6.8) in a 96-well plate, then 10 μ l of alpha-glucosidase (1 U/ml), 20 μ l of different concentrations of MELCF (0.1 to 0.5 mg/ml) was added to it and it incubated at 37 °C for 15 min. Afterwards, as a substrate, 20 μ l P-NPG (4-nitrophenyl- β -D-glucopyranoside) (5 mM) added and incubated further at 37 °C for 20 min. The reaction was stopped by adding 50 μ l sodium carbonate (Na₂CO₃) (0.1 M). The released *p*-nitro phenol's absorbance was measured at 405 nm by spectrophotometer (Spectramax). As a standard various concentrations of acarbose (0.1–0.5 mg/ml) taken. There was a parallel set up without any test substance and the experiment was performed in triplicates. The results are expressed as percentage inhibition and the calculation is done by using the following formula:

$$\text{Percentage of inhibition} = (\text{Absorbance of control} - \text{Absorbance of sample}) / \text{Absorbance of control} \times 100.$$

DPPH radical scavenging assay

The MELCF at different concentrations (40, 80, 160, 200, 400, 600, 800 μ g/ml) was mixed with 0.1 mM DPPH (2,2-diphenyl-1-picrylhydrazyl). In the dark place, the mixture was kept at room temperature for 30 min and against blank the absorbance was measured at 517 nm. Ascorbic acid was employed as standard (Bhattacharya et al. 2010).

$$\text{Percentage of inhibition} = (\text{Absorbance of control} - \text{Absorbance of sample}) / \text{Absorbance of control} \times 100.$$

Nitric oxide scavenging assay

Through Griess Illosvoy reaction, nitric oxide scavenging activity was determined (Bhattacharya and Haldar 2020). The MELCF of different concentrations (40, 80, 160, 200, 400, 600, 800 μ g/ml) were mixed with 2 ml of 10 mM sodium nitroprusside in 0.5 ml phosphate buffer (0.5 M, pH 7.4) the final volume is 3 ml. It was incubated at 37 °C for 60 min and then Griess reagent i.e., N-(1-Naphthyl) ethylenediamine (0.1%) and sulphanilic acid (1%) in H₃PO₄ (5%) was added. Pink chromophore was generated and finally measured spectrophotometrically at 540 nm. As a standard, ascorbic acid was used. The result was calculated according to the formula:

$$\text{Percentage of inhibition} = (\text{Absorbance of control} - \text{Absorbance of sample}) / \text{Absorbance of control} \times 100.$$

Animals

The study was conducted on adult male Swiss albino mice and animals were purchased from the M/s

Chakraborty Enterprise (CPCSCA registered). The age of the animals was around five to six weeks, weighing 35 \pm 5 g were housed in a ambient temperature 25 \pm 2 °C, relative humidity was 45–55% and 12 h in light and 12 h dark cycle was maintained. The animals were kept for two weeks in the laboratory to acclimatize with the environment. The animals were fed normal pellet diet purchased from Hindustan Lever Ltd., India and water ad libitum. As per the Institutional Animal Ethics Committee, Jadavpur University, Kolkata, India all the animal experiments were carried out (Ref. No. AEC/PHARM/1502/09/2015).

Acute toxicity

MELCF was administered orally to the Swiss albino mice for evaluation of the acute toxicity according to the method of Organization for Economic Co-operation and Development (OECD) guideline no. 425 (Anonymous 2008).

Induction of diabetes

The mice were acclimatized for 2 weeks in the laboratory. Afterwards, the animals were fasted overnight. STZ was dissolved in citrate buffer (0.1 M sodium citrate and 0.1 M citric acid, pH 4.5). The diabetes was induced by giving STZ (60 mg/kg) through intraperitoneal injection for 3 consecutive days (Biswas et al. 2011). The blood glucose was measured by taking a drop of blood from

the tail vein with the help of a single touch glucometer (Accu-check, Roche Diagnostic, USA). The mice with blood glucose more than 250 mg/dl were selected for the experiment.

Oral glucose tolerance test (OGTT)

The OGTT was performed in the overnight (12 h) fasted mice. Animals were divided into six groups ($n=6$). Group 1 was treated as normal control which was given water 5 ml/kg. Groups 2, 3, 4 animals were orally given MELCF 100, 200 and 300 mg/kg respectively. Group 5 was orally given metformin 200 mg/kg. After 30 min

of administration of extract and standard drug, glucose solution (2.5 g/kg) was administered orally. After administration of glucose solution 0, 30, 60 and 120 min,

blood glucose level was measured with the help of single touch glucometer (Accu-check, Roche Diagnostic, USA) (Kumar et al. 2011).

Experimental design

The MELCF was evaluated for the antidiabetic activity and the selected animals were divided into six groups ($n=6$) as follows (Das et al. 2011).

- Group 1: Normal control (untreated).
- Group 2: Diabetic/disease control (streptozotocin 60 mg/kg body weight).
- Group 3: Diabetic mice treated with MELCF 100 mg/kg body weight.
- Group 4: Diabetic mice treated with MELCF 200 mg/kg body weight.
- Group 5: Diabetic mice treated with MELCF 300 mg/kg body weight.
- Group 6: Diabetic mice treated with metformin 200 mg/kg body weight.

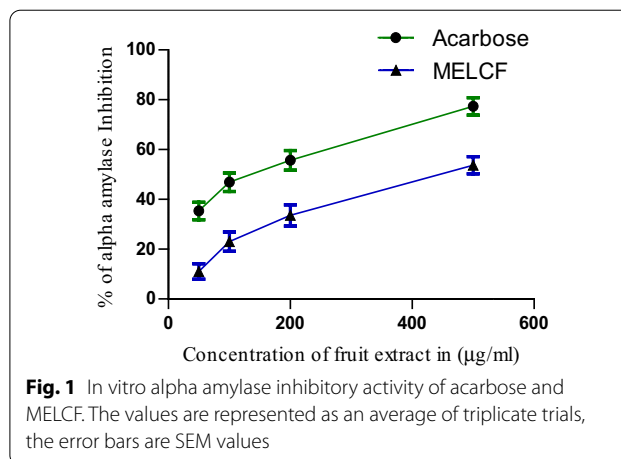
All the animals were treated orally for 28 consecutive days. Fasting blood glucose levels of the mice were monitored in the overnight (12 h) fasted mice on 0, 7, 14, 21 and 28 days of the treatments along with corresponding body weights.

Determination of biochemical parameters

After 24 h of last dosing on the 28th day, the blood samples were collected from the overnight fasted mice in each group by cardiac puncture for determination of glycosylated haemoglobin (HbA1c). For liver function tests aspartate aminotransferase (AST)/serum glutamic oxaloacetic transaminase (SGOT), alanine aminotransferase (ALT)/serum glutamic pyruvic transaminase (SGPT) were performed. For kidney function tests urea, creatinine tests were done. For lipid profile, total cholesterol (TC), triglyceride (TG) were determined by using the reagent kits as mentioned under 'drugs and chemicals' sub-section.

Liver and kidney antioxidant status

On the 29th day, animals of each group were sacrificed by cervical dislocation the liver and the kidney of the sacrificed animals were taken out and homogenized in 10 ml 20 mM phosphate buffer (pH: 7.4). Then it was centrifuged in 12,000 rpm for 10 min in 4 °C the supernatant was collected and used for the estimation of the lipid peroxidation assay (LPO/MDA), reduced glutathione (GSH) and superoxide dismutase (SOD) as per the procedures stated in the respective kits.



Histopathological studies of pancreas, liver and kidney

After 28 days treatment, all animals were sacrificed on the 29th day by cervical dislocation, afterwards pancreas, liver and kidney tissues were isolated from the animals of each group and cut in a small piece measuring about 1 cm and kept it in 10% formaldehyde solution at 4 °C and histology was done in the laboratory, then it was dehydrated gradually by increasing the concentration of ethanol (50–100%), it was cleared with xylene and embedded in paraffin and cut by using a semi-automated rotary microtome (Leica Microsystem, RM-2245, Wetzlar, Germany). Each section was about 5 µm thickness and it was stained with haematoxylin and eosin. The microscopic slides of pancreas, liver and kidney tissues were photographed under 100 × magnification by binocular microscope (Olympus, India).

Statistical analysis

The determinations were carried out in triplicates for in vitro studies and the values are expressed as mean ± standard error of mean (SEM). For in vivo study, statistical significance was analyzed by one way analysis of variance, followed by Dunnett's test. The name of the software used is GraphPad Prism 5.0. (Graph Pad Software Inc, La Jolla, USA). $P < 0.05$ considered as statistically significant.

Results

Preliminary phytochemical screening

From the preliminary phytochemical tests it was revealed that flavonoids, alkaloids, tannins, saponins are present in the methanol extract of *Litsea cubeba* fruit (MELCF).

Acute toxicity

It has been observed that the MELCF is safe up to the dose 1500 mg/kg body weight in mice.

In vitro alpha amylase and alpha glucosidase inhibitory activity

The alpha amylase inhibiting percentage is increasing with the concentration of the plant extract—MELCF (Fig. 1). We can compare the result of the MELCF with the standard drug acarbose. From here we determined that the IC₅₀ value MELCF is 444 µg/ml whereas the reference acarbose exhibits its IC₅₀ value at 147 µg/ml. The alpha glucosidase inhibitory activity is found comparatively less (Fig. 2). The IC₅₀ value of MELCF is 943 µg/ml. The percentage of inhibition of acarbose was not estimated as it may give IC₅₀ value at very low concentration.

In vitro antioxidant activity

DPPH free radical scavenging activity was observed in MELCF as and it was compared with the standard ascorbic acid (Fig. 3). IC₅₀ values were 278 µg/ml and 102 µg/ml respectively. In nitric oxide scavenging assay, seen in MELCF along with the reference ascorbic acid (Fig. 4). Relevant IC₅₀ values were found to be 653 µg/ml and 198 µg/ml respectively.

Oral glucose tolerance (OGTT)

After administration of glucose, change is found in the blood glucose of the normal mice. Treatment with MELCF at 100, 200 and 300 mg/kg and metformin 200 mg/kg significantly reduced blood glucose level after 0, 30, 60, 120 min (Table 1).

Fasting blood glucose

The antidiabetic activity was exhibited on MELCF on STZ-induced diabetic mice (Table 2). We can understand from the results that, the MELCF has dose dependant

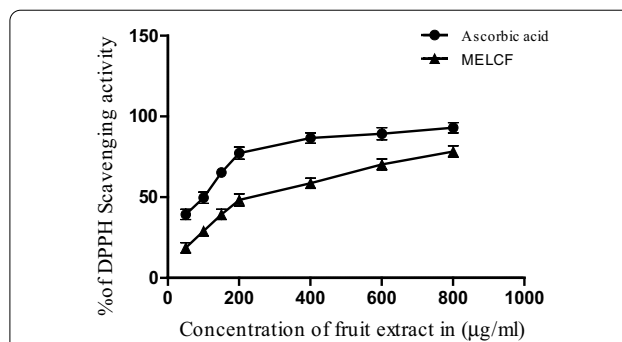


Fig. 3 In vitro DPPH free radical scavenging activity of ascorbic acid and MELCF. The values are represented as an average of triplicate trials, the error bars are SEM values

and significant antidiabetic effect on STZ-induced diabetic mice and the effect of MELCF was comparable with the standard treatment group of metformin 200 mg/kg body weight.

Body weight

After completing 28 days treatment, the body weight of diabetic control group exhibited significant decrease in the body weight where in the treatment groups there were not much decrease in body weight (Table 3).

Glycosylated hemoglobin

The HbA1c level in the treatment groups was decreased as compared to the diabetic control group (Fig. 5). The decrease of the HbA1c level was in a dose dependent manner and the HbA1c level was most decreased and came near to normal level in the groups treated with MELCF 200 and 300 mg/kg body weight and the standard group treated with metformin 200 mg/kg.

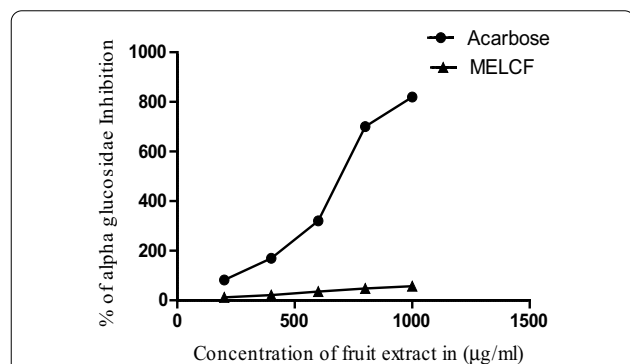


Fig. 2 In vitro alpha glucosidase inhibitory activity of acarbose and MELCF. The values are represented as an average of triplicate trials, the error bars are SEM values

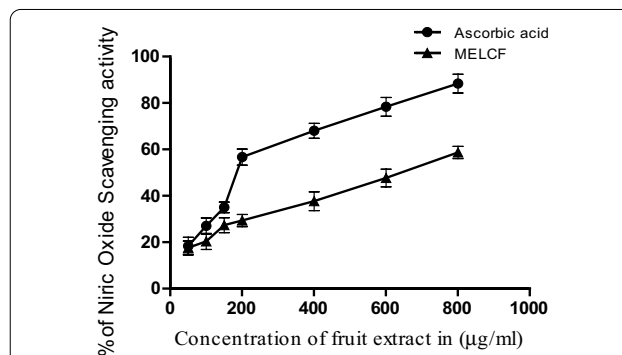


Fig. 4 In vitro nitric oxide scavenging activity of ascorbic acid and MELCF. The values are represented as an average of triplicate trials, the error bars are SEM values

Table 1 Effect of MELCF on OGTT in mice

Treatments	Blood glucose concentration (mg/dl)			
	0 min	30 min	60 min	120 min
Group 1: Normal control	120 ± 4.041	225 ± 2.87	160 ± 3.46	135 ± 2.6
Group 2: MELCF 100 mg/kg	107 ± 4.485	180 ± 4.61*	140 ± 2.32	120 ± 2.30
Group 3: MELCF 200 mg/kg	108 ± 5.77	165 ± 2.88*	135 ± 3.18	110 ± 2.88
Group 4: MELCF 300 mg/kg	121 ± 3.33	140 ± 17.56*	118 ± 11.67*	104 ± 8.45*
Group 5: Metformin 200 mg/kg	111.7 ± 8.81	128 ± 6.09 *	108 ± 4.41*	83 ± 8.5 *

The values represented as mean ± SEM (n = 6). *p < 0.05 when compared with normal control

Table 2 Effect of MELCF on fasting blood glucose level of diabetic mice

Treatments	Fasting blood glucose level (mg/dl)				
	Day 0	Day 7	Day 14	Day 21	Day 28
Group 1: Normal control	145 ± 8.66	142 ± 7.23	123 ± 5.95	131 ± 8.76	119 ± 8.68
Group 2: Diabetic control	335 ± 8.66*	378 ± 10.12*	394 ± 8.66*	424 ± 8.66*	440 ± 6.65*
Group 3: MELCF 100 mg/kg	382 ± 2.96	390 ± 4.66	347 ± 5.04**	343 ± 6.00**	350 ± 8.66**
Group 4: MELCF 200 mg/kg	377 ± 4.93	357 ± 10.79	341 ± 10.12**	309 ± 8.66**	300 ± 9.24**
Group 5: MELCF 300 mg/kg	372 ± 13.02	343 ± 13.02	340 ± 8.66**	301 ± 10.97**	292 ± 6.48**
Group 6: Metformin 200 mg/kg	390 ± 12.13	290 ± 9.53**	266 ± 12.41**	251 ± 7.26**	233 ± 11.67**

The values represented as mean ± SEM (n = 6). *Normal control group vs. diabetic control group (*p < 0.05), **All the treatment group vs. diabetic control group (*p < 0.05) on same day

Table 3 Effect of MELCF on body weight of diabetic mice

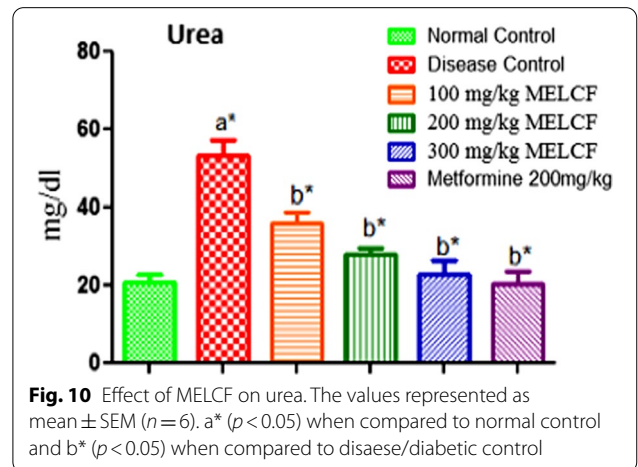
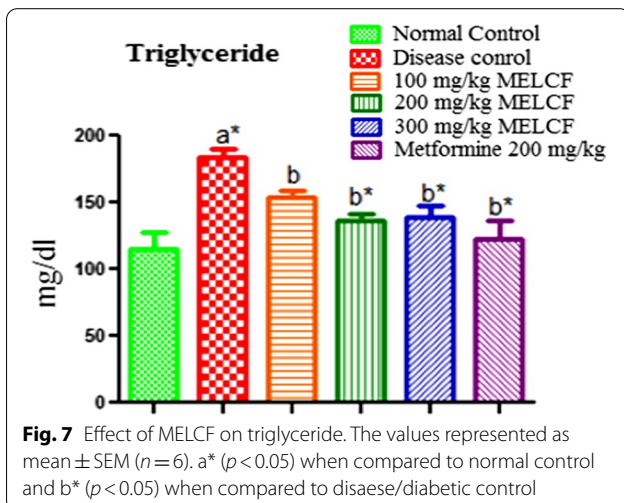
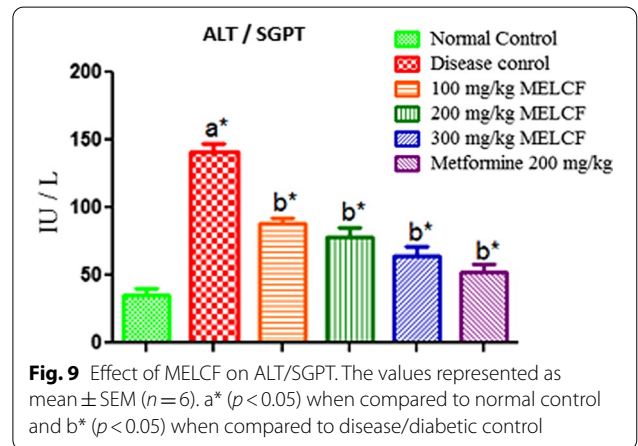
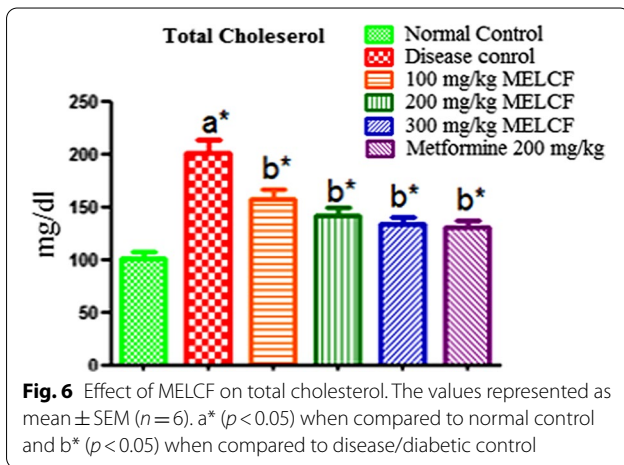
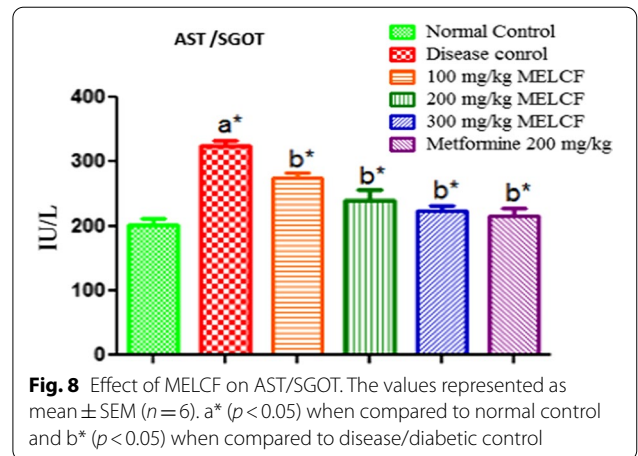
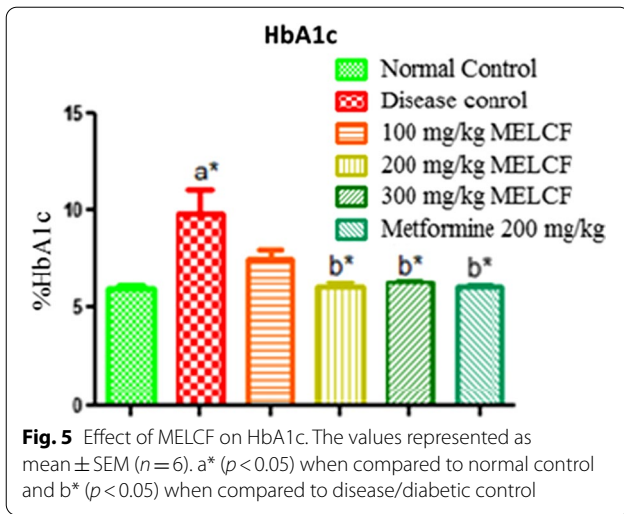
Treatments	Body weight (g)				
	Day 0	Day 7	Day 14	Day 21	Day 28
Group 1: Normal control	24 ± 0.57	26.83 ± 0.47	31.33 ± 0.49	31.33 ± 0.66	34 ± 0.57
Group 2: Diabetic control	29.5 ± 0.76	27.50 ± 0.76	25.5 ± 0.42*	23.83 ± 0.6*	21 ± 0.73*
Group 3: MELCF 100 mg/kg	26.5 ± 0.76	23.83 ± 0.6**	22.23 ± 0.76**	20.5 ± 0.76**	22.5 ± 0.76
Group 4: MELCF 200 mg/kg	23 ± 0.57	23.0 ± 0.73**	20.83 ± 0.6**	21.50 ± 0.76	19.33 ± 0.61
Group 5: MELCF 300 mg/kg	25 ± 0.76	23.83 ± 0.6**	24.17 ± 0.6	23.5 ± 0.76	22.67 ± 0.84
Group 6: Metformin 200 mg/kg	28.5 ± 0.42	28.17 ± 0.48	27.5 ± 0.42	26.17 ± 0.47	26.83 ± 0.83**

The values represented as mean ± SEM (n = 6). *Normal control group vs. diabetic control group (*p < 0.05), **The entire treatment group vs. diabetic control group (*p < 0.05) on same day

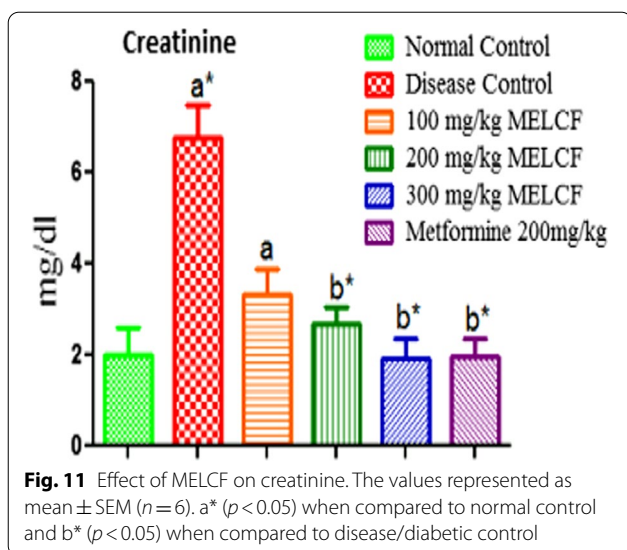
Serum biochemical parameters

After repeated administration of the MELCF for 28 days TC, TG, AST, ALT, urea and creatinine were measured (Figs. 6, 7, 8, 9, 10, 11). In diabetic control group, there was an increase in TC level as compared to normal control group. Total cholesterol level was decreased by MELCF in a dose dependent manner and the minimum total cholesterol was observed in the treatment group of MELCF 300 mg/kg body weight. In the diabetic control group, there was increased TG level where after 28 days of MELCF treatment there was decrease in TG level in a dose dependent manner. Decrease in the TC and TG levels was also found significantly in metformin

(200 mg/kg body weight) treatment. The activities of serum AST/SGOT and ALT/SGPT were also increased in the diabetic control group as compared with the normal control group. After treatment of 28 days with different concentrations of MELCF and metformin, it is seen that there were decrease in both AST and ALT levels in a dose dependent manner which is equivalent to the reference treatment group. The levels of the urea and creatinine were increased in the diabetic control group which is comparable with the normal control group (Figs. 10 and 11). It is observed that, MELCF at 100, 200 and 300 mg/kg body weight can significantly decrease both the urea and creatinine levels in dose



dependant manner respectively which was equivalent to standard treatment group metformin 200 mg/kg body weight.



Tissue antioxidant parameters

The antioxidant effect in vivo was recorded in liver and kidney tissues (Figs. 12, 13, 14). In diabetic control group, the LPO (MDA) level was increased. In the treatment groups, it was decreased in a dose dependent manner. MDA level maximum decreased in MELCF at 300 mg/kg treatment and metformin 200 mg/kg treated groups. The SOD activity and GSH level were decreased in the diabetic control group and

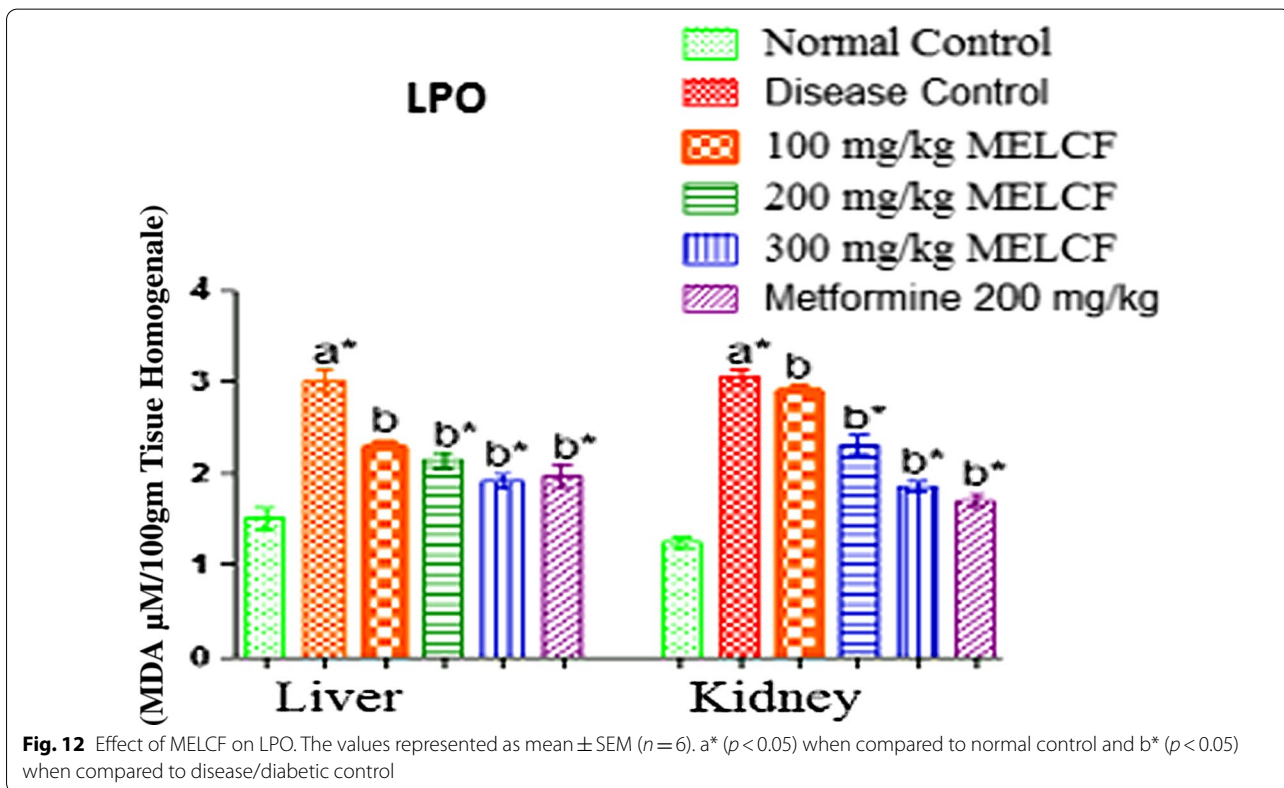
these were normalized in MELCF treated groups in a dose dependent fashion.

Histopathological observations

It is observed that, the normal control showed pancreatic islets of Langerhans in an arranged manner (Fig. 15a) where typical histology of the pancreas is observed. In diabetic control, there is significant destruction of islets of Langerhans (Fig. 15b). In the MELCF 100, 200 and 300 mg/kg treatment groups, there were preserved islets of Langerhans structure (Figs. 15c–e) and in a dose dependent manner and maximum benefit was found in the treatment group of MELCF 300 mg/kg. In standard treatment group, metformin 200 mg/kg, there was also development of islets of Langerhans (Fig. 15f).

Histopathology of normal mice, the normal structure of hepatic portal vein was observed (Fig. 16a). In the diabetic control group, destruction of the portal vein seen in the liver (Fig. 16B). In the MELCF 100, 200 and 300 mg/kg treatment groups, there was preserved portal vein structure in a dose dependent manner (Fig. 16c–e) and maximum benefit is seen in the treatment group 300 mg/kg. In reference treatment group, metformin 200 mg/kg there was also development in portal vein (Fig. 16f).

In the histopathology of normal mice kidney, it is seen there is normal gromeruli of the kidney (Fig. 17a).



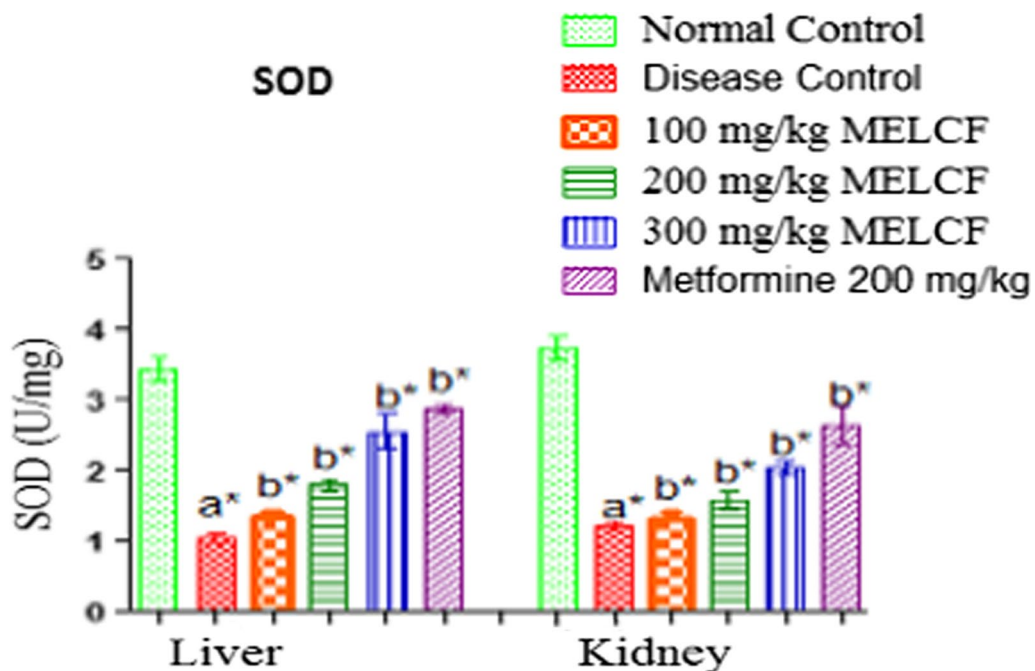


Fig. 13 Effect of MELCF on SOD. The values represented as mean ± SEM (n = 6). a* (p < 0.05) when compared to normal control and b* (p < 0.05) when compared to disease/diabetic control

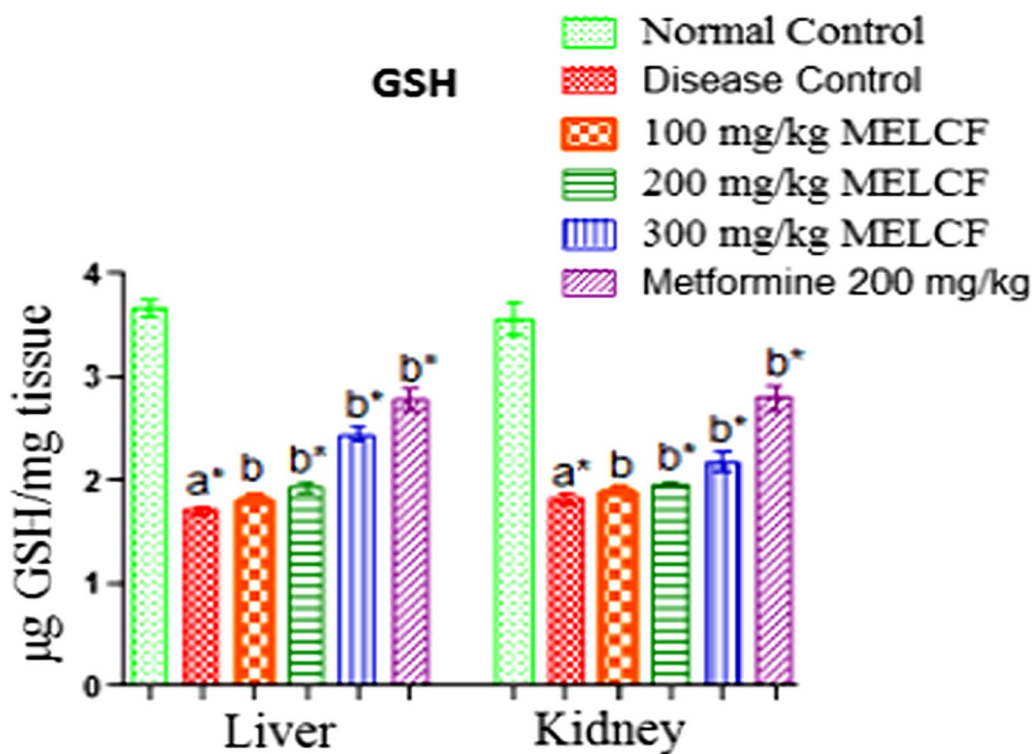


Fig. 14 Effect of MELCF on GSH. The values represented as mean ± SEM (n = 6). a* (p < 0.05) when compared to normal control and b* (p < 0.05) when compared to disease/diabetic control

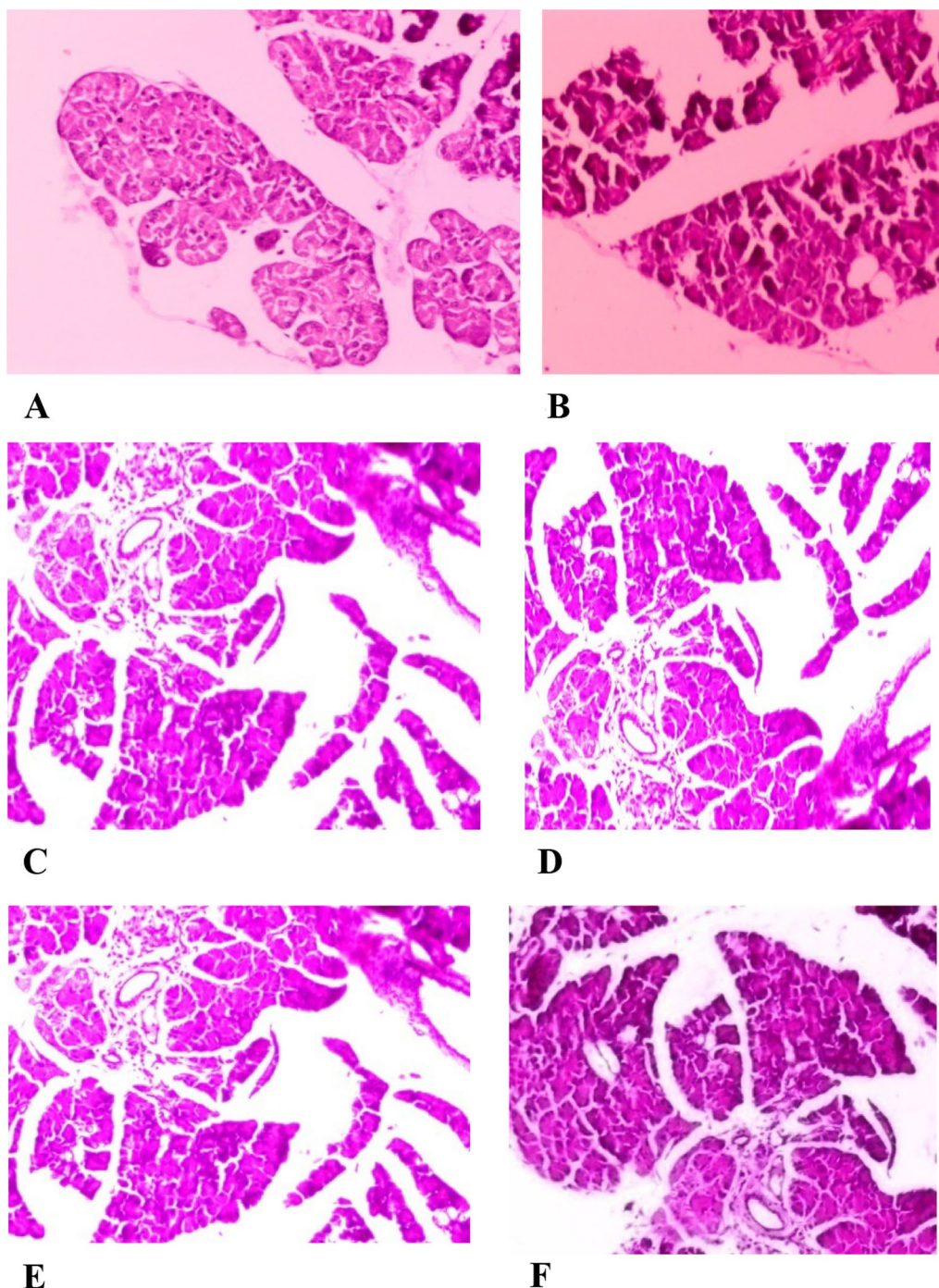


Fig. 15 Hematoxylin and eosin-stained sections of mice pancreas. **a** Islets of Langerhans of normal control mice. **b** Destruction of islets of Langerhans in diabetic mice (**c–e**). Remnants of islets of Langerhans diabetic mice treated with **c** 100 mg/kg and **d** 200 mg/kg **e** 300 mg/kg per day MELCF which reduced the development of STZ-induced alterations in the liver. **f** Islets of Langerhans of metformin 200 mg/kg treated mice

In the diabetic control group, there was thickening the glomeruli (Fig. 17b). In the MELCF 100, 200 and 300 mg/kg treatment groups development was seen in a dose dependent manner (Fig. 17c–e) and maximum

benefit was seen in the treatment group 300 mg/kg. In standard treatment group, metformin 200 mg/kg there was also development of glomeruli of kidney (Fig. 17f).

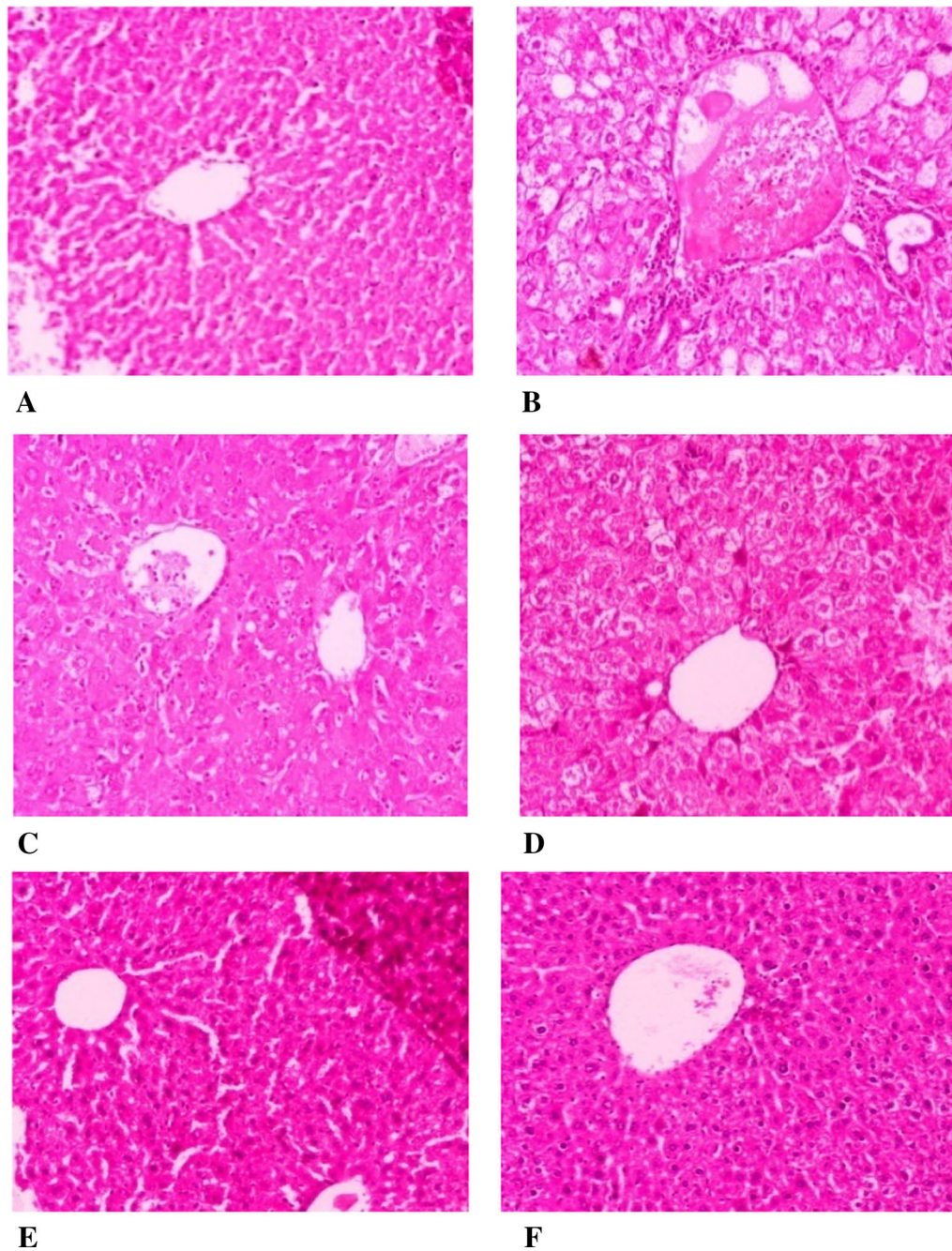


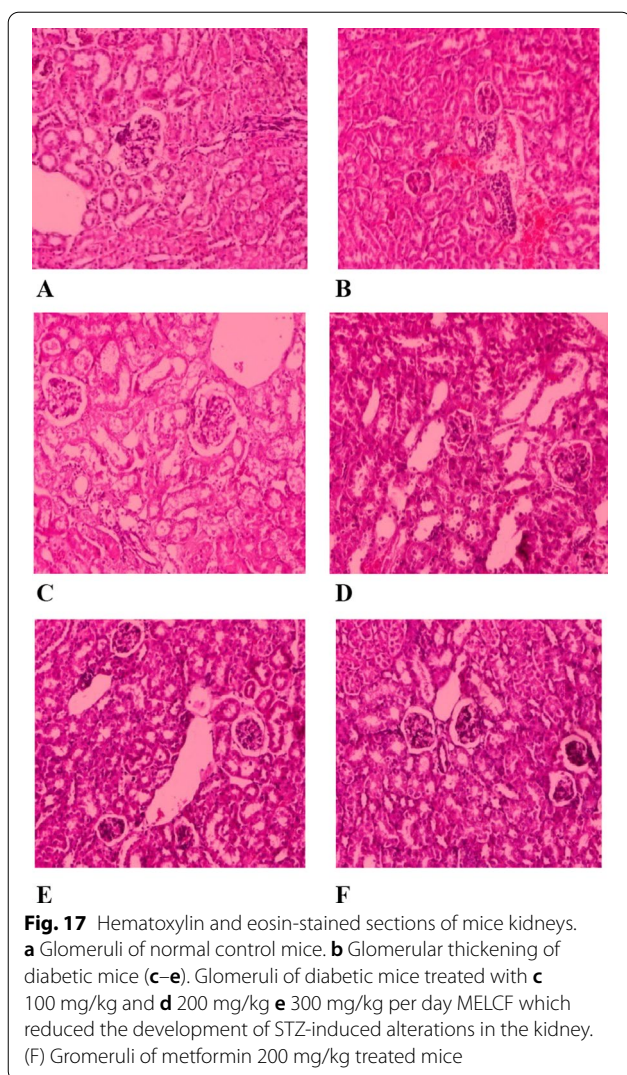
Fig. 16 Hematoxylin and eosin-stained sections of mice liver. **a** Portal vein of normal control mice. **b** Damaging of portal vein of diabetic mice (**c-e**) portal vein of diabetic mice treated with **c** 100 mg/kg and **d** 200 mg/kg **e** 300 mg/kg per day MELCF which reduced the development of STZ-induced alterations in the liver. **f** Portal vein of metformin 200 mg/kg treated mice

Discussion

The present study reports the protective effect of methanol extract from *Litsea cubeba* fruit (MELCF) in diabetes-associated metabolic complications like hyperglycemia and dyslipidemia in male Swiss albino mice where three different doses of MELCF i.e., 100, 200 and 300 mg/kg

body weight were employed. Among them, MELCF at 300 mg/kg showed maximum antidiabetic activity which comes near to the reference treatment group in most of the cases.

It has been documented that, the medicinal plants which have anti-diabetic activity, most of them are rich



in polyphenolic constituents (Bhattacharya 2020). The presence of flavonoid, alkaloid, tannin components were detected in the MELCF. The significant antidiabetic activity of MELCF may be due to the presence of these constituents.

Alpha amylase and alpha glucosidase are the digestive enzymes which break complex carbohydrates present in the food and hydrolyze them in small units like maltose and glucose which increasing the blood glucose. Inhibition of these enzyme reduces postprandial glucose level in blood (Tundis et al. 2010). In the present study, we can record that MELCF has significant alpha amylase and a little alpha glucosidase inhibiting property.

In diabetes, several oxidative and nitrosative free radicals are produced in excess in body, so free radical scavenging activity may be another important property of natural antidiabetic agents (Chakraborty and Mandal

2018). It is seen that, the MELCF has both DPPH and nitric oxide free radical scavenging activity. So from here it can be inferred that, this plant fruit has a moderate antioxidant activity in vitro.

The results of the present study indicated the marked antidiabetic activity of MELCF in STZ-induced diabetes. It significantly lowered the blood glucose level and from the results it may be postulated that, the extract could be responsible for stimulation of insulin release and also possibly due to increase in the peripheral utilization (Gray and Flatt 1998). Hence, MELCF is effective against disturbances in sugar metabolism of diabetic mice.

After induction of diabetes, it was seen that there was rapid decrease in the body weight as compared with the normal control group mice due to muscle waste and tissue protein loss. After treating with MELCF and metformin for 28 days, further there was no loss of body weight where in the diabetic control group there loss of body weight was found. The result was comparable with the standard group of metformin 200 mg/kg body weight.

There is a marker of the glycaemic control, known as glycosylated haemoglobin (HbA1c). The production of glycosylated hemoglobin increases in the diabetic condition (Dash et al. 2021). After the treatment of MELCF and meformin, there is decrease in the glycosylated haemoglobin content which acts by decreasing the binding of glucose with haemoglobin (Patra et al. 2020).

It is estimated in the mice blood serum that, there is remarkable increase in the concentrations of triglyceride (TG), total cholesterol (TC) in the diabetic control group in comparison with the normal control group. There are various metabolic derangements like dyslipidemia found in diabetic state due to insulin deficiency. In the normal state on peripheral fat depot, the lipolytic hormone activated by insulin to prevent the mobilization of free fatty acid. Inactivation of lipoprotein lipase found in the insulin deficiency as a result, liver converts free fatty acids into phospholipids and cholesterol which are discharged into blood and elevated the serum phospholipid levels (Chakraborty et al. 2018). Administration of MELCF in STZ-induced diabetic mice for 28 days decreased the TC and TG levels which came near to the standard metformin treatment group. It can hence be implied that, MELCF may have hypolipidemic activity which helps to decrease the lipid-borne metabolic complications.

Increase in the activities of AST, ALT in the diabetic control group indicates that, hepatic dysfunction led to increase in these enzymes in the blood stream (Bhattacharya and Haldar 2012). After 28 days it is seen that in the treatment groups, there is decrease in these enzymes which means that this plant fruit has good hepatoprotective property. In diabetic condition, there is increase in the concentration of urea and creatinine levels in

the blood stream which indicated dysfunction of kidney, called as diabetic nephropathy. After 28 days it was observed that, in the treatment groups there are decrease in urea and creatinine levels which means that MELCF has a protective effect against diabetic nephropathy.

In diabetic complications, the pathogenesis is protein kinase C isoform is activated by induction of glucose, glucose-derived advanced glycation end product formation is increased, reactive oxygen species (ROS) generation is increased and polyol pathway also found up-regulated (Dash et al. 2021). In diabetes, several tissue damage is happened as a result degenerative complications are seen in many organs specially in liver and kidneys. Tissue damage by lipid peroxides (LPO) is recorded in the development of diabetes. In diabetes, insulin secretion is impaired by this increase the LPO in the biological system. Increased amount of LPO is found in the STZ induced mice (Biswas et al. 2011; KunduSen et al. 2011). In this study it is found that, MELCF at 200, 300 mg/kg and metformin 200 mg/kg body weight decreased the amount of LPO. This indicates that, MELCF has anti-lipid peroxidation activity i.e., antioxidant effect in vivo.

Superoxide dismutase (SOD) is an endogenous scavenging enzyme which removes the toxic superoxide free-radical in biological system and the deleterious effect of reactive oxygen species (ROS), thus it protects all the cells. In diabetes, when there is decline in SOD, there is increased deleterious effect elicited by increased accumulation of superoxide radicals (Bhattacharya and Halder 2013). After administration of MELCF 200, 300 mg/kg and metformin 200 mg/kg, there was increase in the activity of SOD which reverted near to the normal. It means that, MELCF had free radical scavenging activity which helped to restore the oxidative damage incurred by the overproduced superoxide radicals.

Reduced glutathione (GSH) acts as endogenous reducing agent in the biological redox system and known as non-enzymatic antioxidant. With the help of glutathione peroxidase enzyme, detoxification of hydrogen peroxide is done by GSH. The decrease in the GSH concentration in diabetic mice may be due to the decreasing GSH synthesis or by oxidative stress-induced degradation of GSH (Das et al. 2011). After the treatment with MELCF and metformin, it was found that, there is increase the GSH contents in both liver and kidney.

The islets of Langerhans are the groups of pancreatic cells secreting glycaemic hormones viz. insulin and glucagon (Dash et al. 2021). In this study, damage of pancreas seen in STZ-induced diabetic control mice and islets of Langerhans regeneration is seen in standard metformin treated group. The comparable regeneration and restoration of normal cell size of islets of Langerhans was seen in the MELCF treatment groups and the maximum effect

was observed in MELCF at 300 mg/kg body weight treatment group and from here we can deduce that, the antidiabetic effect of MELCF may be attributed to regeneration of islets of Langerhans. Here we can again confirm that, in the management of diabetes MELCF is effective.

It is observed that in normal control group mice, the hepatic cell organelles are preserved. Distortion in the hepatic cell arrangement seen in diabetic mice. It is may be due to the lipid metabolism disorder which is insulin dependent. In diabetic mice, around the central vein liver cells are more vacuolated. Parenchymatous cells are degenerated with sinusoid dilation and necrosis seen in diabetic control mice. After MELCF treatment, the appearance is normal, decreased necrosis and congestion of sinusoids. At 300 mg/kg MELCF and metformin 200 mg/kg treatment groups, similar to the normal hepatic cell arrangement was observed. It is seen in the histopathology of diabetic mice kidney that, glomerular space was increased with tubular regeneration, changes in glomeruli and thickening of the basement membrane. Treatment of MELCF and metformin significantly reduced these aberrations. It may hence be inferred that, MELCF has a protective role against diabetes-induced pancreatic and hepato-renal tissue toxicity.

Conclusions

The results of the present study signifies that the methanol extract of *Litsea cubeba* fruit possesses attenuative potential in diabetes-associated metabolic complications viz. hyperglycemia and dyslipidemia in mice with inherent antioxidant role. The extract also shows moderate in vitro and marked in vivo antioxidant activity as well. Therefore, it can be concluded that, *Litsea cubeba* fruit has significant antidiabetic activity which corroborates its traditional usage in India for diabetes mellitus. To the best of our knowledge, this is the first report of antidiabetic activity of this plant in vivo. However, further study is required to isolate the active compound or to know the possible mechanism(s) of action of the antidiabetic activity.

Abbreviations

MELCF: Methanol extract of *Litsea cubeba* fruit; SEM: Standard error of mean; HbA1c: Glycosylated haemoglobin; TC: Total cholesterol; TG: Triglyceride; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LPO: Lipid peroxidation; GSH: Reduced glutathione; SOD: Superoxide dismutase; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; OGTT: Oral glucose tolerance test; STZ: Streptozotocin; ROS: Reactive oxygen species.

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Authors' contributions

WAG and SJ conducted the experiments and drafted manuscript, RK handled instruments and conducted statistical analysis. SB coordinated the work and

revised the manuscript and PKH conceived and supervised the whole work. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

All animal experiment were performed as per the Institutional Animal Ethics Committee, Jadavpur University, Kolkata, India (vide registration no. AEC/PHARM/1502/09/2015). The written informed consent was obtained to use the animals in this study.

Consent for publication

Not applicable.

Competing interests

The authors do not have any competing interests.

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