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EVALUATION OF ANTIDIABETIC AND ANTIOXIDANT ACTIVITY OF METHANOLIC EXTRACT OF LIMONIA ACIDISSIMA LEAVES IN ALLOXAN-INDUCED DIABETIC WISTER ALBINO RATS: A RANDOMIZED CONTROLLED EXPERIMENTAL STUDY

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ABSTRACT
Objectives: The present study was carried out to find graded-doses antidiabetic and antioxidant activities of methanolic extract of *Limonia acidissima* (MELA) leaves in rats. **Materials and Methods:** Male Wistar rats were randomly divided into 6 groups (n=8): Normal control, Diabetic control (Alloxan-induced), and test groups where varying doses of MELA (100mg/kg, 200mg/kg, and 400mg/kg) or Metformin (100mg/kg) administered post-alloxan induction. Random blood sugar (RBS) levels were estimated at specific intervals over a 28-day period. Moreover, antioxidant markers (malondialdehyde, superoxide dismutase, and reduced glutathione) were assessed on day 28. Histopathological examination of pancreatic tissue was conducted. **Results:** Overall, mean RBS levels decreased at all intervals. There was a significant decrease in median malondialdehyde, an increase in median superoxide dismutase, and reduced glutathione levels (all p-values<0.0001). The change in RBS (p-values<0.0001) and antioxidants (p-value>0.05) levels was greater in Group VI than in Group V. On histopathology, Groups VI and V demonstrated regeneration of necrotic β-cells in the islets, and this was more pronounced than Group IV. **Conclusion:** Results of our study suggested that MELA possesses dose-dependent significant antidiabetic and antioxidant activity.

INTRODUCTION

Diabetes mellitus (DM) is a major metabolic disorder that affects many individuals over time [1]. Approximately 10.5% (537 million) of people are already impacted, and experts predict that number will rise to 12.2% (783 million) by the year 2045 [2]. Though a variety of treatment options are available for type 2 diabetes, however most patients still struggle to attain their glycaemic targets and their disease progresses uncontrolled [3-

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4]. Uncontrolled diabetes causes a variety of micro- and macro-vascular complications, which in turn raise healthcare costs, morbidity, and death [5-7]. Additionally, the available therapies to achieve euglycemia are associated with various adverse events, that further lead to non-compliance, decreased quality of life, and deranged glycaemic levels [8-9]. However, there is evidence that traditional medicinal herbs, besides influencing metabolic pathways, offer considerable anti-diabetic properties with no adverse events [10].

Globally, India is a home to the second largest numbers of adults with DM [2]. The World Health Organization (WHO) has recorded 21,000 medicinal plants, with 2500 of them species being in India. Of these 2500 plants, 800 have been demonstrated to have antidiabetic properties [11]. *Limonia acidissima* (commonly known as 'kaitha' or wood apple) is one of the several therapeutic herbs that are accessible. The genus *Limonia*, which includes the moderately large deciduous tree *Limonia acidissima* Linn or *Feronia limonia* (Family: Rutaceae, subfamily: Aurantioideae), is common in nations of Southeast Asia, including India. All parts of *L. acidissima* have been evaluated and reported to possess antioxidant, anti-bacterial, hepatoprotective, diuretic, antidiabetic, wound healing, anti-nociceptive and anti-cancer properties [12]. Early experimental studies have shown the potential for antidiabetic effects in the ethanolic extract of L. acidissima fruits [13-14].

The fruit of *L. acidissima* has also been reported to have radical scavenging activity [15]. At an oral dose of 1000 mg/kg, the aqueous leaf extract of *L. acidissima*, subsequently demonstrated a statistically significant hypoglycemic effect that was comparable to the effects of glibenclamide (0.9 mg/kg) [16]. However, they did not evaluate the graded dose response of the aqueous leaf extract. Additionally, acute toxicity, antioxidant property, and histopathological pancreatic changes produced by *L. acidissima* leaf extract remains to be evaluated. Thus, the present study was aimed to assess the antidiabetic and antioxidant properties of graded doses of methanolic leaf extract of *L. acidissima*, and to compare its its effects with those of the gold standard drug, metformin.

MATERIALS AND METHODS

Plant material

In December 2020, we gathered leaves of *L. acidissima* from the Aarogydham, Sevagram of Wardha district in Maharashtra. A professional taxonomist confirmed and authenticated the plant's botanical identity.

Drugs and Chemicals

Alloxan monohydrate (S D Fine-Chem Limited, Mumbai), Methanol 99% v/v (Merck), Tablet Metformin 500mg (USV Private Limited, Mumbai), Trichloroacetic acid (TCA) Merck, Thiobarbituric acid (TBA) – Merck, GSH Standard (Mol. wt. = 307.32) – Loba. All these drugs and chemicals were purchased from local shop.

Preparation of methanolic extract of *L. acidissima* (MELA) leaves [17]

The collected leaves of *L. acidissima* were carefully washed, dried in the shade, and ground using an electric mixture. The powder was then stored in a separate airtight container until it was needed to make the methanolic extract. For the preparation of the extract, 99% v/v methanol was used for 18 hours at 60ºC after charging the powder of *L. acidissima* leaves into the thimble of a soxhlet apparatus. Exhaustive extraction was considered complete when a colourless solvent appeared in the siphon tube; further extractions were therefore stopped at that point. Dark green was the hue of the extract.

Experimental animals

Young, healthy male albino rats of wistar strain, weighing 150-250grams, were used and were kept under standard laboratory conditions in a well-ventilated animal house in clean polyvinyl wired cages maintained at 25-26°C temperature and relative humidity 50-70% with a constant 12-hour light/dark schedule. The rats were fed with standard rat pellet chow and clean tap water was made available ad libitum. All the animal procedures were performed after approval from the Institutional Animal Ethics Committee (IAEC) of Mahatma Gandhi institute of medical sciences (MGIMS), Sevagram.

Acute Toxicity Studies [18]

Acute oral toxicity study for the test extract of the *L. acidissima* leaves was carried out as per the guidelines set by Organization for Economic Co-operation and Development (OECD), revised draft guidelines 425 and by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India [18]. The drug was administered in the dose of 2000mg/kg body weight orally to 1 rat and the rat

survived. Then, 5 other rats were dosed sequentially; therefore, a total of 6 rats were tested. The rats were observed individually. During the first 30 min after dosing, periodically during the first 24-hours (with special attention given during the first 4-hours), and daily thereafter, for a total of 14-days. None of the rats died. Thus, we concluded that the median lethal dose (LD50) could be greater than 2000mg/kg. An investigation with 1/20th, 1/10th, and 1/5th of 2000mg/kg i.e., 100, 200, and 400mg was done in pre-screening, and similar doses were used during final screening. Finally, the doses of 100mg/kg, 200mg/kg, and 400mg/kg were selected as working doses for all the experiments.

Induction of diabetes[19]

Before Diabetes induction rats were almost fasted upto 18 hrs period. By holding the rats in dorsal recumbent position and stretching the abdomen and using insulin syringe, the rats then administered 0.3-0.5 ml injection of single dose of a 150 mg/kg body weight solution of alloxan monohydrate dissolved in 0.9% sodium chloride by intraperitoneal (IP) route in right caudal quadrant of abdomen at the level of coxofemoral joint and approximately 5 mm to the right of midline. Subsequently, rats were carefully observed for the first 24 hours for any adverse events. Since alloxan is capable of producing fatal hypoglycaemia, due to massive pancreatic insulin release, animals were treated with 25% glucose solution orally at different time intervals after 6 hours of alloxan injection, and 5% glucose solution was kept in bottles in their cages for the next 24-hours to prevent hypoglycemia. Random blood sugar (RBS) was recorded daily for one week. Animals developed stable hyperglycemia after 4-5 days.

Experimental groups [20]

The experimental study was conducted on 6 groups of animals each with 8 rats. Total 48 male Wistar albino rats were randomly allocated to each of the 6 groups. The groups were treated as follows: Group I consisted of normal rats and served as normal control with no treatment. (Received normal saline 2ml/kg body weight intraperitoneally (IP) only once and then received 10ml/kg body weight distilled water (DW) by gavage once a day for 28-days orally). Group II consisted of diabetic rats and served as diabetic control with no treatment. (Treated with alloxan monohydrate 150mg/kg body weight IP only once). Group III consisted of diabetic rats and served as test group (Received alloxan, 150mg/kg body weight IP only once and then treated with MELA, 100mg/kg body weight, orally by gavage once a day for 28-days). Group IV consisted of diabetic rats and served as test group (Received alloxan, 150mg/kg body weight IP only once and then treated with MELA, 200mg/kg body weight, orally by gavage once a day for 28-days). Group V also consisted of diabetic rats and served as test group (Received alloxan, 150mg/kg body weight IP only once and then treated with MELA, 400mg/kg body weight, orally by gavage once a day for 28-days). Group VI consisted of diabetic rats and served as standard control group (Received alloxan 150mg/kg body weight IP only once and then treated with Metformin 100mg/kg body weight, orally by gavages once a day for 28-days).

Biochemical parameters estimation

Random blood sugar (RBS) levels

Random blood sugar levels were measured with the help of a digital Gluco Chek glucometer (Aspen Laboratories Pvt Ltd) as per the user's manual. Before using it for estimation of actual RBS levels, it was calibrated & validated using both level 1 & level 2 control solutions. Blood was withdrawn from the retro orbital sinus under ether inhalation anaesthesia or tail vein. Sugar level was estimated using glucose test strips at 48-hours after alloxan injection. Then rats were treated with test extract i.e., MELA (100, 200 and 400mg/kg doses) or standard drug i.e., Metformin for 28-days and RBS was estimated at an interval of 1st, 3rd, 7th, 14th, and 28th day after administration of test/standard drug i.e., 0-day.

Serum malondialdehyde (MDA) level

Serum MDA concentration was measured according to process mentioned by Buege and Aust SD [21]. Serum of test samples (100µl) were taken in the test tubes and mixed with DW (400µl). To this diluted sample, 1 ml of TCA-TBA-HCL (15% Trichloroacetic acid + 0.375% Thiobarbituric acid + 0.25N Hydrochloric acid) reagent was mixed. This mixture was then heated in a boiling water bath for 15mins. The solution was cooled and then centrifuged at 1000 rpm for 10 minutes. The pink coloured supernatant was taken out and optical density was taken in 535nm using spectrophotometer. Using 1.56×10^5 $M^{-1}cm^{-1}$ as the molar extinction coefficient, results were expressed as micro moles of MDA/Litre (µmol/L) of serum.

Serum superoxide dismutase (SOD) activity

Serum SOD activity was measured according to Marklund and Marklund [22]. The assay mixture in a 3ml volume consisted of

100µL each of pyrogallol (0.2mM), EDTA (1mM), DTPA (1mM), and test serum (100µL) in equilibrated tris-HCl buffer (50mM; pH 8.2). Pyrogallol was added after the addition of all other reagents to start the reaction. Initial 10s period was considered as induction period of the enzyme. So, after 10s, change in absorbance at 420nm at 10s intervals was recorded by spectrophotometer for a period of 4min. The average change in the absorbance per minute was calculated. One unit of enzyme SOD was defined as the amount of enzyme received to cause 50% inhibition of pyrogallol auto-oxidation. Accordingly, the activity of the enzyme in different standards was expressed in units/ml.

Reduced glutathione (GSH) levels

Reduced glutathione concentration was measured according to method described by Beutler et al [23]. Whole blood (200µl) was mixed thoroughly with DW (1.8ml) and 3ml of Precipitating reagent solution (Metaphosphoric acid 1.67gm +Di-sodium EDTA 0.2gm+ NaCl 30gm to make up to 100ml with double DW) and allowed to stand for 5min at room temperature. Subsequently, it was filtered and two test tube were marked as test and blank. In tube marked as test, clear filtrate (2ml) was added from above mixture to Di-sodium phosphate buffer (8ml), and then DTNB reagent (1ml) was added to it, and mixed well. The colour developed rapidly, and remained stable for 10min. A reagent blank was made using DW (2ml), phosphate buffer (8ml) and DTNB reagent (1ml). Readings were taken at 412nm in the spectrophotometer. The curve was plotted taking absorbance at 412nm on the Y-axis and concentration on the X-axis. The concentration of the test samples was calculated by using standard curve. Reduced glutathione concentration in blood sample was expressed in mg/dl.

Histopathological examination (HPE) [24]

Pancreatic tissues from all groups were subjected to HPE. The whole pancreas from each animal was dissected out on 28th day after sacrificing the animal under anaesthesia. Pancreatic tissues were then fixed in 10% formalin solution and sent to histopathology laboratory. These tissues were then cleared in xylene and embedded in paraffin wax. Sections of 5µm thickness were cut. Haematoxylin and Eosin (H&E) dye was used for staining. First the slides were stained in Haematoxylin followed by wash under tap water. Counterstaining was done in Eosin dye followed by dehydration. Finally, these slides were examined under light microscope with 10x magnification.

Statistical Analysis

The data was analysed with SPSS (IBM, Armonk, NY, USA) version 23.0 for windows. Variables with a normal distribution are represented as mean ± standard deviation (SD). Skewed variables are presented as the median (interquartile range). Within group analyses were done with repeated measures ANOVA followed by post-hoc analysis by Bonferroni's test. For normally distributed variables, between group analyses was done with one-way analysis of variance (ANOVA) followed by post-hoc analysis by Tukey's test. While, for skewed variables, between group analyses was done with Kruskal-Wallis H test followed by post-hoc analysis by Dunn's test. A two-tailed probability value of <0.05 was considered as statistically significant [26].

RESULTS

Acute toxicity

The graded doses of MELA (100, 200, 400, 800, 1000, and 2000mg/kg), administered in six groups with six rats in each group, did not produce death, any adverse event, or significant change in behaviour of rats. The rats were physically active, suggesting that the LD50 could be >2000mg/kg body weight.

Random blood sugar levels

There was no significant difference between the groups in terms of mean baseline RBS levels (p-value=0.518). Thus, the groups were homogenous. Between group analysis, for the change in mean RBS levels, suggested an incremental decrease in RBS levels. Overall, compared to the mean RBS levels at 48 hours following Alloxan injection, mean RBS levels decreased significantly at all the intervals (all p-values<0.0001). However, at the end of study, the decrease in mean RBS was significantly greater with Metformin followed by MELA 400, MELA 200, and MELA 100 (all p-values<0.0001) (Table 1, Figure 1).

In Group I, administration of DW (PO) did not result in a significant change in the mean RBS levels (p-value=0.341) at any of the time intervals evaluated. While, in Group II, administration of DW (PO), resulted in a statistically significant decrease in the mean RBS levels (p-value=0.001). Similarly, in Group III, IV, V, and VI administration of MELA 100, 200, 400, and Metformin 100 resulted in a statistically significant decrease in the mean RBS levels (all p-values<0.0001), respectively (Table 2).

Table 1: Between group comparison of baseline and change in mean random blood sugar at various intervals

Groups	Baseline RBS (mg/dl)	Change in RBS (mg/dl)				
		48-hrs–Day 1	48-hrs–Day 3	48-hrs–Day 7	48-hrs–Day 14	48-hrs–Day 28
Group I	118.0±5.2	-5.0±4.1***	-7.3±3.4***,$,$$$	-4.5±2.9***,$$$,^	-4.00±4.3***,$$$	-4.7±3.5***,$$$,^
Group II	116.0±3.7	25.9±13.9*	32.13±17.9***	40.6±29.1***,$$$,@	-15.9±10.4***,$$$	-86.1±19.8***,$$$
Group III	119.5±4.5	29.3±15.0**	-33.8±22.3***	-55.8±25.8***,$$	-151.0±21.3***,$$$	-250.3±24.2***
Group IV	116.3±5.2	13.0±12.6***	-54.8±15.0***	-92.0±18.3***	-187.5±16.9***	-269.8±17.9***
Group V	116.5±6.2	-16.0±8.9***	-91.5±16.2***	-178.0±17.5***	-252.5±16.9***	-308.0±15.3***
Group VI	114.8±5.4	-41.8±15.1	-149.5±18.8	-251.5±11.4	-289.0±16.0	-322.0±14.6
p-value	0.518	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

* - One-way ANOVA followed by post-hoc analysis by Tukey's test; p-value<0.05 was considered as statistically significant; 48-hrs – RBS levels 48 hours after administration of Alloxan (IP); Post-hoc analyses – **48-hours–Day 1:** *p-value<0.05 Vs Group I, **p-value<0.01 Vs Group I, ***p-value<0.0001 Vs Group VI; **48-hours–Day 3:** ***p-value<0.0001 Vs Group VI, Vs Group V, $p-value<0.05 Vs Group III, $p-value<0.0001 Vs Group IV; **48-hours–Day 7:** ***p-value<0.0001 Vs Group VI, Vs Group V, $$$p-value<0.0001 Vs Group IV, $$p-value<0.001 Vs Group IV, ^p-value<0.0001 Vs Group III, @p-value<0.001 Vs Group I; **48-hours–Day 14:** ***p-value<0.0001 Vs Group VI, Vs Group V, $$$p-value<0.0001 Vs Group IV, Vs Group III; **48-hours–Day 28:** ***p-value<0.0001 Vs Group VI, Vs Group V, $$$p-value<0.0001 Vs Group VI, Vs Group III, ^p-value<0.0001 Vs Group II.

Table 2: Within group comparison of mean random blood sugar at various intervals

Time intervals	Group I	Group II	Group III	Group IV	Group V	Group VI
At 48-hours	114.0±4.7	457.9±20.8	450.0±20.7	451.8±15.2	475.5±14.9	462.5±13.9
Day 1	112.0±6.9	483.5±15.3*	474.3±9.6	462.3±11.1	440.5±7.8***	420.8±6.4**
Day 3	113.3±7.3	490.0±9.3*	420.8±9.9$$$	397.0±10.1***,$$$	366.0±10.7***	313.0±13.1***,$$$
Day 7	112.0±4.3	498.5±14.0	394.3±11.6**,$$$	359.8±7.4***,$$$	279.5±10.8***,$$$	211.0±7.6***,$$$,^
Day 14	111.0±3.0	454.0±9.2**,$$,^	299.0±6.6***,$$$,^,@	264.3±5.6***,$$$,^,@	205.0±5.6***,$$$,^,@	173.5±4.6***,$$$,^,@
Day 28	113.8±3.5	371.8±5.9***,$$$	199.8±6.5***,$$$,^	182.0±5.5***,$$$,^	149.5±3.3***,$$$,^	140.5±2.6***,$$$,^
p-value*	0.341	0.001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

* - Repeated measures ANOVA followed by post-hoc analysis by Bonferroni's test; p-value<0.05 was considered as statistically significant; Post-hoc analyses – **Group II:** *p-value<0.05 Vs 48-hours, **p-value<0.001 Vs Day 1, ***p-value<0.0001 Vs 48-hours and Day 1, $$p-value<0.001 Vs Day 3 and Day 7, $$$p-value<0.0001 Vs Day 3 and Day 7, ^p-value<0.0001 Vs Day 28; **Group III:** **p-value<0.001 Vs 48-hours, ***p-value<0.0001 Vs 48-hours, $$$p-value<0.0001 Vs Day 1, ^p-value<0.0001 Vs Day 3 and Day 7, @p-value<0.0001 Vs Day 28; **Group IV:** ***p-value<0.0001 Vs 48-hours, $$$p-value<0.0001 Vs Day 1, ^p-value<0.0001 Vs Day 3 and Day 7, @p-value<0.0001 Vs Day 28; **Group V:** ***p-value<0.0001 Vs 48-hours and Day 1, $$$p-value<0.0001 Vs Day 3, ^p-value<0.0001 Vs Day 7, @p-value<0.0001 Vs Day 28; **Group VI:** **p-value<0.001 Vs 48-hours, ***p-value<0.0001 Vs 48-hours, $$$p-value<0.0001 Vs Day 1, ^p-value<0.0001 Vs Day 3 and Day 7, @p-value<0.0001 Vs Day 28.

Antioxidant activity

Between group analysis suggested that the median MDA levels decreased significantly (p-value <0.0001). The decrease in median MDA levels was maximum in Group VI followed by Group V, Group IV, and Group III **(**Figure 2). However, there was no significant difference between Group VI and V (p-value >0.05). Further analyses revealed statistically significant increase in the median SOD and GSH levels (p-value <0.0001). The rise in median SOD and GSH levels was maximum in Group VI followed by Group V, Group IV, and Group III (Figure 3, Figure 4). However, there was no significant difference between Group VI, V, and IV (both p-values >0.05) (Table 3).

Table 3: Between group comparison of antioxidant activity

Groups	Median MDA levels (µmol/dl)	Median SOD levels (units/ml)	Median GSH levels (mg/dl)
Group I	3.7 (3, 3.9)	2 (2, 2)***,$$	3 (3, 3)***,$
Group II	9.1 (8.7, 10.4)***,$,^^^	1 (1, 1)***,$$$,^^^	1 (0.6, 1.3)***,$$$,^
Group III	5.1 (4.9, 5.3)**,^	3 (3, 4)*	3.5 (3.1, 4)**
Group IV	4.6 (4.5, 4.6)*	5.5 (5, 6)	4 (4, 4.5)
Group V	4 (3.9, 4.2)	6 (6, 7)	5.3 (5, 5.8)
Group VI	3.3 (3, 3.7)	7 (7, 8)	6 (6, 6.4)
p-value*	< 0.0001	< 0.0001	< 0.0001

Data represented as median (Interquartile range); * - Kruskal-Wallis H test followed by post-hoc analysis by Dunn's test; p-value<0.05 was considered as statistically significant; Post-hoc analyses – **MDA levels:** *p-value<0.05 Vs Group VI, **p-value<0.001 Vs Group VI, ***p-value<0.0001 Vs Group VI, $p-value<0.001 Vs Group V, ^p-value<0.05 Vs Group I, ^^^p-value<0.0001 Vs Group I; **SOD levels:** *p-value<0.05 Vs Group VI, ***p-value<0.0001 Vs Group VI, $$p-value<0.001 Vs Group V, $$$p-value<0.0001 Vs Group V, ^^^p-value<0.0001 Vs Group IV; **GSH levels:** **p-value<0.05 Vs Group VI, ***p-value<0.0001 Vs Group VI, $p-value<0.05 Vs Group V, $$$p-value<0.0001 Vs Group V, ^p-value<0.05 Vs Group IV.

Histopathology findings

Group I which consisted of normal rats had regular, normal appearance of islet cells. Group II had mild inflammation with destruction and atrophy of the β-cells of islet of Langerhans. Group III revealed findings similar to that of Group II except the fact that there was absence of any inflammatory signs with presence of marked decrease in number islets of Langerhans. Group IV demonstrated occasional islet cells and increase in volume and density of islets suggestive of regeneration. Group V revealed restoration or regeneration of pancreatic islet cells. The regeneration of necrotic β-cells was especially more pronounced after treatment with 400mg/kg of MELA extract than in the group treated with 200mg/kg. Additionally, apparently normal structure of pancreas was observed in diabetic rats compared to the diabetic control group. Similarly, Group VI revealed restoration or regeneration of pancreatic β-cells in the islets. Compared with the diabetic control, almost normal structure of pancreas was seen in diabetic rats treated with metformin (Figure 5)**.** Effects of MELA (400mg/kg) were comparable to that of Metformin (100mg/kg).

DISCUSSION

In this study, we used Alloxan as a diabetogenic agent which induces chemical diabetes in experimental animals through the selective destruction of beta cells of islets of Langerhans of the pancreas. Alloxan gets reduced to dialuric acid and thus reactive oxygen species are formed which destroy these cells [25]. It results in a weakened antioxidant defence system due to an increase in lipoxygenase and a reduction in superoxide dismutase (SOD) activity, as well as lower protein content in their blood serum [27]. In this in-vivo study, it was observed that MELA can reverse metabolic derangements occurring in alloxan induced diabetic rats. Crude extract of *L. acidissima* plant parts such as bark, leaf, rind, pulp and seed showed the presence of alkaloids, flavonoids, steroids, saponins, glycosides, phenols, gum and mucilage, fixed oils and fats, resins and tannins. The preliminary phytochemical analysis of MELA leaves showed presence of alkaloids, saponins, phenols and fixed oils [28]. Active phytochemical ingredients act through various mechanisms and are responsible for specific therapeutic properties of the plant. In the present study antidiabetic activity of MELA leaves was evaluated and it was found that administration of MELA, in a dose of 100, 200, and 400mg/kg markedly improved the glucose levels in alloxan-induced diabetes in rats. We observed that the onset of action was early with dose of 400mg/kg than 100 and 200mg/kg. On Day 1, 400mg/kg dose led to reduction in the mean RBS levels, while with 100 and 200mg/kg dose, the mean RBS levels increased. Additionally, the change in mean RBS levels and effect size of MELA 400mg/kg was significantly greater than MELA 100 and 200mg/kg. Thus, a dose-dependent RBS reduction was observed.

The decrease in mean RBS levels was significantly less with all the 3 doses of MELA than Metformin (100mg/kg). Additionally, at all the time intervals, the decrease in mean RBS was greatest with Metformin (100mg/kg). Thus, the sequence of decrease in mean RBS levels was Metformin 100mg/kg > MELA 400mg/kg > MELA 200mg/kg > MELA 100mg/kg.

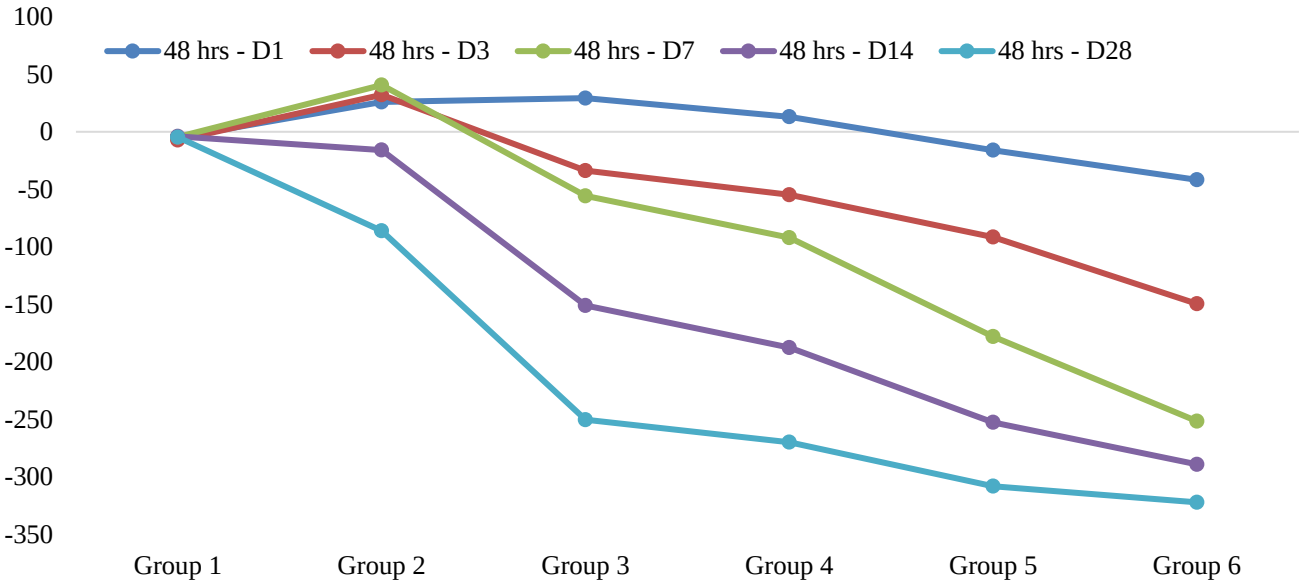

Figure 1. Between group comparison of change in random blood sugar at various intervals.

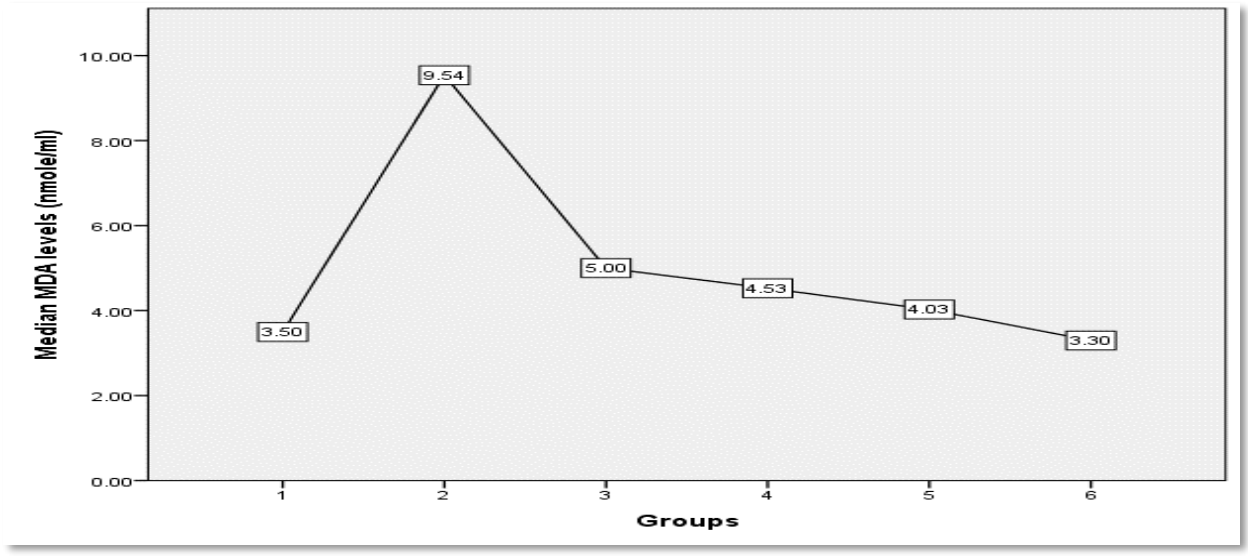

Figure 2. Between group comparison of MDA levels

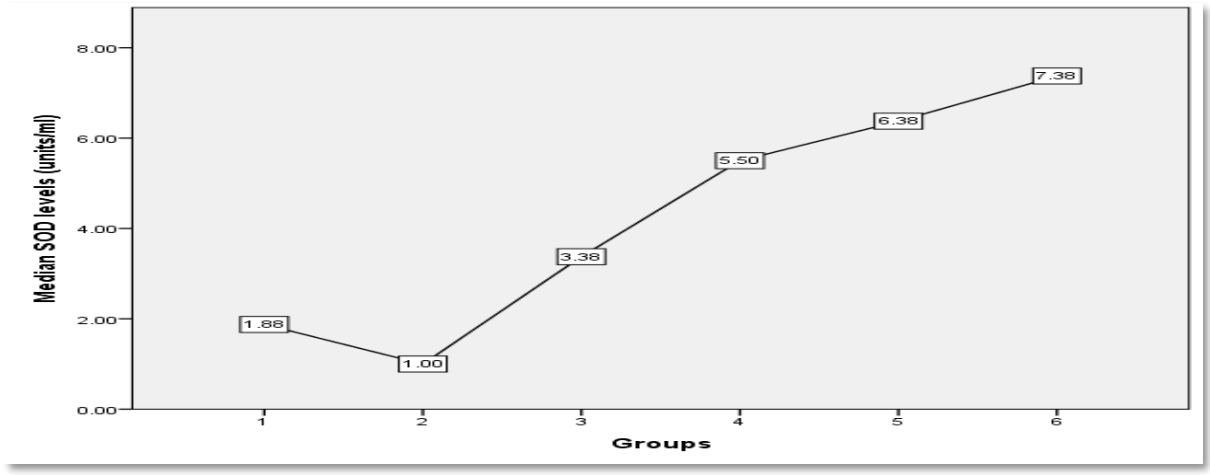

Figure 3. Between group comparison of SOD levels

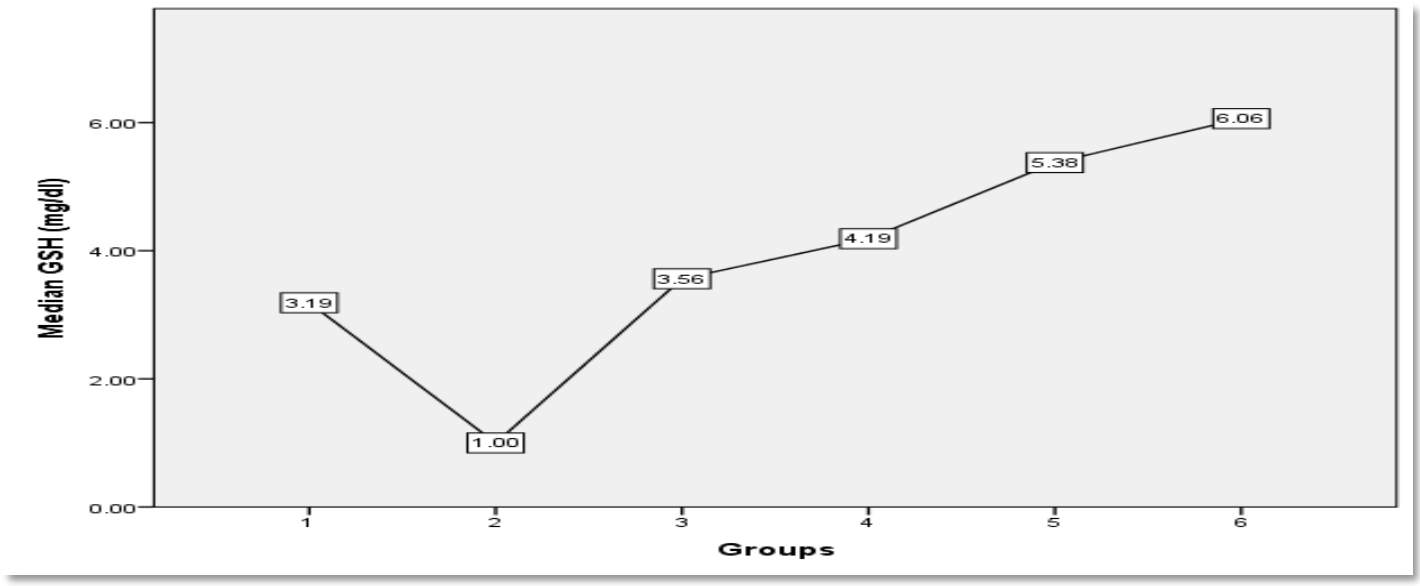

Figure 4. Between group comparison of GSH levels

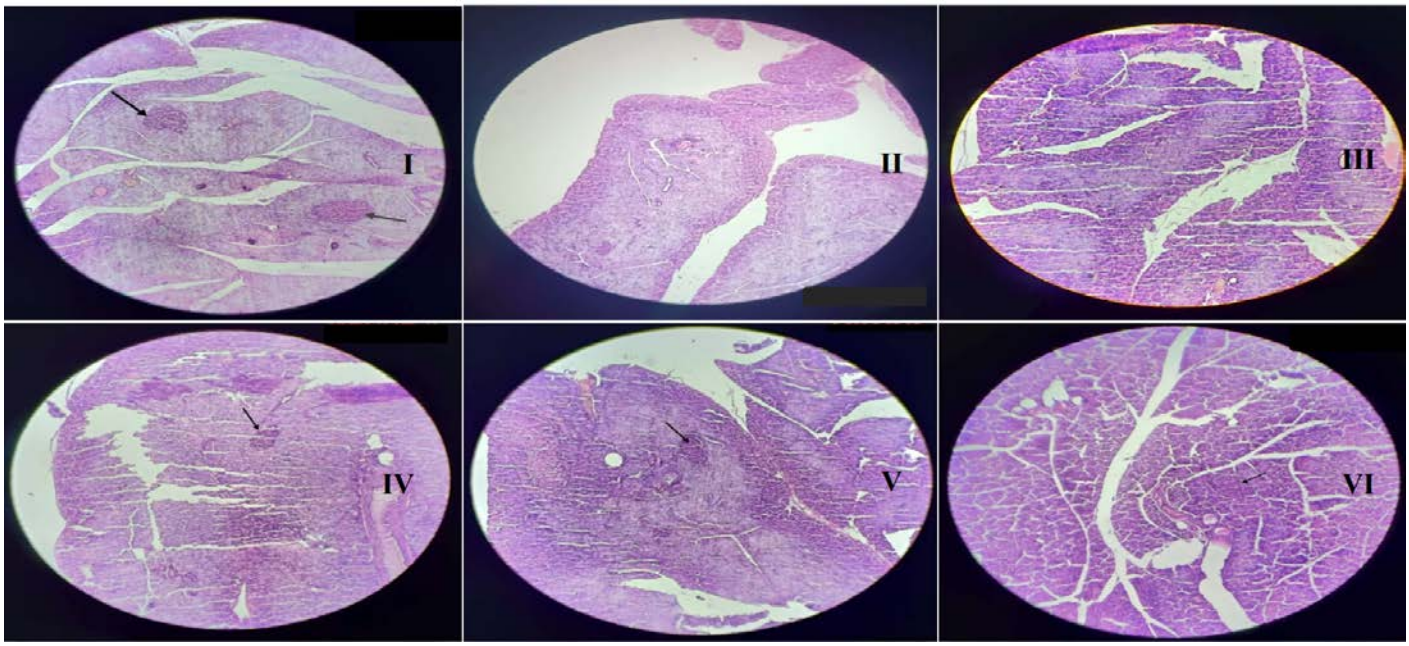

Figure 5: Photomicrograph of pancreas on Day 28 (H&E 10×). **Group I:** Normal rat; **Group II:** Rat treated with Alloxan 150 mg/kg body weight; **Group III:** Rat treated with MELA 100 mg/kg body weight; **Group IV:** Rat treated with MELA 200 mg/kg body weight; **Group V:** Rat treated with MELA 400 mg/kg body weight; and **Group VI:** Rat treated with Metformin 100 mg/kg body weight.

The possible mechanisms by which MELA may exert its antihyperglycemic action in alloxan-induced diabetic rats involve both pancreatic and extrapancreatic pathways. These mechanisms may include:

1) Potentiation of Pancreatic Insulin Secretion: MELA may enhance insulin secretion from intact beta cells in the islets of Langerhans. This could increase insulin availability, helping regulate blood glucose levels more effectively.

2) Extrapancreatic Mechanisms: Decreased Glycogenolysis: MELA might reduce the breakdown of glycogen into glucose (glycogenolysis) in the liver similar to metformin. This would contribute to lower blood glucose levels by limiting the release of glucose into the bloodstream.

Enhanced Glycogenesis: The phytochemical compounds like alkaloids/saponins in MELA leaves may promote glycogenesis, the process of converting glucose into glycogen in the liver. This would serve to store excess glucose, preventing its release into the bloodstream.

Enhanced Glucose Transport: MELA may facilitate the transport of blood glucose to peripheral tissues, such as muscle cells and adipose tissue. This could enhance glucose utilization and contribute to reducing hyperglycemia.

3) Direct Effect on Islet Regeneration or Repair: The histopathological studies suggest that MELA may have a direct effect on the regeneration or repair of the islets of Langerhans in the pancreas. This is evidenced by the restoration of the architecture of these islets.

These multifaceted mechanisms collectively contribute to the antihyperglycemic effects of MELA in diabetic rats. The direct impact on the regeneration or repair of pancreatic islets, as observed in histopathological studies, adds an additional layer to the understanding of MELA's potential therapeutic action in diabetes. Nevertheless, further research is essential to validate and elucidate the specific molecular pathways involved in these observed effects.

The oxidative stress induced by alloxan arises due to a compromise in natural antioxidant mechanism and an increase in oxygen free radical production. Antioxidants are the substances that neutralize free radicals before they can attack cells and hence prevent damage to cell proteins, lipids, and carbohydrates [25]. In their in-vitro studies, Attarde et al. and Merinal et al. demonstrated antioxidant activity of extracts from leaves of *L. acidissima* linn [29][30]. Here, we evaluated the antioxidant activity of MELA leaves and observed that MELA (400mg/kg) resulted in a significant decrease in MDA compared to diabetic control and the activity did not differ significantly with Metformin (100mg/kg). We also observed that MELA (400 and 200mg/kg) led to a significant rise in the levels of Free radical scavenging enzymes such as superoxide dismutase (SOD). The reduced activities of SOD seen in Group II suggest their excessive utilisation in neutralizing free radicals generated during the metabolism of alloxan. Nonenzymatic antioxidant (reduced glutathione) acts synergistically to scavenge the free radicals formed in the biological system, thus preventing cells from oxidative damage [32]. In Group II, the reduced GSH level may be due to its increased utilisation during oxidative stress to scavenge free radicals. However, the reduced GSH level was reversed with the administration of MELA to the diabetic rats. This may suggest that MELA maintained the levels of GSH that are utilised during diabetes and hence also stabilised lipid peroxidation. It is also to be noted that MELA (400 and 200mg/kg) and Metformin (100mg/kg) did not differ significantly in the rise of SOD and GSH levels. Thus, in accordance with the literature, we observed antioxidant activity of MELA and this activity was similar to that of Metformin. Various studies have shown that *L. acidissima* extract does not cause acute toxicity [31][33]. Similarly, in the present study, we did not observe any acute toxicity to MELA leaves upto 2000mg/kg. Thus, supporting the safe medicinal use of *L. acidissima*.

CONCLUSION

The study indicates that MELA leaves, at different doses (100, 200, and 400mg/kg), exhibits significant antidiabetic and antioxidant activity, suggesting its potential as a natural source for antihyperglycemic and antioxidant agents. Although the exact mechanism for the antihyperglycemic property of MELA leaves remains unidentified, further confirmatory studies are needed to explore this therapeutic property and identify the main antihyperglycemic compounds.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

AUTHOR CONTRIBUTION

This study was done under the guidance of Leena Chimurkar. Ashishkumar Baheti, Leena Chimurkar & Kanchan Mohod planned and designed the experiment. Ashishkumar Baheti performed the experiments, collected data and prepared the first draft of manuscript. Leena Chimurkar and Kanchan Mohod contributed in conducting literature survey, collecting the data and manuscript review. All authors read and approved the final manuscript.

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