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Antidiabetic and antihyperlipidemic effects of alcoholic and aqueous stem bark extracts of *Limonia acidissima*, linn in alloxan induced diabetic rats

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Abstract

The present study was designed to investigate the antidiabetic and antihyperlipidemic effects of the alcoholic and aqueous extract obtained from the stem bark of *Limonia acidissima*Linn (Rutaceae) using alloxan induced diabetic model in rats for 14 days. The alcoholic extract (200mg/kg) showed significant activity (P<0.01) in lowering blood glucose level than the aqueous extract (200mg/kg) which was comparable to glibenclamide (5mg/kg), a standard antidiabetic drug. Different biochemical parameters i.e. TGL(triglycerides), HDL, LDL, VLDL and total cholesterol level were also carried out which show that both alcoholic and aqueous extracts has significantly (p<0.001) reversed the diabetes-included hyperlipidemia compared to standard drug. Concurrent histological studies of the pancreas of these animals showed comparable regeneration by alcoholic and aqueous extracts which were earlier, necrosed by alloxan. The data suggests that the alcoholic extracts (200 mg/kg) may be effectively utilized as antidiabetic and antihyperlipidemic agents and supports its traditional usage in the control of diabetes. However, further pharmacological evaluations are required to isolate and identify the active antidiabetic and antihyperlipidemic principles in the plant as well as elucidating their mechanisms of action.

Keywords: *Limonia acidissima* Linn, Antidiabetic activity, Antihyperlipidemic activity, Alloxan, Histopathology.

Introduction

Limonia acidissima, Linn. belonging to family Rutaceae synonomicaly known as Feronia Limonia Swingle[1], Feronia elephantum Correa[2], Schinus Limonia Linn[3] and commonly also called as wood apple and elephant apple is native and common in the wild in dry plains of India and Ceylon and cultivated along roads and edges of fields and occasionally in orchards. It is also grown throughout Asia tropical, Asia temperate, Southern America and northern Malaysia [4,5,6]. It is an aromatic, slow growing deciduous tree up to 9m tall grows all over India in dry and warm areas up to 450m elevation, Often polygamomonoecious tree with rough, spiny bark, The spines axillary, short, straight, 2-5 cm long on some of the zigzag twigs[7]. The leaves are deciduous, alternate, dark-green, leathery, 3 to 5 inch long. Small fragrant light red flowers 1/2 inch wide are borne in small, loose, terminal or lateral panicles. The fruit is berry round to oval, globose, large, 2 to 5 inch wide, with a hard, woody rind, which is grayish-white, scurfy rind about 6 mm thick. The pulp is sticky brown, aromatic odorous, resinous, astringent, acid or sweetish, white seeds scattered through it [2,3,7,8].

The bark mainly contains marmesin, Bergapten, Psoralen, acidissimin, (-)-(2S)-5,3'-dihydroxy-4'-methoxy-6",6"-dimethylchromeno-(7,8,2"3")- flavanone, alkaloid, coumarins (luvangetin, xanthotoxin and marmesin), lignan, steroids (sitosterol and sitosterol-o-beta- d-glucoside) and triterpenoid (lupeol and limonin)[9,10].

The fruit is much used in India as a liver and cardiac tonic, and when unripe, as an astringent means of halting diarrhea and dysentery and effective treatment for hiccough. A decoction of the bark is effective for uterine disorders, bark cures bilious disorders. The gum is demulcent and constipating, and is used in treatment of diarrhoea, dysentery, gastropathy, haemorrhoids and diabetes [2,8]. The bark of plant is reported to have antidiarrhoeal properties [11].

It is well known that the incidence of diabetes mellitus is high all over the world especially in Asia. Diabetes is characterized by derangements in carbohydrates, protein and fat metabolism caused by the complete or relative insufficiency of insulin secretion. Pharmacological studies are of much importance and only the way for evaluating any herbs/drug efficacy, therefore, considering its importance we have planned here, stepwise evaluation of antidiabetic potential of alcoholic and aqueous extracts of powdered

stem bark of *Limonia acidissima*, Linn. including biochemical parameters.

Materials and Methods

Plant Material

The stem barks of *Limonia acidissima*, Linn. were collected from the ABS garden, Karipatti, Salem, Tamilnadu in the month of June-July 2008 which were identified and authentified as *Limonia acidissima*, Linn. by Dr. P. Jayaraman, Botanist, Director, Plant Anatomy Research Centre (PARC), West Tambaram, Chennai. (Certificate no. PARC/2008/206). The voucher specimen was kept at Dept. of Pharmacognosy, Padamavathi College of pharmacy, Dharmapuri, T.N., India.

Preparation of Extract

The stem bark were washed with water and dried in sunlight for one hour and then it was dried under shade. By the help of wood grinder the dried stem bark was powered and passed through the sieve no. 60. Dried course powder of the stem bark was extracted with alcohol (90%) and water by using soxhlet apparatus separately until the extraction solvents becomes colourless. The extracts were then concentrated, dried and stored in refrigerator. Obtained dark green alcoholic and aqueous extract were suspended in 1% tween 80 solution for the pharmacological study [12,13].

Experimental animals

Wister strain albino rats of either sex weighing between 200-250 gms were used and were kept in a well ventilated animal house in clean polypropylene cages maintained at 27±2°C temperature and relative humidity 55±10% with a constant 12 hour light/dark schedule. The animals were fed with standard rat pellet diet (Hindustan Lever Ltd., Mumbai, India) and clean drinking water was made available ad libitum. All the animal procedures were performed after approval from the Institutional animal ethics committee, Periyar college of pharmaceutical sciences for girls, tiruchirapalli (T.N.), India (CPCSEA/265).

Chemicals and reagents

Alloxan monohydrate and Glibenclamide were obtained from Loba Chemicals, India. estimation of Blood glucose, TGL(triglycerides), HDL, LDL, VLDL and total cholesterol level by using Secomam semi auto-analyzer (Analytical Automation Pvt. Ltd., Mumbai). Standard rat pelleted diet obtained from Hindustan Lever Ltd., Mumbai, India and plant extracts (alcoholic and aqueous).of the stem bark of Limonia acidissima, Linn were harvested from the ABS garden, Karipatti, Salem, Tamilnadu, India. All other reagents and chemicals used were of analytical/ Pharmacopoieal grade purchased from Loba Chemicals, India.

Acute Toxicity Studies

The acute oral toxicity study was carried out as per the guidelines set by OECD 423 (Acute toxic class method) [9]. Animals (n=3) were fasted overnight prior to dosing (food but not water was withheld). The dose level to be used as the starting dose was 5mg/kg body waight. Animals were observed individually after dosing during the first 30 minutes, periodically during the first 24 h, with special attention given during the first 4 h, and daily thereafter [14,15].

Induction of diabetes

Hyperglycemia was induced by single intraperitonial injection of freshly prepared aqueous solution of alloxan monohydrate 150mg/kg, to overnight fasted rats. After 48 hrs of alloxan injection, the animals which did not developed hyperglycemia i.e. glucose level>200mg/dl, were rejected /replaced with new animals. Immediately after induction of diabetes, rats were classified into five groups of six rats each [16,17,18].

Experimental design

Evaluation of antidiabetic effect of plant extracts was done by taking randomly six rats in each five groups as following[17,18].

Group I: Served as normal control (Received normal saline 2 ml/kg body weight).

Group II: Served as diabetic control (Treated with alloxan monohydrate 150 mg/kg body weight).

Group III: Received alcoholic extract, 200mg/kg body weight which is prepared in 2% carboxy methyl cellulose (CMC) was given orally.

Group IV: Received aqueous extract, 200mg/kg body weight which is prepared in 2%carboxy methyl cellulose (CMC) was given orally.

Group V: Served as reference standards (Glibenclamide, 5mg/kg body weight orally).

The various treatments were started after induction of diabetes and considered as day 0 of diabetes. Drugs were administered orally and treatment was continued for 14 consecutive days. The doses employed for all drugs were within therapeutic range to suit the experimental animal used i.e. the rat.

Sample collection

Before the treatment (0 day) and at the end of 7th and 14th day, blood samples were collected from the tip of the tail of each rat under mild ether anesthesia in 1ml Eppendrof tubes containing 50µl of anticoagulant (heparin). Rats were sacrificed by cervical dislocation under mild ether anaesthesia after day 14 and tissues were used for Histopathological studies.

Estimation of biochemical parameters

Blood glucose estimation was done on the day 0, 7 and 14 of the study. Blood glucose levels were estimated by glucose oxidase

method using Secomam semi auto-analyzer. Serum separated by centrifugation of blood at 4000rpm for 10mins which is stored in a refrigerator until analyzed and the serum was subjected for the estimation of TGL(triglycerides), HDL, LDL, VLDL and total cholesterol level using Secomam semi auto-analyzer (Analytical Automation Pvt. Ltd., Mumbai) following manufacturers instruction [19,20].

Statistical Analysis

Values are expressed as mean ± standard error of mean (SEM) and analyzed by using statistical package for social sciences (SPSS) version 7.5 using one way analysis of variance (ANOVA) followed by student 't' test. Data were considered statistically significant at $P<0.001$ and $P<0.01$[21].

Histopathological studies

Rats were sacrificed by cervical dislocation under mild ether anaesthesia after day 14. The whole pancreas from each animal was removed after killing the animals, was placed in 10% formalin solution and immediately processed by the paraffin technique. Sections of 5 mm thickness were cut and stained by haematoxylin and eosin (H & E) for histological examination [22,23].

Result and Discussion

Acute Toxicity Studies

Acute oral toxicity studies were performed according to the OECD (Organization of Economic Co-operation and Development) guidelines 423(Acute toxic class method). In acute toxicity study no toxic symptoms were observed. All animals behaved normally. No neurological or behavioral effects could be noted and no mortality was found for alcoholic and aqueous extracts of stem bark of *Limonia acidissima* Linn. up to dose 2000mg/kg body weight, it is considered as LD50. Thus, 1/10th of LD50 is taken as the effective dose. As a result, doses of 200 mg/kg body waight was taken as an effective dose for the study. Body weights of the rats before and after termination were noted and any change in skin and fur, eyes, mucous membrane, respiratory, circulatory, autonomic and central nervous system, somatomotor activity behavior pattern were observed and also signs of tremors, convulsions, salvation, diarrhoea, lethargy, sleep and coma were noted. The onset of toxicity and signs of toxicity was also to be noted if any. The results of toxicity studies and gross behavior were reported in Table 1 and 2 respectively.

Table 1: The Results Of Acute Toxicity Class Method.

S. No	Group	Dose	Weight of animal in gms		Signs of Toxicity	Onset of toxicity	Reversible/ Irreversible	Duration of observation
			Before test	After test (on4th day)				
1.	Aqueous extract	2 gm/kg	244	241	No signs of toxicity	Nil	Nil	3 days
2.	Aqueous extract	2 gm/kg	244	242	No signs of toxicity	Nil	Nil	3 days
3.	Aqueous extract	2 gm/kg	236	233	No signs of toxicity	Nil	Nil	3 days
4.	Alcoholic extract	2 gm/kg	239	237	No signs of toxicity	Nil	Nil	3 days
5.	Alcoholic extract	2 gm/kg	238	235	No signs of toxicity	Nil	Nil	3 days
6.	Alcoholic extract	2 gm/kg	225	225	No signs of toxicity	Nil	Nil	3 days
As no toxicity or death was observed for these dose levels, the same dose level was tried again.								
7.	Aqueous extract	2 gm/kg	238	235	No signs of toxicity	Nil	Nil	3 days
8.	Aqueous extract	2 gm/kg	244	242	No signs of toxicity	Nil	Nil	3 days
9.	Aqueous extract	2 gm/kg	225	222	No signs of toxicity	Nil	Nil	3 days
10.	Alcoholic extract	2 gm/kg	234	230	No signs of toxicity	Nil	Nil	3 days
11.	Alcoholic extract	2 gm/kg	228	226	No signs of toxicity	Nil	Nil	3 days
12.	Alcoholic extract	2 gm/kg	235	233	No signs of toxicity	Nil	Nil	3 days

Table 2: The Results Of Gross Behavioral Studies Of The Animal.

Time	Alert	Passive	Groo-ming	Restless-ness	Aggre-ssive	Touch response	Pain response	Convulsion	Gripping strength	Pin reflex	Corneal reflex	Writ-hing	Pupil size	Saliv-ation	Skin colour	Lacrimatio	Respiration
1st hour	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2nd hour	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3rd hour	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
4th hour	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
24th hour	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- = Normal ++ = Moderate effect
+ = Mild +++ = Marked effect

Antidiabetic effect

Blood glucose levels in alloxan (150 mg/kg) induced diabetic rats were in the range of 250-358 mg/kg, which was considered as sever diabetes. In the standard drug (Glibenclamide 5mg/kg) and alcoholic extract (200 mg/kg) treated groups the peak values of blood sugar significantly decreased to 90.4 mg/kg and 99.8 mg/kg respectively on the 14th day. Thus the alcoholic extract was found to be almost significant as standard drug in lowering blood glucose level (Fig. 1). Where as the aqueous extracts (200mg/kg) treated group showed 112.6 kg/mg blood glucose level, which is comparatively less to alcoholic extract and standard drug. The results are presented in Table 3.

Antihyperlipidemic effect

The alcoholic and aqueous extract of the stem bark of *Limonia acidissima*, Linn has significantly ($p<0.001$) reversed the diabetes-included hyperlipidemia compared to standard drug (Fig. 2). Alcoholic extracts with 200mg/kg significant reduction of total cholesterol level (110.94 mg/dl), TGL (97.73mg/dl), HDL (38.84mg/dl) VLDL (20.13mg/dl) and LDL (50.86 mg/dl.) was comparative to standard drug (Glibenclamide 5 mg/kg) treated groups total cholesterol (84.64 mg/dl.), TGL (84.58 mg/dl), HDL (33.82 mg/dl), VLDL (16.92 mg/dl), LDL (33.9A mg/dl.) reached normal when compared to aqueous extract treated group with 200kg/mg total cholesterol (130.4mg/dl), TGL (105.76mg/dl), HDL (42.68 mg/dl), VLDL (21.87 mg/dl), LDL (72.85 mg/dl), which was comparatively less the observed ratio of TGL, HDL, VLDL, LDL and cholesterol. Different biochemical parameters is tabulated in Table 4

Table 3: Effect Of Aqueous And Alcoholic Extract On Blood Glucose Level.

Drug treatment	Dose	Blood glucose (mg/dl)		
		0 day	7th day	14th day
Control (Normal Saline)	2 ml/kg	90.3±4.6	90.8± 3.6	90.8±6.6
Diabetic control (Alloxan)	150 mg/kg	263.3 ±18.4	262.1 ±19.2	262.4 ±17.7
Aqueous Extract	200 mg/kg	265.5 ±15	169.7±12.3*	112.6 ±7.6**
Alcoholic Extract	200 mg/kg	264.8 ±17.4	122.4±8.4**	99.8 ±6.2**
Glibenclamide	5 mg/kg	260.3 ±13.5	109.6± 6.7**	90.4±7.2**

- Data are expressed as mean ± SEM; n=6 animals in each group.
- Values are statistically significant at *P<0.01 Vs Control and **P<0.001 Vs Control by Student't' test.

Table 4: Effect Of Aqueous And Alcoholic Extract On Levels Of Total Cholesterol, Tgl, Hdl, Ldl And Vldl.

Drug treatment	Total cholesterol (mg/dl)	TGL (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
Control (Normal saline 2 ml/kg)	82.53±7.21	75.15±5.03	25.6±1.83	41.9± 4.37	15.03±1.006
Diabetic control (Alloxan 150 mg/kg)	219.3±10.49	116.48±8.5	24.93±6.52	171.07±2.27	23.30±1.7
Aqueous Extract (200 mg/kg)	130.4±7.52	105.76±5.54	42.68*±4.12	72.85**±2.72	21.87±2.33
Alcoholic Extract (200 mg/kg)	110.94±6.42	97.73*±4.63	38.84*±3.34	50.86**±3.24	20.13±2.24
Glibenclamide (5 mg/kg)	84.64 ±6.79	84.58*±6.7	33.82*±2.79	33.9**±2.66	16.92±1.34

Data are expressed as mean ± SEM; n=6 animals in each group by student't' test.
Values are statistically significant at * P<0.01 and ** P<0.001 Vs Control.

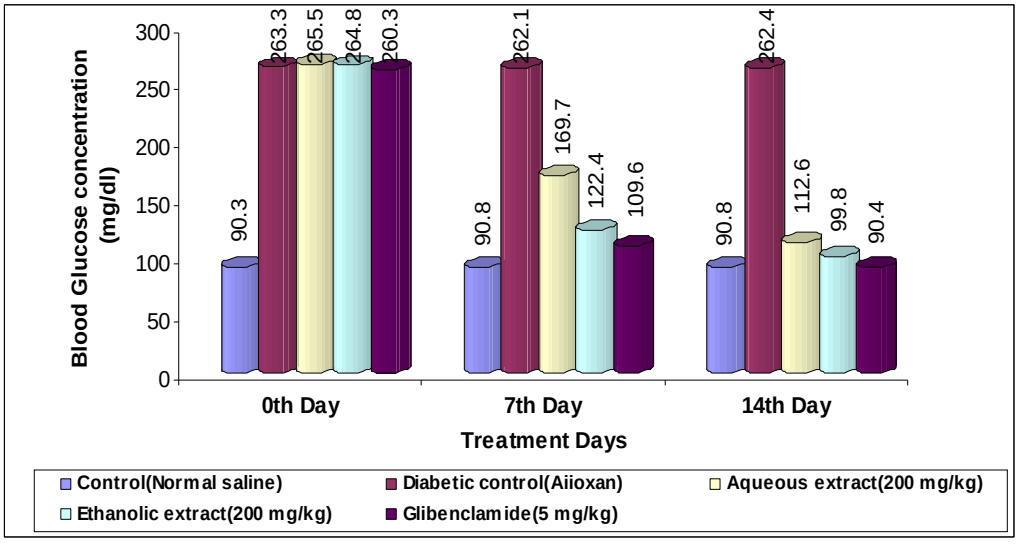

Fig 1: Graphical representation of Effect of aqueous and alcoholic extract of Stem bark of Limonia acidissima, Linn on blood glucose level.

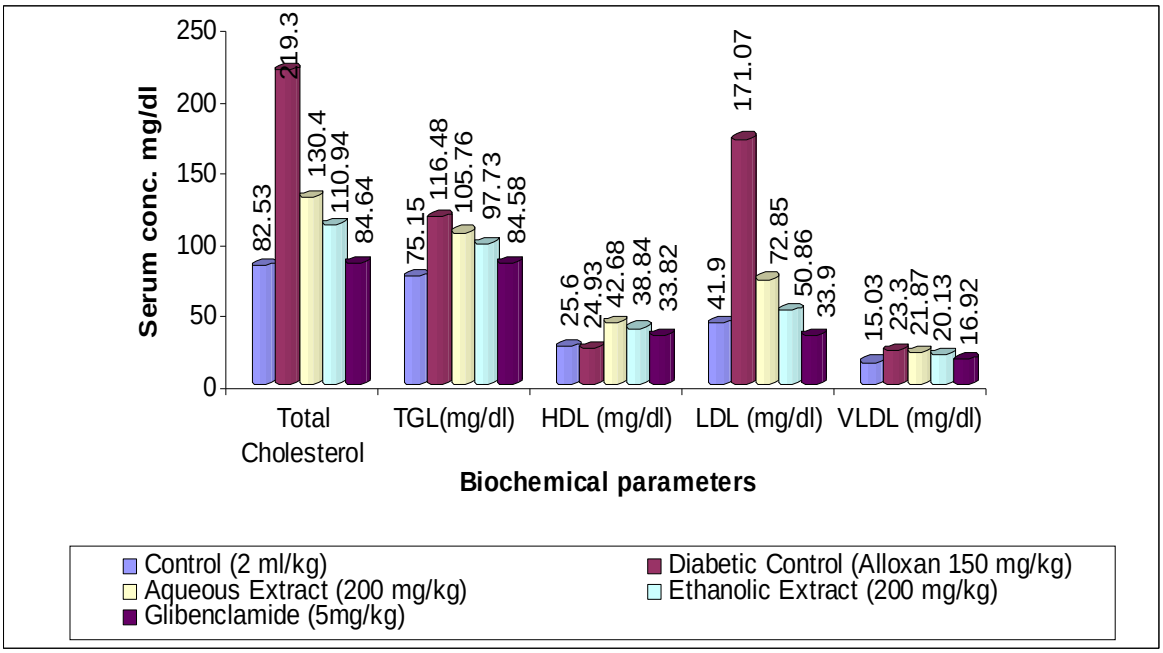

Fig 2: Graphical representation of effect of aqueous and alcoholic extract of stem bark of Limonia acidissima, Linn on levels of Total Cholesterol, TGL, HDL, LDL and VLDL.

Histopathological studies

Multiple section of pancreas were taken and studied for any histological changes. The pancreas present in the group of animals treated with standard drug showed normal appearance of pancreatic lobules, acini and cell. The islets were normal in size, shape and number. The pancreas in the group of animals treated with the alcoholic extract 200 mg/kg showed normal appearance of pancreatic lobules, acini and cells. The islets were normal in size, shape and number comparatively similar to that of standard treated. Where as pancreas present in the group of animals treated with aqueous extract 200 mg/kg showed normal appearing exocrine pancreas but islets number was found less compared to alcoholic extract treated and size was also reduced. And pancreas of diabetic control animal showed partially damaged or even destroyed pancreatic lobules, acini, cells, islet size, shape and number. The photomicrographs of histopathological section of pancreas are presented in figure (3-7).

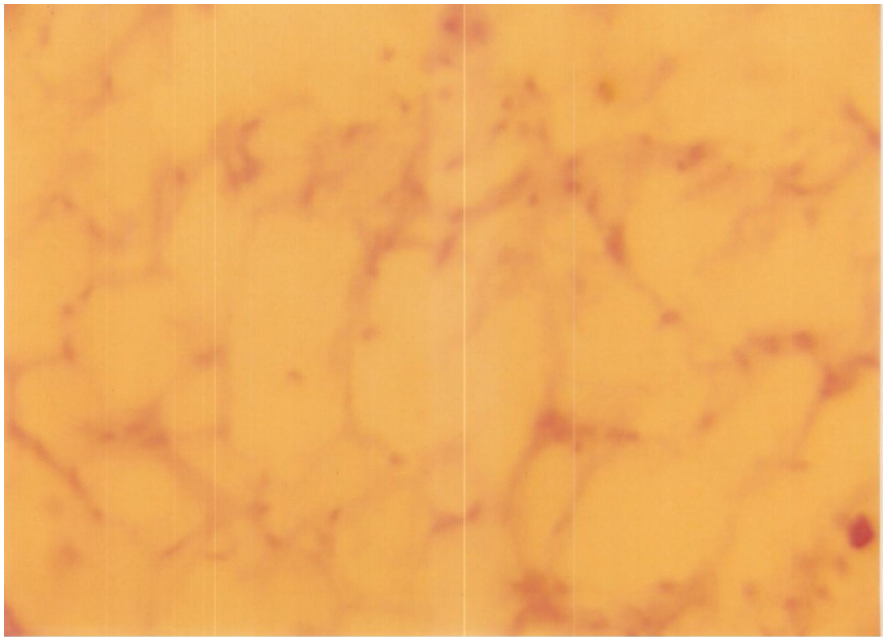

Fig. 3: Histopathological section of pancreatic tissue of normal control rats.

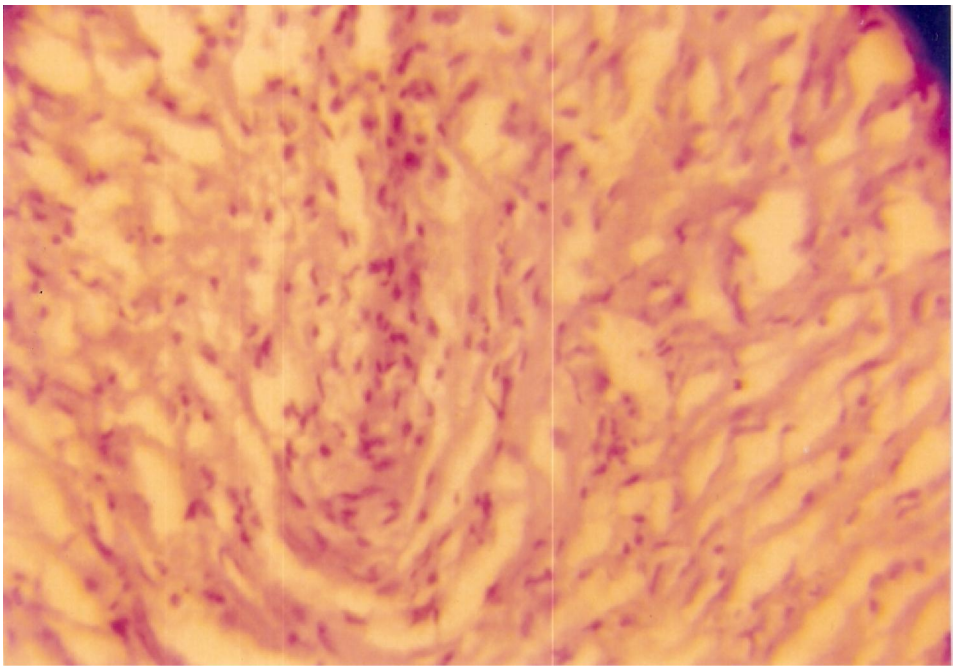

Fig. 4: Histopathological section of pancreatic tissue of Alloxan diabetic control rats.

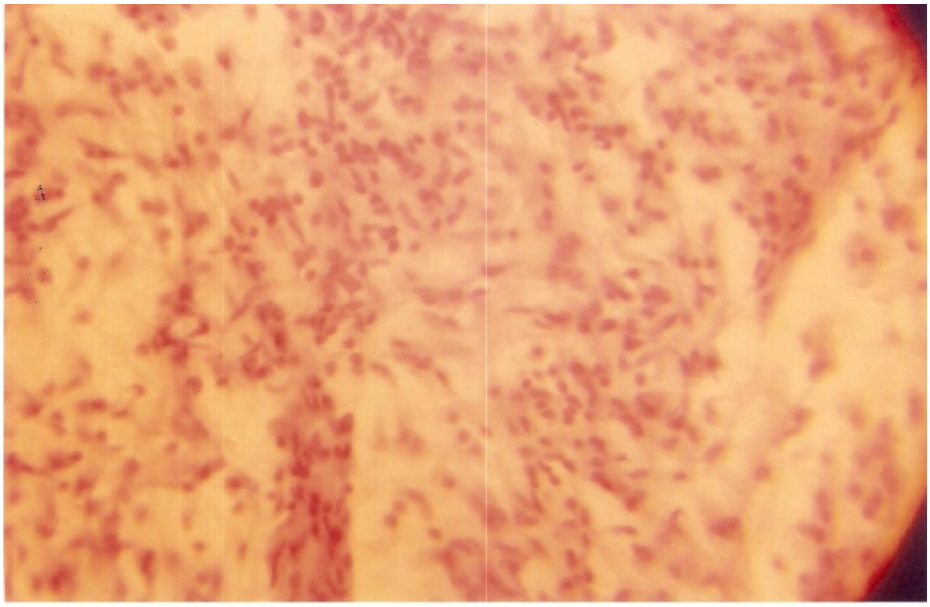

Fig. 5: Histopathological section of pancreatic tissue of rats treated with Alcoholic Extract of stem bark of Limonia acidissima, Linn.

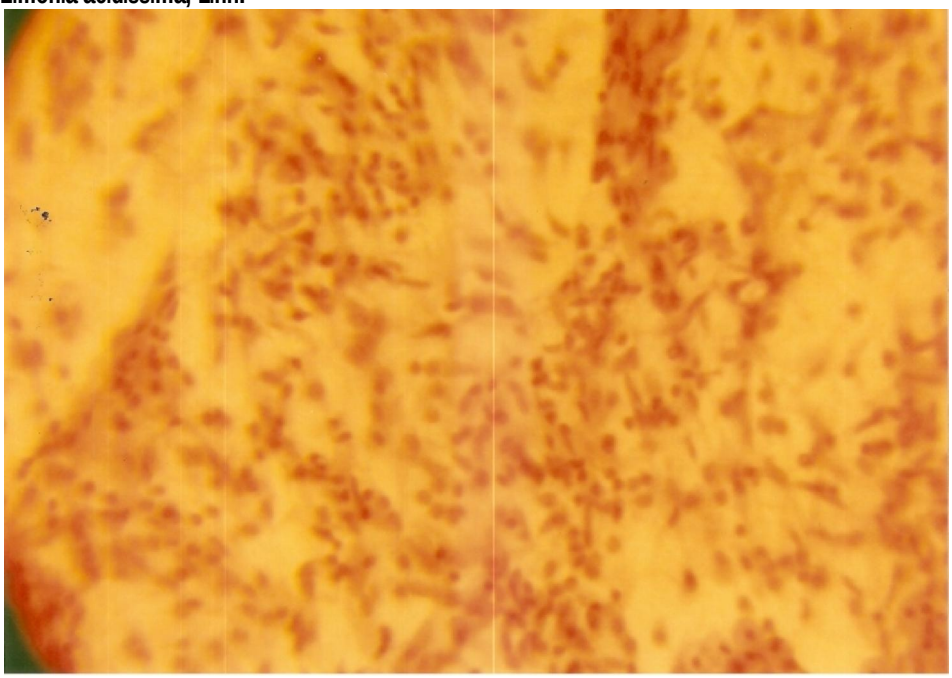

Fig. 6: Histopathological section pancreatic tissue of rats treated with Aqueous Extract of stem bark of Limonia acidissima, Linn.

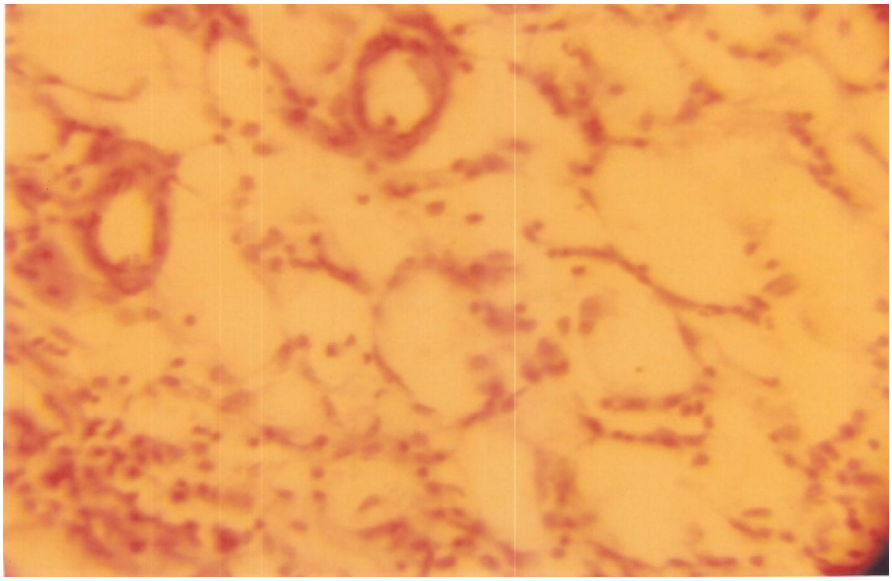

Fig. 7: Histopathological section of pancreatic tissue of Glibenclamide treated rats showing normal pancreatic morphology with mild degeneration.

Conclusion

In the present study, alcoholic and aqueous extracts of stem bark of *Limonia acidissima* Linn were evaluated on blood sugar levels and serum biochemical analysis in alloxan-induced diabetic rats for its antidiabetic and antihyperlipidemic activity. The acute toxicity studies revealed that LD50> 2000mg/kg for the alcoholic and aqueous extracts. The alcoholic extract (200mg/kg) showed significant activity ($P<0.01$) in lowering blood glucose level than the aqueous extract (200mg/kg) which was comparable to glibenclamide (5mg/kg), a standard antidiabetic drug. Different biochemical parameters i.e. TGL(triglycerides), HDL, LDL, VLDL and total cholesterol level were also carried out which show that both alcoholic and aqueous extracts has significantly ($p<0.001$) reversed the diabetes-included hyperlipidemia compared to standard drug. Concurrent histological studies of the pancreas of these animals showed comparable regeneration by alcoholic and aqueous extracts which were earlier, necrosed by alloxan. The data suggests that the alcoholic extracts (200 mg/kg) may be effectively utilized as antidiabetic and antihyperlipidemic agents and supports its traditional usage in the control of diabetes. However, further pharmacological evaluations are required to isolate and identify the active antidiabetic and antihyperlipidemic principles in the plant as well as elucidating their mechanisms of action.

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