



Antidiabetic and antioxidant activity of *Limonia acidissima* linn. in alloxan induced rats.

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Abstract

The present work is carried out to study the effect of *Limonia acidissima*.Linn (Rutaceae) on blood glucose levels and antioxidant enzymes levels in Alloxan induced diabetic rats. Alloxan (120 mg/kg, i.p) induced diabetic rats were treated with *Limonia acidissima* methanolic extract at a dose levels of 200 and 400 mg/kg for 21 days. Glucose level was measured in blood serum and antioxidant enzymes levels viz. Superoxide dismutase (SOD), Catalase (CAT) and Lipid Per oxidation (LPO) were measured in pancreatic homogenate, methanolic extract of fruit pulp of *Limonia acidissima*.Linn significantly ($P<0.01$) lowered the Alloxan induced hyperglycemia. It also produced a significant ($P<0.01$) decrease in peroxidation products viz. MDA in blood serum. The activity of antioxidant enzymes such as SOD, CAT was found to be higher in the blood serum of diabetic animals treated with the *Limonia acidissima* extract. This confirms the antihyperglycemic and antioxidant activities of *Limonia acidissima* in Alloxan induced diabetic rats.

Keywords: *Limonia acidissima*, antidiabetic activity, alloxan, antioxidant enzymes, histopathology.

Introduction

Diabetes mellitus (DM) is the most common endocrine disorder. It affects more than 100million persons world wide and its incidence is increasing steadily with changes in lifestyles. Complications of diabetes are many and include diabetic nephropathy and retinopathy [1]. Diabetes was discovered as early as 700-200B.C.Until the time insulin was not invented, this disorder was managed principally by the traditional practices by using medicinal plants. Many indigenous Indian medicinal plants have been reported by various authors to have anti diabetic properties.Ethnobotanical interventions about medicinal plants having beneficial effects on diabetes are reported in approximately 800 plants. [2]

Reactive oxygen species (ROS) are an important part of the defense mechanisms against infection, but excessive generation of free oxygen radicals may damage tissue. The role of ROS in tissue damage in various human diseases such as cancer, ageing, neurodegenerative disease, diabetes and atherosclerosis has been recognized [3].

Limonia acidissima Linn, syn. *Feronia limonia* is a moderate sized deciduous tree grown throughout India. The fruits are woody, rough and used as a substitute for bael in diarrhea and dysentery. The bark and leaves of the plant are used for vitiated conditions of vata and pita. The fruits are used for tumors, asthma, wounds, cardiac debility and hepatitis [4]. It was found that this part of the fruit contains flavanoids, glycosides, saponins and tannins [5]. There are reports that some coumarins [6] and tyramine derivatives [7] were isolated from the fruits of *Limonia*. The leaves were reported to possess hepatoprotective activity [8]. The fruit shells were reported to have antifungal compounds namely psoralene, xanthotoxin, 2, 6-dimethoxybenzoquinone and ostenol [9]. The stem bark of plant yielded(-)-(2S)-5,3'-dihydroxy-4'-methoxy-6",6"-dimethylchromeno-(7,8,2",3")- flavanone along with several known compounds including an alkaloid, five coumarins, a flavanone, a lignan, three sterols and a triterpene which were found to possess antimicrobial activity[10].

With regard to the above literature the present study of Anti-Diabetic and anti oxidant activity was undertaken to prove the ethno pharmacological claim of the plant part.

Materials and Methods

Plant material

Fruits of *Limonia acidissima* (LA) were purchased from koyambedu market in Chennai during the Month of August 2008 and separated into pulp and shell and authenticated by Prof.P. Jayaraman, Botanist at Plant Anatomy Research Centre (PARC), Tambaram, and Chennai. A Voucher specimen was deposited in the department of Pharmacognosy (SRMCP/07/08), SRM College of Pharmacy for future reference. The fruit pulp was shade dried, coarsely powdered using a cutter mill and stored in an air-tight, light resistant container for further use.

Preparation of L.acidissima alcoholic extract

The coarsely powdered fruit pulp was defatted with hexane using soxhlet apparatus. The defatted marc was further extracted with 95% alcohol using soxhlet and the extract obtained was concentrated using rotary flash evaporator. The extract value was found to be 23% w/w on dried weight basis and stored in vacuum desiccator for further pharmacological studies.

Experimental Animals

Inbred adult male Wistar albino rats (150-200 g) and albino mice were obtained from the animal house of SRM College of Pharmacy. The animals were maintained in a well-ventilated room at a temperature of $25\pm 1^{\circ}\text{C}$ with 12:12 hour light/dark cycle in polypropylene cages. Standard pellet feed (Hindustan lever, Bangalore) and tap water were provided *ad libitum* throughout the experimentation period. Animals were acclimatized to laboratory conditions 10 days prior to initiation of experiments. The project proposal was

approved by IAEC (Institutional Animal Ethical Committee) and the approval number being IAEC/30/2007.

Acute toxicity studies

Acute oral toxicity of methanolic Extract of *Limonia acidissima* (MELA) was determined using nulliparous, non-pregnant female mice. Albino mice were fasted for 3 hr prior to the experiment and were administered single dose of MELA dissolved in water and observed for mortality up to 48 hrs. Based on the short term toxicity, the dose of the test animals were determined as per OECD guidelines 423. All the animals were observed for lethal or toxic signs up to 2000mg/kg.

Experimental Design

Diabetes was induced by 120 mg/kg of Alloxan administered to overnight fasted rats' i.p. in water for two days at the interval of 24hrs. Twenty four hours after last injection; blood glucose levels were measured to confirm the induction of diabetes. Rats with blood glucose level above 200 mg/dL alone were selected as diabetic induced rats and were included in the experiment.

MELA was administered to the diabetes induced rats (200 and 400 mg/kg I.P) according to the toxicity studies. This dose was then converted to an equivalent dose in rats using the dose conversion table .

The animals were divided into 5 groups of 6 animals each and the grouping was as: Normal control, diabetic control, diabetic rats treated with 200 and 400 mg/kg of MELA and diabetic rats treated with Standard drug Glipizide (0.5 mg/kg. p.o. daily) for 21 days.

Every week (from 0week to 3rdweek) on 1st, 7th, 14th, 21st day blood samples were collected by retro-orbital puncture under light ether anesthesia, then the serum was separated by centrifugation at 2000rpm for 15min in a cooling centrifuge and blood glucose levels were measured. On 21st day, glucose level was finally measured and antioxidant enzymes levels were measured in pancreatic homogenate collected by sacrificing the animals by euthanasia.

Effect of MELA on Blood Glucose levels in alloxan induced diabetic rats

Blood glucose was measured by using commercially available GOD POD Kit from Span Diagnostics, Germany using Semi auto analyzer (Maysun-500e).

Effect of MELA on per oxidation product and antioxidant enzymes

The level of per oxidation product viz. Tissue Malondialdehyde (MDA) was measured [14] in pancreas homogenate where the reaction depends on the formation of a colored complex between Malondialdehyde (MDA) and thiobarbituricacid reactive substances (TBARS) having an absorption maximum at 532 nm. After estimating MDA in pancreas homogenate the remaining solution was further used to check the activities of antioxidant enzymes.

Super oxide dismutase (SOD) activity [11] was measured in pancreas homogenate. Epinephrine can be autooxidised to adrenochrome by super oxide radicals. The ability of SOD to inhibit the auto oxidation of epinephrine to adrenochrome has been used as the basis for the assay of this enzyme. Catalase (CAT) was measured [12] in pancreas homogenate

where the rate of decomposition of hydrogen peroxide by Catalase was measured spectrophotometrically at 230 nm.

Histopathological studies

A portion of the autopsied pancreas of Experimental animals were washed in normal saline and fixed in 10 % formalin solution, dehydrated with 50% ethanol. Sections of the pancreas were stained with hemotoxylin and eosin and evaluated for histopathological changes under light microscope.

Statistical analysis

The data are expressed as mean \pm SEM (n=6). Statistical significance was determined by one way ANOVA followed by Dunnet's test with $p < 0.05$ considered significant.

Results and Discussion

Table.1 Effect of methanolic extract of *Limonia acidissima* on Blood Glucose Level (mg/dl) in Alloxan induced diabetic rats

Groups	0 week	1 st week	2 nd week	3 rd week
Normal Control	101.46 \pm 3.81	132.92 \pm 2.86	124.70 \pm 3.23	120.45 \pm 2.23
Diabetic Control	190.32 \pm 2.01	314.41 \pm 1.91	382.25 \pm 2.86	413 \pm 2.20**
Standard	407.20 \pm 2.48	308.34 \pm 3.47	236.99 \pm 5.91	160.47 \pm 2.37 £
MELA (200 mg/kg)	396 \pm 1.83	376.47 \pm 1.81	326.51 \pm 1.23	292.39 \pm 2.91££
MELA (400 mg/kg)	388.80 \pm 2.73	346.12 \pm 1.92	276.51 \pm 2.98	190.39 \pm 1.36££

Each value is represented as mean \pm SEM, No of animals (n) = 6

** $p < 0.01$ Vs Normal Control

££ $p < 0.01$ Vs Diabetic Control, One-way ANOVAs Followed by Dunett's Test.

Table.2 Effect of methanolic extract of *Limonia acidissima* on total protein, total cholesterol, Serum Creatinine, and BUN in Alloxan induced diabetic rats.

Groups	Total Protein (G/DL)	Total Cholesterol (mg/dl)	Serum Creatinine	BUN
Normal Control	6.96±0.02	81.28±1.79	0.50±0.01	42.66±1.22
Diabetic Control	3.77±0.05**	181.60±1.15*	0.79±0.008**	70.79±0.88**
Standard	6.368±0.08 ^{££}	91.948±0.56 ^{££}	0.63±0.008 ^{££}	50.83±0.87 ^{££}
MELA200 mg/kg	5.022±0.15	107.784±1.34 [£]	0.71±0.007 [£]	61.83±1.35 [£]
MELA400 mg/kg	6.05±0.09 ^{££}	97±1.64 ^{££}	0.69 ±0.008 ^{££}	54.52±1.47 ^{££}

Each value is represented as mean± SEM, No of animals (n) = 6

* p<0.05 Vs Normal Control

** p<0.01 Vs Normal Control

£ p<0.05 Vs Diabetic Control

££ p<0.01 Vs Diabetic Control, One-way ANOVA Followed by Dunett's Test.

Table.3 Effect of methanolic extract of *Limonia acidissima* on Antioxidants level in pancreatic homogenate of Alloxan induced diabetic rats

Groups	SOD (U/mg protein)	CAT (k/mg protein)	LPO (µ mole/g protein)
Normal Control	16.54±0.15	6.32±0.15	0.43±0.07
Diabetic Control	11.324±0.18	2.680±0.19	1.891±0.13
Standard	15.148±0.33**	5.225±0.14**	0.34±0.01**
MELA 200 mg/kg	14.38±0.13**	3.946±0.16	0.295±0.04
MELA 400 mg/kg	13.859±0.21**	4.653±0.14**	0.234±0.01**

Each value is represented as mean± SEM, No of animals (n) = 6

D.c vs std ** P<0.01; D.c vs ext ** P<0.01, One-way ANOVAs Followed By Dunett's Test.

Histopathological studies

Section from the non-diabetic rats showed normal acini and islets while the section from diabetic control rats showed minute and reduced number of islets. Section from MELA extract treated diabetic rats showed good number of regenerating tiny islets, which could be comparable to that of non diabetic rats (Fig A,B,C,D).

Magnification-400x

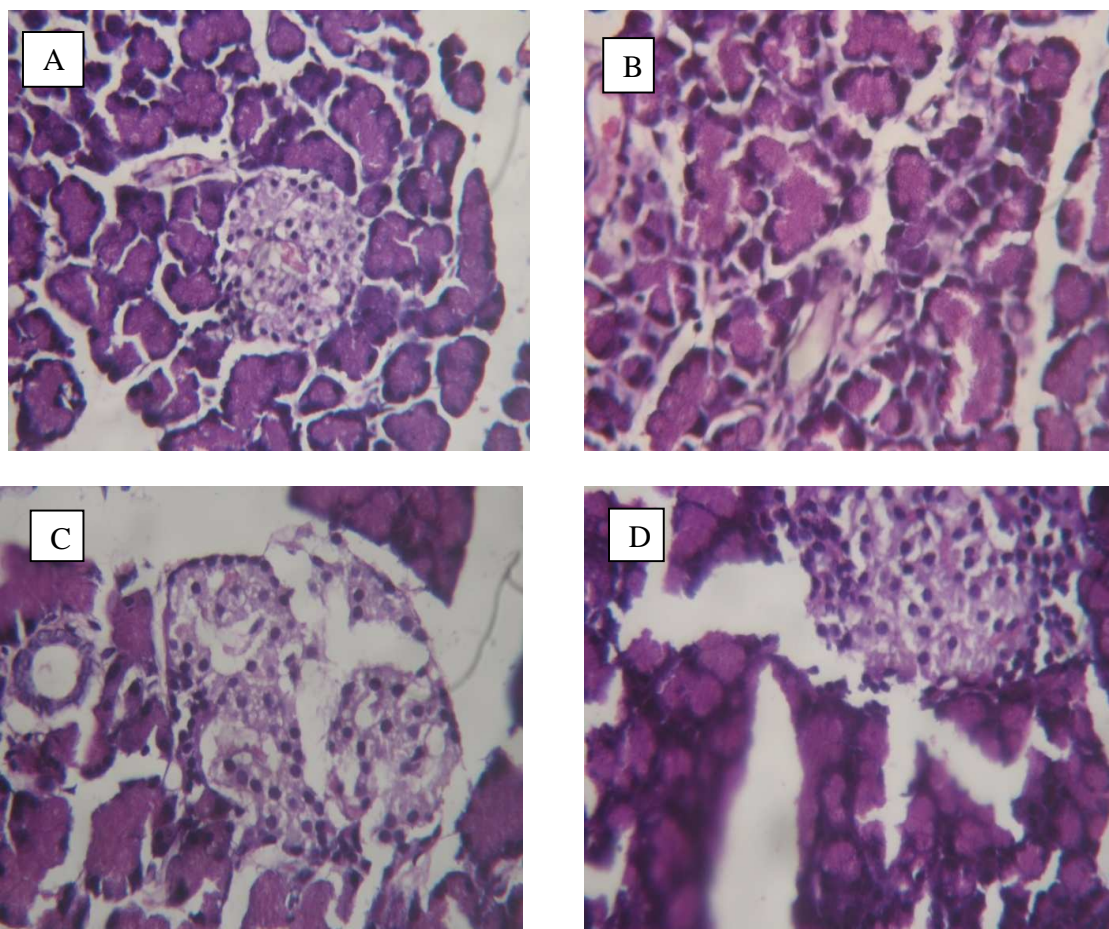


Fig1: Photograph of Pancreatic islets with its acinar tissue (A) control rat (B) diabetic control (C) diabetic rat after standard drug (Glipizide) therapy (D) diabetic rats after MELA treatment

Type 1 diabetes is one of the most common chronic childhood illnesses, affecting 18 to 20 per 1,00,000 children a year in the United Kingdom. The American Diabetes Association committee recommends the term type 1A diabetes for immune mediated diabetes with its destruction of the islet β cells of the pancreas. Non immune mediated diabetes with severe insulin deficiency is termed type 1B. At present, the development of type 1 diabetes is a life

sentence to a difficult therapeutic regimen that is only partially effective in preventing acute and chronic complications.

The expression of diabetes relates auto antibodies in young children monitored from birth indicate that these markers are a major risk factor for the future development of type 1 diabetes. No treatment has been shown to safely prevent type 1 diabetes in humans, although islet transplantation and new immunosuppressive regimens can be cured [13].

In alloxan-induced diabetic rats, increased food consumption and decreased body weight were observed. This indicates polyphagic condition and loss of weight due to excessive breakdown of tissue proteins [14]. Decreased body weight in diabetic rats could be due to dehydration and catabolism of fats and proteins. Increased catabolic reactions leading to muscle wasting might also be the cause for the reduced weight gain by diabetic rats. [15]

It has been shown that *Limonia acidissima* extract both 200 and 400mg/kg markedly improved the glucose tolerance in alloxan induced diabetes in rats as compared to control ($p < 0.01$). Extract showed dose dependent effect, 200 and 400 mg/kg dose shows reduction in glucose level. More over *Limonia acidissima* extract showed significant reduction in blood urea and creatinine in treated rats as compared to diabetic rats ($p < 0.01$) but significantly increased total protein level.

Possible sources of oxidative stress and damage to proteins in diabetes induced free radicals generated by autoxidation reactions of sugars and sugar adducts to protein and by autoxidation of unsaturated lipids in plasma and membrane proteins. The oxidative stress may be amplified by continuing cycle of metabolic stress, tissue damage, and cell death, leading to increased free radical production and compromised free radical inhibitory and scavenger systems [16] Under conditions of severe oxidative stress, free radical generation leads to protein modification. Proteins may be damaged directly by specific interaction of oxidants or free radicals with particularly susceptible amino acids. They are also modified indirectly, with reactive carbonyl compounds formed by the auto-oxidation of carbohydrates and lipids, with eventual formation of advanced glycation/lipoxidation end products [17].

Lipid peroxidation is a free-radical mediated propagation of oxidative insult to polyunsaturated fatty acids involving several types of free radicals and termination occurs through enzymatic means or by free radical scavenging by antioxidants[18]. Lipid peroxidation end products measured as thiobarbituric acid reactive substances and hydro peroxides were seen increased in plasma of alloxan-diabetic rats in this study. Drug with antioxidant properties may supply endogenous defense reactive oxygen species.

In diabetic mellitus, a variety of proteins is subjected to non-enzymatic glycation and is thought to contribute to the long term complications of the disease [19]. The level of total plasma protein was found to be decreased in this study. This could be due to increase lipid peroxidation in the diabetic rats. The decreased in plasma protein may also be ascribed to (i) decreased amino acid uptake. (ii) Greatly decreased concentration of variety of essential

amino acids, (iii) increased conversion of glycogenic amino acid to CO₂ and H₂O and (IV) reduction in protein synthesis secondary to a decreased amount and availability of mRNA [20]. Decreased protein content of blood serum in diabetic patients were reported [21] indicating elevated lipid per oxidation and reduce SOD and CAT activity and decreased antioxidant defensive system. *Limonia acidissima* extract significantly reduced the elevated LPO and significantly improved SOD and CAT activity in the treated animals.

In histopathological study, the light microscopic photograph islets from control rat appeared circular with the granulated beta cells appearing darker. Small and shrunken islets and destruction of beta cells were observed in the diabetic rats. Well –formed islets and increased cell number were observed in diabetic rats, after MELA therapy. The data presented in electron micrograph of the beta cell of normal and treated rats showed evidence for increased secretory granule synthesis and there by increased insulin secretion after the administration of extract of *Limonia acidissima* suggesting possible regeneration /repair of the islets of langerhans in Alloxan treated rats.

In diabetes, there is always a relationship between glucose homeostasis, lipid metabolism later renal function and enzyme activities [22]. We found that a 21 day administration of *Limonia acidissima* shows equal effectiveness in controlling diabetics when compared with diabetic rats treated with standard drug (Glipizide). Methanolic extract of *Limonia acidissima* proved to have a hypoglycemic effect on Alloxan –induced diabetic rats, a fact that indicated that there was a repair/regeneration of the beta cells of the islets of langerhans. As a result, there was an increase in insulin level, which brought about homeostasis in the above mentioned biochemical parameters such as Cholesterol, Urea, Creatinine, Total protein, and in the enzyme activities.

Conclusion

The present study indicated a significant dose dependant anti-diabetic effect for the methanolic extract of *Limonia acidissima* (200 and 400 mg/kg) and supports its traditional usage in the control of diabetes. It is also concluded that the extract have strong antioxidant potential activity by *in vivo* studies.

Further studies are under progress in our laboratory for the detailed studies in isolation of the compounds and pharmacological investigations of the constituents, which are responsible for the pharmacological activity reported traditionally and its exact mechanism of action.

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