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Investigation on anti-diabetic and anti-hyperlipidemic activities of *Blepharis edulis* root extract in wistar albino rats

Shashi Kant, Jagdeep Singh Dua and Viney Lather

Abstract
In the present work deals with the extraction of methanolic extract of *Blepharis edulis* and investigation of antidiabetic effects in male Wistar albino rats. Intraperitoneal injection of streptozotocin solution (60 mg/kg body weight) was given to the fasted animals to induce diabetes. The samples were collected to analyze blood glucose level and plasma insulin levels at the end of the study. Blood glucose levels were measured using glucometer and enzyme-linked immunosorbent assay (ELISA) method was used for the determination of insulin levels. Insulin level, triglyceride level, cholesterol level, low-density lipoproteins (LDL) level, serum glutamic oxaloacetic transaminase (SGOT) level and serum glutamic pyruvic transaminase (SGPT) level were also examined at the end of the study. The liver and pancreas of the treated animals were isolated for histopathology examinations. The phytochemical screening of *Blepharis edulis* methanolic extract indicated the presence of alkaloid, flavonoids and phenolic groups. The results of in vivo study showed a significant decline in fasting blood glucose levels. The extract at high dose significantly ($p< 0.05$) normalized plasma insulin level. The results revealed that the methanolic extract significantly ($p< 0.05$) normalized plasma lipid status and decreased cholesterol, triglyceride and LDL levels. A significant ($p< 0.05$) increase in SGPT level was observed. However the effect on SGOT level was non-significant. The histopathologic study results revealed that the treatment of animals with pure glibenclamide (10 mg/kg) and *Blepharis edulis* plant extract showed a decreased pattern of liver enzymatic activities.

Keywords: anti-diabetic, biochemical parameters, *Blepharis edulis*, histopathology, methanolic extract

1. Introduction
Medicinal plants and traditional medicines has a long history, it is practices based on the theories, principles and knowledges of different cultures and eras. It is more accessible to most of the population. About 65 to 90% of the populations of every country of the developing world trust on herbal or indigenous forms of medicine [1]. *Blepharis* is the largest genus of Acanthaceae, recently monographed as containing 129 species. Various species of *Blepharis* has showed medicinal values like anti-inflammatory, antiarthritic, antimicrobial, antifungal, antioxidant and cytotoxic activities. For the present study species of *Blepharis* was selected. *Blepharis edulis* Linn. (Family Acanthaceae) is a small gray pubescent or nearly glabrate perennial plant, found in Western Rajasthan, Punjab, Pakistan, Iran and Malwa region of MP. In Hindi language the plant is known as Utangan or Chaupatia. It is primarily used in treatment of asthma, fever, cough, and inflammation of throat [2]. It is act as appetizer, astringent to bowels [3]. Seeds contain Allantoin, a bitter glycoside and Blepharin, a glucoside. Catechol and tannin are also known to be present in this plant. Dihydrofurano-dihydrocoumarin was first detected as occurring in nature in this plant [4]. In the ethnomedicinal literature of India, *B. edulis*is used as food to increase sperm count and as aphrodisiac plant [5]. on the basis of classification Acanthaceae ranks eighth among the 17 families with high representation of more than 160 species of endemics in India. It has 382 taxa in India, of which 224 are endemic with 56.9% endemism. It is also third among the six families having high degree of endemism in Peninsular India, with a total number of 146 endemic taxa and the percentage of endemism is 38.8% [6].

2. Materials and Methods
2.1 Materials collection identification and authentication of plant material:
Plant of *Blepharis edulis* collected from northern part of the India. The collection was done in the month of August and September. Streptozotocin (STZ) and glibenclamide were purchased from Sigma Chemicals (St. Louis, MO, USA). Other chemicals and reagents used in this study were of analytical grade.

2.2 Preparation of extract

Extract of *Blepharis edulis* roots was prepared with a slight modification in the method of Lee *et al.* (Lee *et al.* 2002). Briefly, dried powdered *Blepharis edulis* root material (200 g) was placed in a conical flask with 65% ethanol and extracted at 65 °C for 2.5 h. Filtration was carried out and remaining extracting solvent was evaporated using rotary film evaporator (Buchi, India) at low pressure. The residue obtained was freeze dried and stored at - 20°C. Further, the extract was eluted with n-hexane, dichloromethane, ethyl acetate and n-butanol in a stepwise manner. After the stepwise elution of extract, the fractions were collected and the remaining solvents were removed. The extracted material was dissolved in dimethyl sulfoxide (DMSO) and diluted with phosphate buffer saline (pH 7.4) [7].

2.3 Physicochemical determination of *Blepharis edulis* roots

Blepharis edulis roots were tested for following physiochemical parameter:

2.3.1 Determination of total ash

Four grams of the powdered extract was weighed and placed in a previously ignited and tared silica crucible. The material was spread in an even layer from surface and ignited by gradually increasing the heat to a temperature of 550–650°C until it was burn fully until no carbon is left, indicating the absence of carbon with white appearance of the material. The material was cooled in a desiccator and weighed. The content of total ash was recorded in mg/g with reference to air-dried material [8].

2.3.2 Determination of acid insoluble ash

Ten milligrams of the ash was taken in the crucible and hydrochloric acid (20 ml) was added to it. The material was covered with a watch glass and boiled gently for 4 min. The insoluble matter was collected on an ash less filter paper and washed with hot water until the filtrate was neutralized. The insoluble matter left on the filter paper was transferred to the original crucible, dried on a hot plate and ignited to constant weight. The residue was allowed to cool in a suitable desiccator for 20 min and weighed. The content of acid-insoluble ash was calculated [8].

2.3.3 Determination of water soluble ash

The obtained ash was placed in the boiling water for 5 min. Then the insoluble material was put on the ash less filter paper. The weight of the insoluble material was subtracted from the weight of the drug ash. The resultant weight represented the water soluble ash. Finally, the percentage of water-soluble ash with reference to the air dried drug was calculated [8].

2.3.4 Determination of foreign matter

Sixty gram of drug sample was weighed and spread out a thin layer. The foreign matter was detected by inspection with the unaided eye, separated and weighed, if any, and calculated the percent foreign present [8]. Drug undertaken for further study were free from moulds, insects, animal faecal matter and other contamination such as soil, stones and extraneous material.

2.3.5 Determination of loss on drying

The percentage loss on drying was determined of *Blepharis edulis*. The method used was by gravimetry in which the air dried extract was weighed accurately and placed within a dried weighing bottle and then sample was dried in an oven at 100 to 105°C unless the two consecutive weights were same. With the given formula percent loss on drying was calculated [8].

$$Percentage\ loss\ on\ drying = \frac{Wa - Wb}{Wa} X100$$

Where, Wa is the weight on air dried and Wb is the weight after dried in oven

2.4 Preliminary phytochemical screening and quantitative estimation of phytoconstituents (carbohydrates, alkaloids, flavonoid, tannins and phenolic)

The dried powdered plant material was successively extracted in alcohol (75% v/v) using a Soxhlet apparatus. The extract obtained from powder by successive solvent extraction was subjected to qualitative examination for the phytoconstituents like alkaloids, flavonoids, saponins and phenolic compounds. The preliminary phytochemical studies were performed for testing of different chemical groups present in the drugs solution (20% w/v) of extract was taken unless otherwise mentioned in the respective individual test [9].

2.4.1 Carbohydrate

Molisch's test: To 2-4 ml aqueous dispersion of extract, few drops of alpha-naphthol solution in alcohol were added and shaken well for mixing. Concentrated sulphuric acid was added from sides of the test tube. The appearance of violet ring was observed between two layers of liquid.

Barfoed's test: Equal volume of Barfoed's reagent and test dispersion were mixed and heated for 2 min on boiling water bath. Formation of red color precipitate confirms the presence of carbohydrate.

2.4.2 Alkaloid

To extract the dilute hydrochloric acid were added and filtered. The filtrate was collected and analyzed using following tests:

Murexide test for purine alkaloids: To 3-4 ml test dispersion, 3-4 drops of concentrated sulphuric acid was added and evaporated to dryness. Residue was cooled; two drops of ammonium hydroxide were added and observed for the appearance of purple color.

Wagner's test: To 2-3 ml filtrate, few drops of Wagner's reagent were added and observed for the appearance of reddish brown color precipitate.

Hager's test: To 2-3 ml filtrate, few drops of Hager's reagent were added and observed for the appearance of yellow color precipitate.

Mayer's test: To2-3 ml filtrate, few drops of Mayer's reagent was added and observed for the appearance of precipitate.

Dragendroff's test: To 2-3 ml of filtrate, few drops of Dragendroff's reagents was added and observed for the appearance of orange- brown precipitate.

2.4.3 Tannins and phenolics compounds

To 2-3 ml of alcoholic dispersion of mucilage, few drops of 5% ferric chloride solution were added, and the reaction mixture was observed for the appearance of deep blue-black color.

2.4.4 Flavanoids

Shinodatest: To dry powder or extract, 5 ml of 95 percent ethanol was added to it then after few drops of concentrated hydrochloric acid was added then after 0.5 g of magnesium turnings the observation of pink color confirms the presence of flavonoids.

2.5 *In vivo* studies

2.5.1 Animal model

The experimental animals were male *Wistar rats* weighing between 150 to 170 g. The animal experimental protocol was approved by the Institutional Animal Ethical Committee (No. JCDMCOP/IAEC/06/17/39). The animals were handled as per the guidance of the Committee for the Purpose of Control and Supervision on Experimental animals (CPCSEA), New Delhi, India. During this phase the animal had free access to food, water and kept at constant temperature (22±3° C) and alternate cycle of the light/ dark 12h.

2.5.2 Induction of diabetes

Male albino rats were injected intraperitoneally with streptozotocin (STZ) dissolved in 0.1 M citrate buffer (pH 6.5) at 60 mg/kg. Animals of control group received equal volume of vehicle. After 48 h of STZ injection, blood glucose level of the diabetes induced rats was estimated. The rats depicting values ranging fasting blood glucose ≥ 240 mg/dL, were considered to be diabetic animal.

2.5.3 Experimental design

Total 30 animals were utilized for the different biochemical and histopathological studies and randomly divided into five groups (n = 6) as:

Group I – Normal rats

Group II – Diabetic control (administered with STZ)

Group III – Diabetic control + *Blepharis edulis* (200 mg/kg body weight)

Group IV – Diabetic control + *Blepharis edulis* (400 mg/kg body weight)

Group V – Diabetic control + Glibenclamide (1 mg/kg body weight)

2.5.4 Administration of extract

The *Blepharis edulis* root extract was administered to the respective group via oral route using intragastric tube for 28 days. Tis was executed by inserting an infant oral feeding tube, which was connected to a syringe containing the extract, into the gastric region of the rat. The animals were fasted 30 min before and after the treatment to ensure maximum bioavailability [10].

2.5.5 Determination of blood glucose level and plasma insulin level

Rats of the different groups were kept on fasting overnight and the blood was withdrawn by retro orbital puncture with light anesthesia. Blood was withdrawn from the rats on the 1st, 14th and 28th day after the induction of diabetes to assess the blood glucose [11] and plasma insulin level by glucose oxidase modified method of Herbert [12]. The alteration in the body weight was observed throughout the therapy in the experimental animals.

2.5.6 Determination of total cholesterol, triglyceride and low density lipoprotein

The lipid parameters *viz* total cholesterol and triglycerides were evaluated according the methods of Zlatkis [13]. Level of serum low density lipoprotein (LDL) and cholesterol was estimated by Friedewald formula [14]. Serum was collected by the snip-cut at the tip of the tail under mild anesthesia. The blood was collected in EDTA vial and centrifuged to extract out blood serum for the estimation of biomedical parameter such as total cholesterol, triglyceride, LDL.

2.5.7 Biochemical serum analysis

Blood from each rat was withdrawn from carotid artery at the neck and collected in previously labeled centrifuging tubes and allowed to clot for 30 min at room temperature (25°C). Serum was separated by centrifugation at 3000 rpm for 15 min. The separated serum was used for the estimation of some biochemical parameters. The samples were analyzed for the estimation of serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) [15].

2.5.8 Histopathology of liver and pancreas

After 28 days of treatment, the animal was fasted for 12 h before sacrificing. After sacrificing, the liver and pancreas were picked out and placed in deep freezer maintaining the temperature - 70 to – 80 °C until the start of analysis.

2.5.8.1 Histopathological studies of liver

The liver was taken and washed gently with phosphate buffer solution (7.4 pH) in triplicate manner in order to avoid sticking of blood clot on the organ surface. Further it was placed in 10% formaline solution. Washing was carried out using analytical grade alcohol followed by washing with xylene after. The washed tissue was placed in paraffin wax. Further, the tissue was cut with semi-automatic microtome (Leica RM2245) in size width of 6 µm and side by side it is stained with Hematoxylin-eosin. The tissues were examined under the contrast microscope on optical zoom with 40X (Optec B300) and the images were taken using computer assisted analyzer. The observations were done to check tissue inflammation (connective tissue portal region), tissue necrosis, sinusoidal hyperemia, the observation was thrice independently for each parameter the score were made on that basis as 0 for no change and normal condition, + for mild change, ++ for moderate change, and +++ for severe change.

2.5.8.2 Histopathological studies of pancreas

After putting out the pancreas from the deep freezer it was washed with ice cold saline. Then the pancreatic tissues were placed in 10% neutral formalin solution to fix the tissues. Further it is embedded on paraffin wax and tissues were cut with microtome of thickness 6 µm. Then it was stained with the Hematoxylin-eosin and examined under the microscope [16].

3. Results and Discussion

3.1 Physicochemical determination *Blepharis edulis* roots

The percentage yield of the crude extract was calculated to be 4.15 g. The acid insoluble ash and water soluble ash values of *Blepharis edulis* root were 0.4 and 5 % w/w of the total powdered extract. Total ash value was estimated as 4.5 % w/w. The loss on drying was 1.30 % w/w. It was observed that there was no foreign matter presented in the crude dried extract (Table 1).

Table 1: Physiochemical estimation of powdered root of *Blepharis edulis.*

Parameters	Results
Total ash	4.5 ± 0.8 % w/w
Acid insoluble ash	0.4 ± 0.052% w/w
Water-soluble ash	5 ± 0.2% w/w
Foreign matter	Nil
Loss on drying	1.3 ± 0.035% w/w

3.2 Phytochemical screening

The results of phycochemical screening tests are presented in Table 2. From the table it is clear that the extract contained alkaloid, flavonoids and phenolic groups.

Table 2: Phytochemical screening test result showing presence and absence of different chemicals.

Phytochemical tests	Observations
Alkaloids	+
Flavonoids	+
Tannins	-
Carbohydrate	-
Phenolic	+
+ indicate presence, and - indicate absence	

3.3 Blood glucose level

The blood glucose level in the animals has been presented in Figure 1. The intraperitoneal administration of STZ resulted in nearly threefold increase of the fasting blood glucose levels in the animal models. The blood glucose level was measured at different intervals of time such (1st, 14th and 28th day). It was observed that with the gradual increase in dose of the *Blepharis edulis*, the blood glucose level was improvised. At the end of 28th day, *Blepharis edulis* treated animals showed a significant ($p < 0.05$) reduction in blood glucose level nearly to the normal level compared with the diabetic animals. Application of one way analysis of variance (ANOVA) showed a significant difference between the groups at 95% confidence interval ($p < 0.05$) as the calculated F value was higher than the tabulated F value (Table 3).

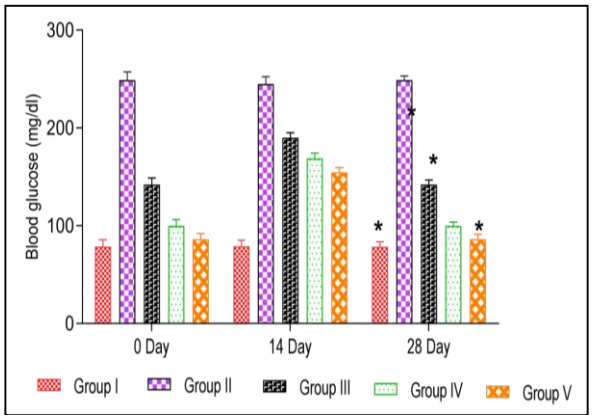

Fig 1: Blood glucose level on day 0 and day 14 of dose administration

Table 3: Results of one way ANOVA on the blood glucose level of animals treated with different dose of plant extract ($p < 0.05$)

Source of Variation	SS	DF	MS	F (DFn, DFd)	Significant
Interaction	15413	4	3853	F (4, 50) = 15.24	Yes
Row Factor	19789	1	19789	F (1, 50) = 78.29	
Column Factor	190203	4	47551	F (4, 50) = 188.1	

3.4 Plasma insulin level

The plasma insulin levels were measured at 1st, 14th and 28th day and presented in Figure 2. A significant lowering of insulin level was observed in animals treated with *Blepharis edulis* root extract. Further, after comparison it is noticed that there is significant increase in the insulin level in the Group III and Group IV and it is concluding that this extract *Blepharis edulis* had anti diabetic activity. Application of two way analysis of variance (ANOVA) showed a significant difference between the groups at 95% confidence interval ($p < 0.05$) as the calculated F value was higher than the tabulated F value (Table 4).

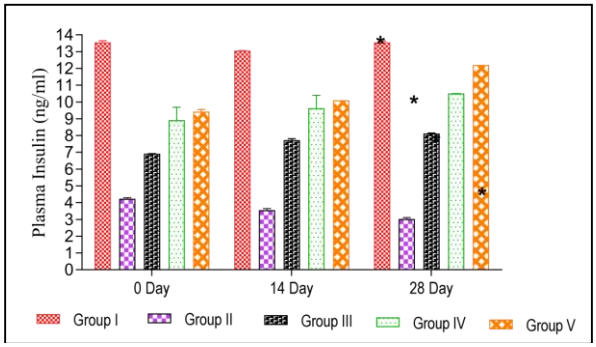

Fig 2: Plasma insulin level after administration on day 0, day 14 and day 28

Table 4: Results of one way ANOVA on the insulin level of animals treated with different dose of plant extract ($p < 0.05$).

Source of variation	SS	DF	MS	F (DFn, DFd)	Significant
Interaction	30.14	8	3.767	F (8, 75) = 7.642	Yes
Row Factor	12.51	2	6.253	F (2, 75) = 12.69	
Column Factor	960.4	4	240.1	F (4, 75) = 487.1	
Residual	36.97	75	0.493		

3.5 LDL and triglyceride and cholesterol level

Significant decrease in LDL, triglyceride and total cholesterol levels was observed in animals treated with *Blepharis edulis* root extract (Figure 3, 4 & 5). Application of two way analysis of variance (ANOVA) on the results of LDL, triglyceride level, and total cholesterol level showed a significant difference between the groups at 95% confidence interval ($p < 0.05$) as the calculated F value was higher than the tabulated F value (Table 5, 6 & 7).

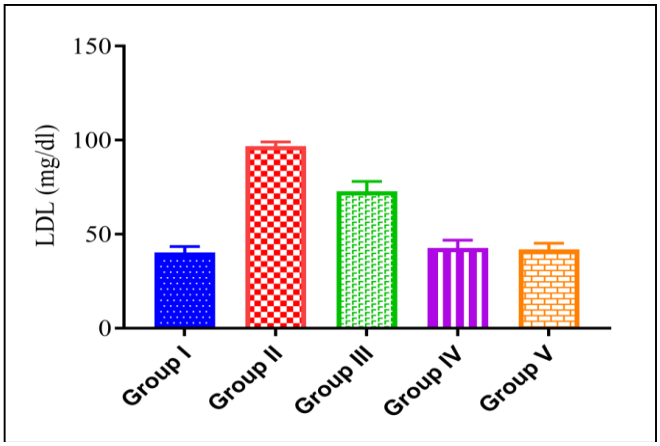

Fig 3: Graphical representation of LDL level in different group animals

Table 5: Results of one way ANOVA on the LDL level of animals treated with different dose of plant extract ($p < 0.05$).

Source of variation	SS	DF	MS	F (DFn, DFd)	Significant
Treatment (between columns)	15141	4	3785	F (4, 25) = 44.92	Yes
Residual (within columns)	2107	25	84.26		
Total	17247	29			

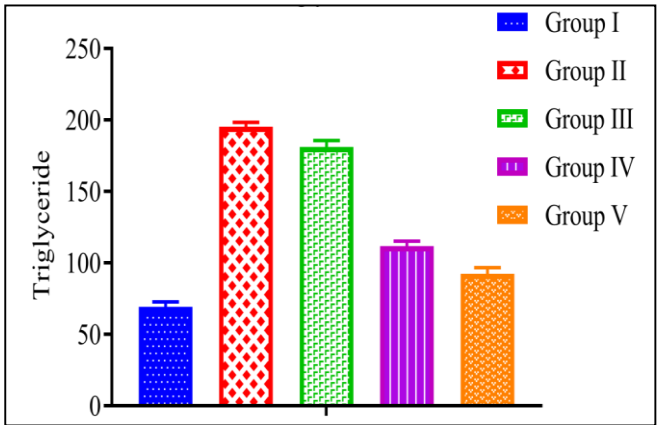

Fig 4: Graphical representation of triglyceride level in different group animals

Table 6: Results of one way ANOVA on the triglyceride level of animals treated with different dose of plant extract ($p < 0.05$).

Source of variation	SS	DF	MS	F (DFn, DFd)	Significant
Treatment (between columns)	73788	4	18447	F (4, 25) = 201.6	Yes
Residual (within columns)	2287	25	91.48		
Total	76075	29			

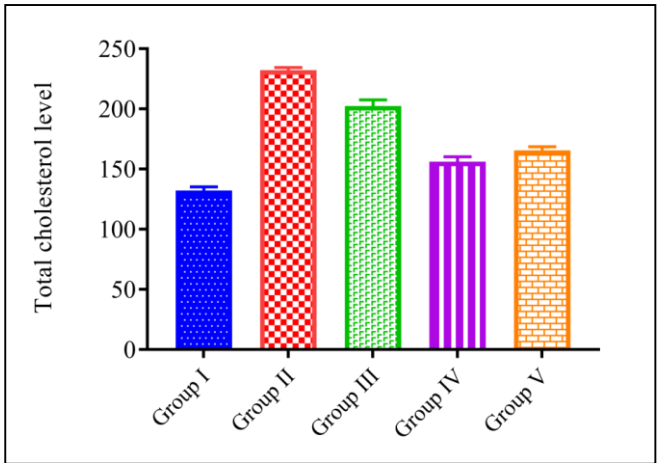

Fig 5: Graphical representation of total cholesterol level in different group animals

Table 7: Results of one way ANOVA on the total cholesterol level of animals treated with different dose of plant extract ($p < 0.05$).

Source of variation	SS	DF	MS	F (DFn, DFd)	Significant
Treatment (between columns)	37533	4	9383	F (4, 25) = 111.4	Yes
Residual (within columns)	2107	25	84.26		
Total	39639	29			

3.6 Biochemical analysis SGPT and SGOT

While statistical treatment of data it is seen that the SGPT is decreased on the dose of 400 mg/kg body weight by continuous treating the animal for 28 days (Figure 6 and 7). When the comparison occurred with the diabetic animal it is seen that there is significant change in the SGOT level of the different group rats so the extract of *Blepharis edulis* is very suggestive to the medicinal use and mainly for anti-diabetic action. Application of one way analysis of variance (ANOVA) on the SGPT data showed a significant difference between the groups at 95% confidence interval ($p < 0.05$) as the calculated F value was higher than the tabulated F value (Table 8). However, in case of SGOT level no significant ($p < 0.05$) difference between the groups was observed as the calculated value was lower than the tabular value (Table 9).

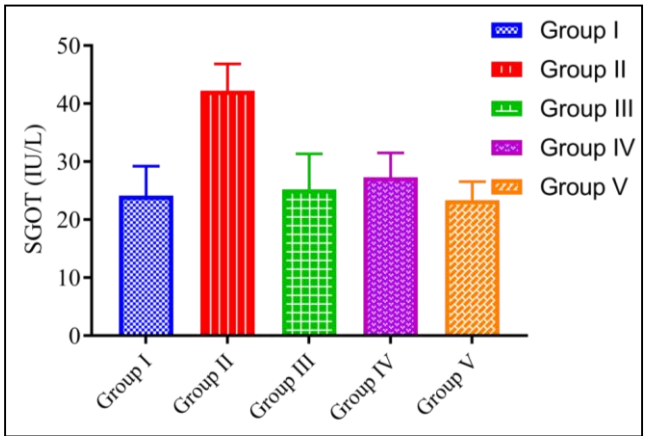

Fig 6: SGOT level in different animal group after continuous treatment of 28 days

Table 8: Results of one way ANOVA on the SGOT level of animals treated with different dose of plant extract ($p < 0.05$).

Source of variation	SS	DF	MS	F (DFn, DFd)	Significant
Treatment (between columns)	1478	4	369.4	F (4, 25) = 2.727	No
Residual (within columns)	3386	25	135.5		
Total	4864	29			

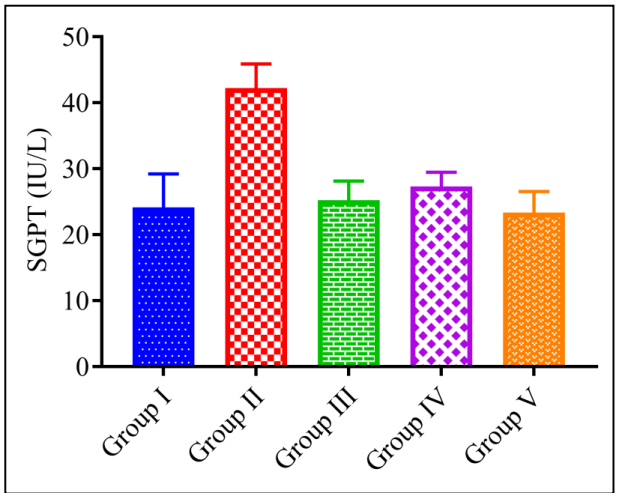

Fig 7: SGPT level in different animal group after continuous treatment of 28 days

Table 9: Results of one way ANOVA on the SGPT level of animals treated with different dose of plant extract ($p < 0.05$).

Source of variation	SS	DF	MS	F (DFn, DFd)	Significant
Treatment (between columns)	1478	4	369.4	F (4, 25) = 4.887	Yes
Residual (within columns)	1890	25	75.59		
Total	3367	29			

3.7 Result of histopathology of liver

Changes in the histopathology of the liver are reported in Figure 8 and quantitative estimation results are presented in Table 10. A well stated arranged hepatocytes and sinusoid are clearly visible in normal non-diabetic rats. Where as in the diabetic rats the several areas are disorganised with hepatocytes and also found necrosis with increase in the thickness of wall of sinusoids. In the animals treated with plant extract and glibenclamide, we observed that due to the treatment with *Blepharis edulis* and glibenclamide prevented such distortion in the liver cells.

Table 10: Quantitative analysis for the change in histopathology of liver on the basis of different parameters.

Parameters	Control	Diabetic	200 mg/kg Body weight (*Blepharis edulis*)	400 mg/kg Body weight (*Blepharis edulis*)	600 µg/kg Body weight Glibenclamide
Inflammation in tissue	0	+++	++	0	0
Sinusoidal hyperemia	0	+	++	++	0
Tissue necrosis	0	++	+	++	+

0: no change and normal condition, +: mild change, ++: moderate change, +++: severe change

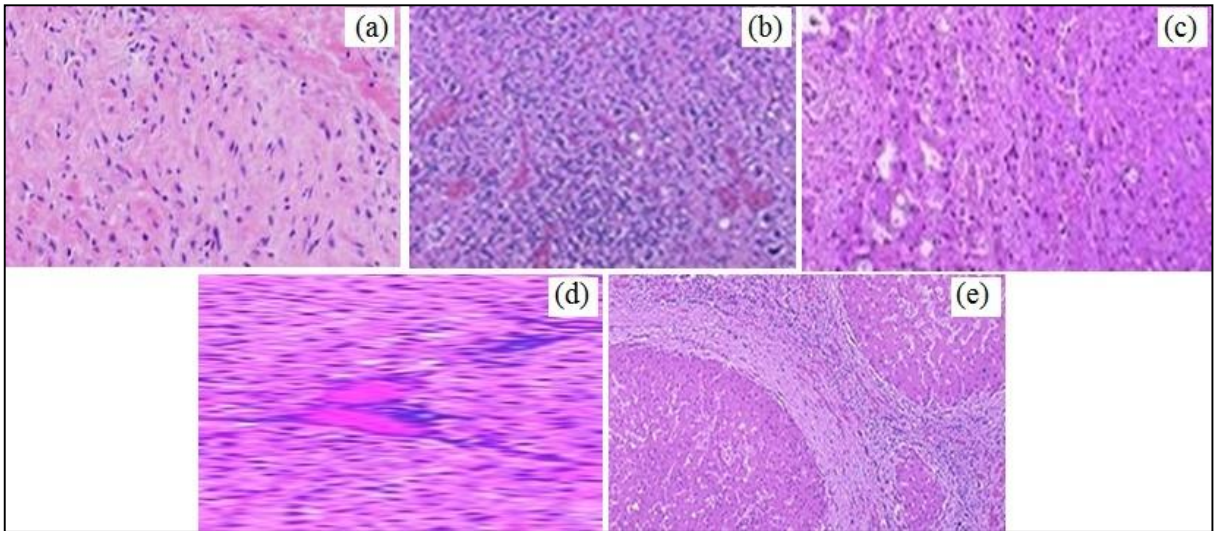

Fig 8: Results of histopathology study of liver in normal control rats (a), diabetic control rats (b), rats treated with *Blepharis edulis* root extract (200 mg/kg body wt) (c), rats treated with *Blepharis edulis* root extract (400 mg/kg body wt) (d) and rats treated with glibenclamide (600 µg/kg body wt)

3.8 Result of histopathology of pancreas

The islets of langerhans in the normal pancreatic tissues were distributed and of variable size in the sane lobule of pancreas (Figure 9a). The stained acinar cells were arranged well in the nuclei. The islets were griped with acinar cell and capsules are on its surroundings (Figure 9b). The islets cells are seen to be normal state in the case of treated with the glibenclamide (Figure 9c). The islets are very compact as seen in the treated group with *Blepharis edulis* 200 mg/kg body weight and counting of islets number in comparison to the normal is lesser (Figure 9d). In comparison of last group treated with *Blepharis edulis* 400 mg/kg body weight is said to be shrieked and hydrolysis in comparison to the control group (Figure 9e).

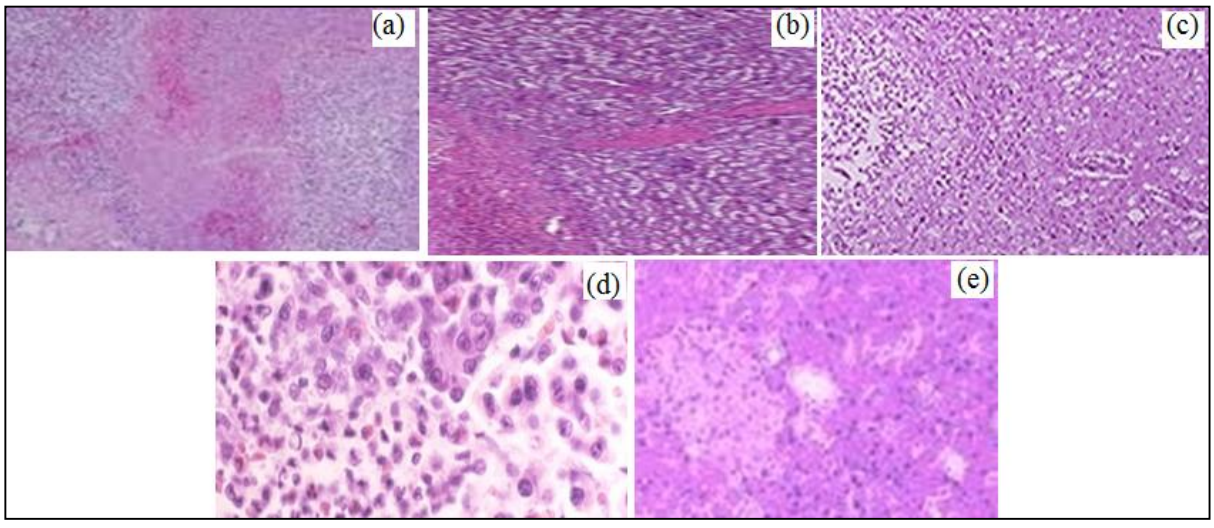

Fig 9: Results of histopathology study of pancreas in normal rats (a), diabetic control rats (b) rats treated with glibenclamide (600 µg/kg body wt) (c). rats treated with *Blepharis edulis* root extract (200 mg/kg body wt) (d) rats treated with *Blepharis edulis* root extract (400 mg/kg body wt)

4. Conclusion

In this study, methanolic extract of *Blepharis edulis* root was oral administered to chemical induced diabetic male Wistar albino rat models for 4 weeks. The extract showed significant anti-hyperglycemia activity and normalized triacylglycerol, total cholesterol, and low density lipoprotein; and serum enzymes such as alanine aminotransferase and aspartate aminotransferase when compared to chemical induced diabetic control Wistar albino rats.

5. Conflict of interest

All authors confirm no conflict of interest involved with this manuscript.

6. References

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