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Hyperglycemic and oxidative stress management efficacies of hydro-methanol extract of *Ipomoea mauritiana (Jacq.)* root tuber in streptozotocin (STZ) induced diabetic male rats: Dose selection study

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ABSTRACT: Diabetes mellitus needs efficient therapeutic strategies with nominal undesirable effects. This comparative dose-dependent study aimed to search out the hyperglycemic and oxidative stress corrective efficacies of hydro-methanol (60:40) extract of root tuber of *Ipomoea mauritiana (Jacq.)* (IMHME) in streptozotocin (STZ)-induced diabetic male rats. The *in-vivo* study has been designed to identify the most potent dose for such therapeutic outcomes. Single dose of STZ (4 mg/100 g body weight (BW)) was administered intramuscularly in rats to induce diabetes. Subsequently, rats were allowed for treatment at doses of 5, 10, 20, and 30 mg per 100 g BW of the IMHME for 4 weeks. Blood glucose level in fasting, insulin level, and activities of glucose-6-phosphatase, hexokinase, catalase, and superoxide dismutase were assessed to interpret the hypoglycemic and anti-oxidative properties of IMHME. The IMHME demonstrated a dose-dependent increase in insulin level with a concomitant decrease in fasting blood glucose level and rectification of said enzyme activities. Additionally, IMHME revealed potent anti-oxidative effects, as evidenced by a significant correction in oxidative metabolic biomarkers, i.e., thiobarbituric acid reactive substances, along with increase in pancreatic islet diameter. The outcome of this comparative dose-dependent investigation focused on the 10 mg dose of IMHME, which has the optimal therapeutic efficacy in improving diabetes by mitigating oxidative stress. No significant difference was noted among the groups under the treatment of 10, 20, and 30 mg doses. The promising results form the basis for exploration of the underlying mechanism and the translation of this into clinical research.

KEYWORDS: *Ipomoea mauritiana (Jacq.);* Anti-diabetic; Oxidative stress; Hexokinase; Pancreatic islet.

1. INTRODUCTION

Diabetes is a highly prevalent endocrine disorder with chronic complications that drastically impact health and quality of life. An estimated 537 million individuals, from 20 to 79 years old, are diabetic, as per the report of the International Diabetes Federation (IDF). It is predicted that the graph will rise to 653 million in the year 2030 and rise to 783 million in 2045. Approximately 1.2 million people, including children and adolescents, are affected by type 1 diabetes. The mortality rate of diabetes was 6.7 million in 2021 [1]. Diabetes-related Disability Adjusted Life-Years (DALYs) increased up to 27.6% in 2019 since 1990. Type 1 diabetes is categorized by insufficient insulin production and usually requires daily insulin therapy, which is known as juvenile, insulin-dependent, and childhood-onset diabetes [2]. Glucose is not utilized for energy in the case of type 2 diabetes patients because insulin resistance elevates glucose levels. Oxidative stress has a strong association with diabetes and its related complications. In a hyperglycemic state, superoxide radicals are produced excessively, resulting in nuclear DNA fragmentation, and activation of the poly-ADP-ribose polymerase-1 (PARP-1) enzyme which inhibits the activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), favours hydrogen peroxide (H_2O_2) production. This geared up oxidative stress by decreasing glutathione peroxidase, superoxide dismutase, and catalase activities [3]. Similarly, glyoxal produced by glucose autooxidation is a precursor for the production of advanced glycation end products (AGEs), increases the generation of ROS, and imposes oxidative stress at cellular and sub-cellular levels, that

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ultimately causes genomic and epigenomic instability, disrupts normal cellular metabolism, and causes cellular injuries [4]. The primary source of therapeutic compounds is medicinal plants. Nowadays, drug resistance is a common issue, along with numerous side effects for diabetic treatment [5]. An alternative approach to use phytomedicine for the treatment purpose of diabetes based on their well-known radical scavenging activity for preventing the progression of oxidative chain reactions. The phytocompounds may delay the oxidation of biomolecules and prevent the development of chronic metabolic disorders linked with ROS [6].

Ayurveda is a traditional medicine practice that originated in prehistoric India around 1000 BC. It is based on religious and spiritual treatment, and its main sources of medicine are plants, herbs, and natural ingredients. Ayurvedic treatments for chronic illnesses, including diabetes, hypertension, and cancer, are in high demand [7]. *Ipomoea mauritiana (Jacq.)* is a perennial climber plant belonging to the 'Convolvulaceae' family. This plant is widespread worldwide and provides significant evidence in ethnomedicine. The plant is correlated with 'Kshiravidari' in the 'Indian Ayurvedic Pharmacopoeia' [8]. Nearly 45 Ayurvedic formulations use tubers of this plant. It is used as phytomedicine and has a significant role in the well-known ayurvedic remedy 'Chyavanaprasha' [9]. Local communities in various regions used the plant to treat a variety of illnesses, including spinal cord pain, increasing breast milk production and sperm count, preventing tuberculosis, and purifying blood in jaundice. Also, the plant has been used to treat biliary disorders and has shown anti-amnesic, anti-asthmatic, and hepatoprotective properties. [10]. In the tribal areas, folk medicine practitioners use this plant leaf to treat leukorrhea, diabetes with obesity [11], and infrequent urination [12]. The callus extract of this plant significantly lowers blood glucose level. Although this plant is used in wide pathophysiological domains as ethnomedicine for diabetes management, specially in Bangladesh [13], there is no evidence of its antidiabetic activities at the molecular level. So, the primary objective of this study is to find a potent dose of Ipomoea mauritiana (Jacq.) hydro-methanol (60:40) extract (IMHME) of root tuber for diabetes and oxidative stress management.

The *in-vivo* experiment uses streptozotocin (STZ)-induced male rats to mimic the pathophysiological conditions of diabetes. Blood glucose level in fasting, level of insulin in serum, carbohydrate metabolic enzyme activities, anti-oxidative enzyme activities, toxicity sensors, and lipid peroxidation end-products were assessed to evaluate the anti-diabetic and anti-oxidative efficacies of IMHME. Additionally, the investigation explores the histopathological assessment of pancreatic islets, providing a protective effect of IMHME on pancreatic tissue integrity. As the scientific community continues to explore novel avenues for diabetes management, this study aims to shed insights on the therapeutic capability of IMHME, offering a natural and potentially safer substitute for traditional anti-diabetic interventions.

2. RESULTS

2.1. Evaluation of fasting blood glucose (FBG) level

The FBG level was increased significantly ($p<0.05$) in the vehicle-treated diabetic (VT diabetic) group in contrast to the vehicle-treated control group (VT control). In Table 1, IMHME administration at doses of 5, 10, 20, and 30 mg resulted in significant ($p<0.05$) recovery of FBG level directed to the VT control group. Significant ($p<0.05$) decrease in FBG level between the doses of 5 and 10 mg was observed, though no significant ($p<0.05$) difference in the level of FBG was noted among the groups treated with doses of 10, 20, and 30 mg. In this concern, it was stated that the group receiving a minimum dose of 10 mg had the highest level of recovery compared to other treated doses.

2.2. Serum insulin level

The VT diabetes group had a considerably ($p<0.05$) reduced serum insulin level in comparison to the VT control group. Continuous 28 days of treatment with IMHME at the mentioned doses corrected the serum insulin level significantly ($p<0.05$) in the direction to the VT control group. Figure 1 presented that insulin level increased in the treated rats with 5 and 10 mg doses. However, there was no further recovery in this parameter at a significant level ($p>0.05$) when the applied doses were 20 and 30 mg in respect to the 10 mg dose-treated group.

Table 1. Improvement in FBG level after treatment at different doses of IMHME in STZ-induced diabetic rats

Group	Fasting blood glucose level (mg/dl)					
	0 day	1st day (7th day of STZ injection)	8th day	15th day	22nd day	29th day
VT control	70.16±2.54^{a}	71.83±2.22^{a}	70.83±3.05^{a}	72.53±2.18^{a}	71.48±2.46^{a}	70.09±2.03^{a}
VT diabetic	72.66±2.45^{a}	343.16±5.56^{b}	349.50±6.36^{b}	352.38±7.03^{b}	352.53±5.63^{b}	341.69±4.25^{b}
5 mg IMHME treated diabetic group	71.50±2.59^{a}	352.33±4.05^{b}	292.14±6.83^{c}	239.43±4.29^{c}	205.66±6.52^{c}	179.83±4.70^{c}
10 mg IMHME treated diabetic group	71.50±2.59^{a}	339.33±4.73^{b}	284.16±6.13^{c}	215.43±4.67^{d}	162.66±6.18^{d}	110.83±5.68^{d}
20 mg IMHME treated diabetic group	70.33±2.84^{a}	347.83±7.03^{b}	273.83±4.06^{d}	212.16±3.80^{d}	159.83±5.12^{d}	105.49±5.45^{d}
30 mg IMHME treated diabetic group	71.33±2.76^{a}	344.50±5.59^{b}	276.84±4.74^{d}	192.98±4.02^{d}	155.33±5.91^{d}	100.65±4.43^{d}

Note: Data were presented as Mean ± SEM (n = 6), ANOVA followed by multiple comparison of Students' two-tail 't' test. Values with different superscripts (a, b, c, and d) within each column significantly differ from each other, ($p<0.05$).

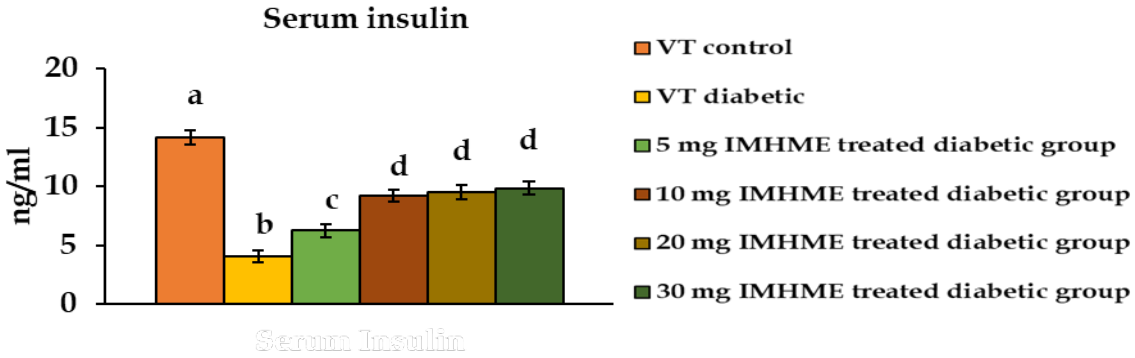

Figure 1. Serum insulin level in IMHME-treated diabetic rats. The results were presented as bar diagrams with Mean ± SEM, n = 6. ANOVA followed by a multiple comparison Students' two-tail 't' test. Bars with different superscripts (a, b, c, and d) indicate significant differences among different groups ($p<0.05$).

2.3. Activities of hexokinase and gulose-6-phosphatase in liver and skeletal muscle

A significant ($p<0.05$) inhibition in hexokinase and elevation in glucose-6-phosphatase activity resulted in the VT diabetic group when evaluated in respect to the VT control group. Indicated dose-dependent delivery of the aforementioned plant extract executed a significant ($p<0.05$) decrease in the activity of glucose-6-phosphatase and increase in the activity of hexokinase relative to the VT control group. Significant ($p<0.05$) corrections in the enzymatic activities were seen at the 10 mg dose in comparison to the 5 mg dose. However, no significant ($p>0.05$) changes in enzymatic activities were detected at the 20 and 30 mg doses in comparison to the 10 mg dose treated group. Ten mg dose was considered as the threshold dose, the minimum dose, but with maximum efficacy (Figure 2).

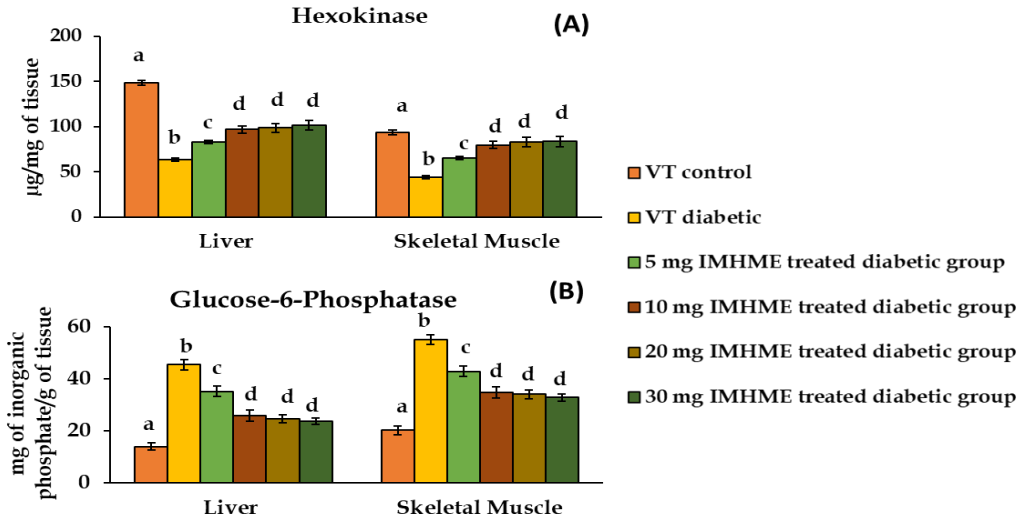

Figure 2. Hexokinase (A) and glucose-6-phosphatase (B) activities were recovered in diabetic rats following treatment with different doses of IMHME. Bar graphs were shown as Mean ± SEM, n = 6, ANOVA, followed by a multiple comparison Students' two-tail 't' test. Columns with different superscripts a, b, c, and d differ from each other significantly, ($p<0.05$).

2.4. Activities of antioxidative enzymes in hepatic and renal tissue

The significant inhibition ($p<0.05$) in the activities of catalase and superoxide dismutase (SOD) in liver and kidney tissue was noted in the VT diabetic group in contrast to the VT control group. The enzyme activities after the treatment of extract were significantly ($p<0.05$) elevated at the said doses in contrast to the VT control rats. An increase in enzyme activities was detected at the 10 mg dose in comparison to the 5 mg dose. No significant ($p>0.05$) changes in the said enzyme activities were detected in groups treated with 20 and 30 mg doses against the 10 mg treated group (Figure 3).

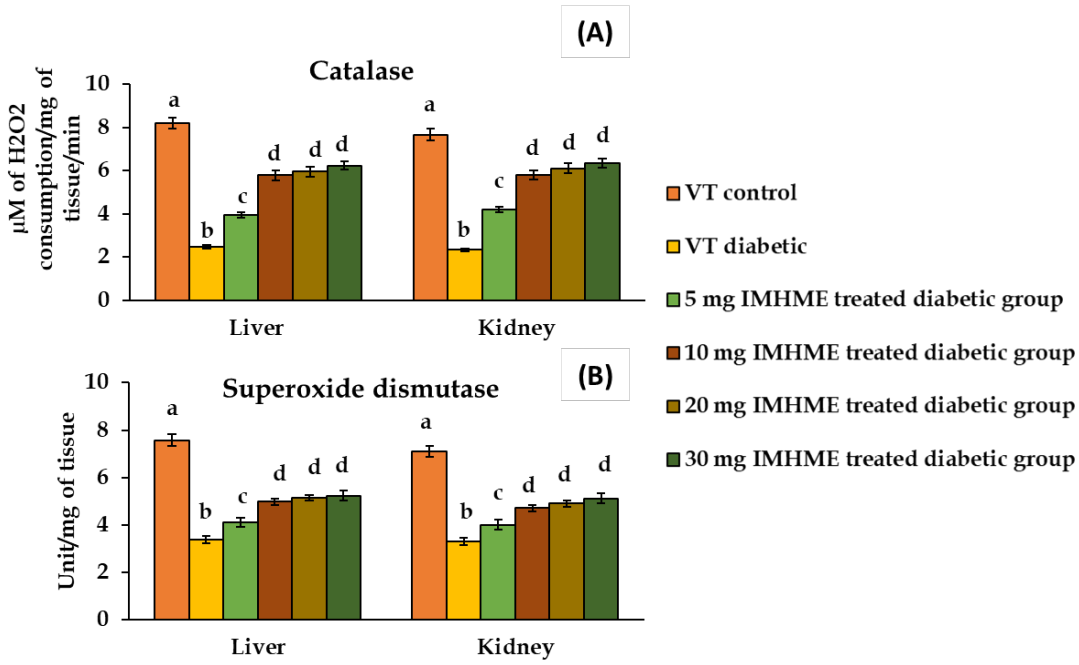

Figure 3. Catalase (A) and superoxide dismutase (B) activities in the liver and kidney tissue following administration of IMHME in different doses in diabetic rats. Bar diagrams were presented as Mean ± SEM, n = 6, ANOVA, followed by a multiple comparison Students' two-tail 't' test. Bars with different superscripts (a, b, c, and d) significantly differ from each other, ($p<0.05$).

2.5. Level of lipid peroxidation end product

The amount of lipid peroxidation end product, i.e., thiobarbituric acid reactive substances (TBARS), both in hepatic and renal tissues were significantly ($p<0.05$) higher in the VT diabetes group in contrast to the VT control group. Following IMHME treatment, the TBARS levels in both the tissues resulted in significant recovery ($p<0.05$) towards the VT control group in all the dose-treated groups. The recovery of TBARS levels in 10 mg dose was more significant ($p<0.05$) than in the 5 mg dose-treated group. The 10 mg dose had the most promising result because the higher doses, i.e., 20 and 30 mg, had no significant ($p>0.05$) recovery in contrast with the 10 mg dose treated group (Figure 4).

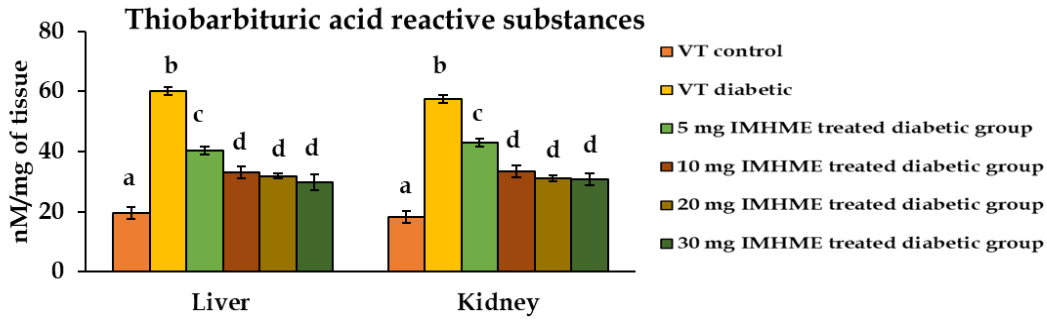

Figure 4: Level of TBARS in liver and kidney tissue after treatment with different doses of IMHME in diabetic rats. Bar diagrams were presented as Mean ± SEM, n = 6, ANOVA, followed by a multiple comparison Students' two-tail 't' test. Bars with different superscripts (a, b, c, and d) significantly differ from each other, ($p<0.05$).

2.6. Histoarchitectural study

Histoarchitectural view of the pancreatic islets' diameter and islets' cell population density in the qualitative aspect were significantly ($p<0.05$) lesser in the VT diabetic group against the VT control group. Oral administration with said doses of plant extract increased the diameter of the islet and islets' cell density significantly. No significant ($p>0.05$) recovery was noted in the said parameters in 20 and 30 mg dose-treated groups against the 10 mg dose-treated group (Figure 5).

2.7. Hepatic toxicity assessment

Activities of glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) enzymes were elevated significantly ($p<0.05$) in VT diabetic rats in contrast to VT control group. The IMHME extract in the above-mentioned doses diminished the said enzyme activities towards the VT control group. Considerable diminution in enzyme activities was recorded in 5 and 10 mg dose-treated groups, but no significant ($p>0.05$) improvement was noticed in 20 and 30 mg dose-treated groups against the 10 mg dose-treated group (Figure 6).

2.8. Acute toxicity test

There was no mortality, behavioural abnormalities, including anorexia, and drowsiness in any dose-treated group. Every rat has a normal tendency for food and water consumption and was not affected by the IMHME administration.

2.9. Levels of creatinine, urea, and albumin test

Levels of serum creatinine, urea, and albumin were elevated significantly ($p<0.05$) in the VT diabetic group against the VT control group. Treatment of IMHME in the above doses resulted in significant ($p<0.05$) improvements in the said sensors towards the direction of VT control rats. Significant ($p<0.05$) diminution in levels of all the above sensors was noted in 5 and 10 mg dose-treated groups. No further recovery was observed in the above sensor levels in the groups treated with 20 and 30 mg compared to the 10 mg dose treated group (Table 2).

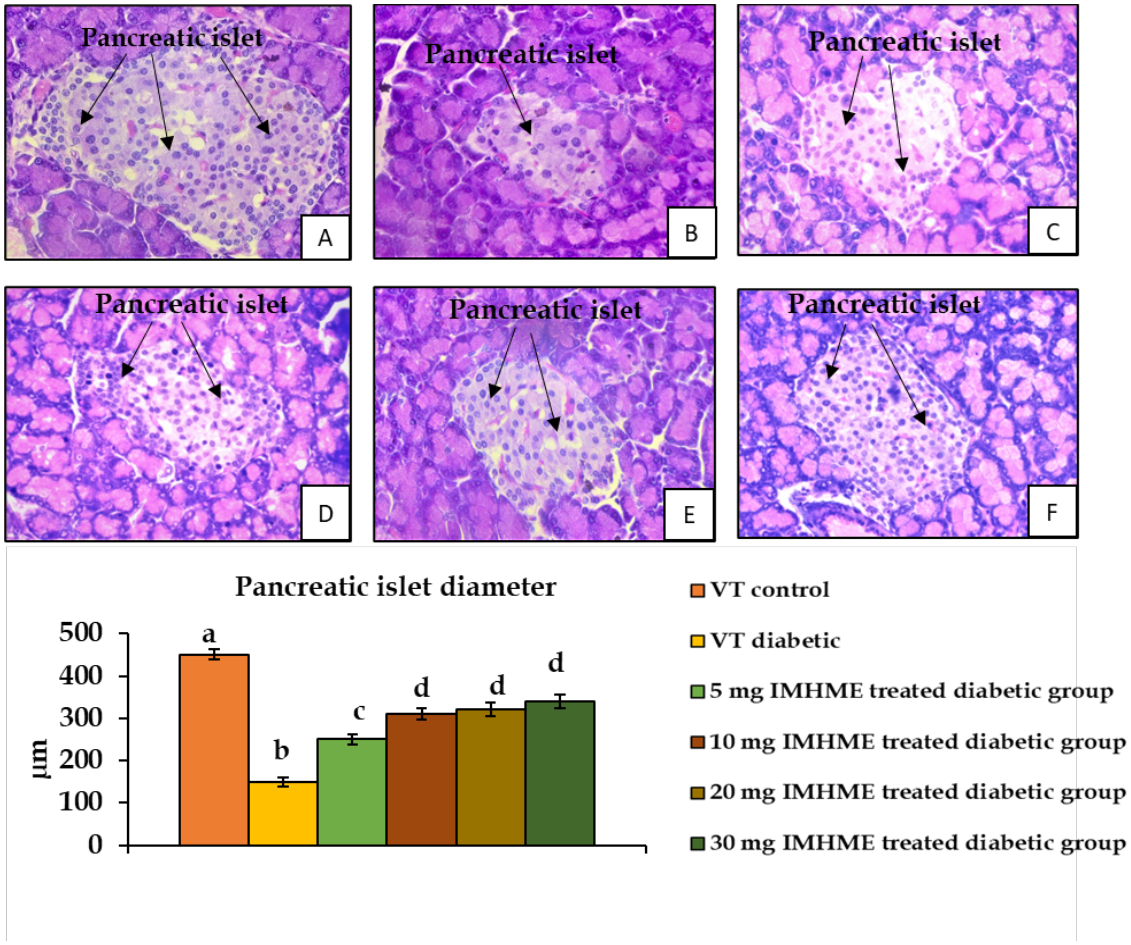

Figure 5. Histometric study of pancreatic islet after treatment with different doses of IMHME in diabetic rats, vehicle-treated control (A), vehicle-treated diabetic (B), 5 mg IMHME treated (C), 10 mg IMHME treated (D), 20 mg IMHME treated (E) and 30 mg IMHME treated (F) diabetic groups. Haematoxylin-eosin stain, original magnification 400X. Bar diagrams were presented as Mean ± SEM, n = 6, ANOVA, followed by a multiple comparison Students' two-tail 't' test. Bars with different superscripts (a, b, c, and d) significantly differ from each other, ($p<0.05$).

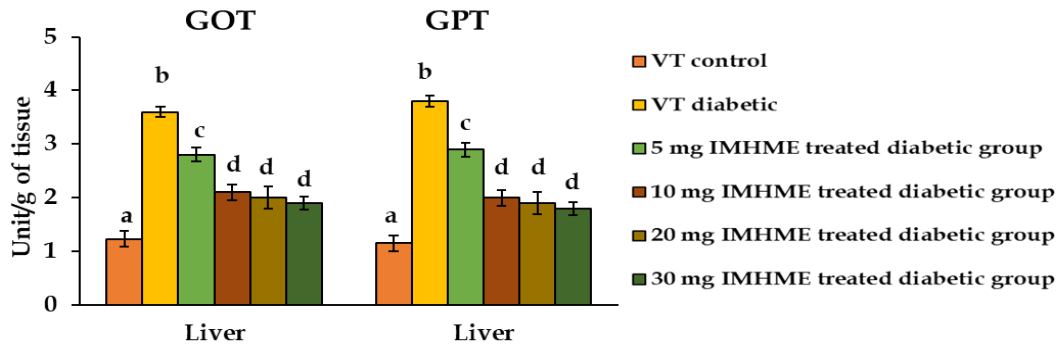

Figure 6. Assessment of GOT and GPT activities in liver tissue after treatment with different doses of IMHME in diabetic rats. Bar diagrams were presented as Mean ± SEM, n = 6, ANOVA, followed by a multiple comparison Students' two-tail 't' test. Bars with different superscripts (a, b, c, and d) significantly differ from each other, ($p<0.05$).

Table 2. Protective effect of IMHME on serum urea, creatinine, and albumin levels in STZ-induced diabetic rats

Group	Creatinine (mg/dl)	Urea (mg/dl)	Albumin (g/dl)
VT control	0.7±0.054[a]	142.481±6.22[a]	3.83±0.09[a]
VT diabetic	3.0±0.15[b]	329.351±7.90[b]	7.40±0.16[b]
5 mg IMHME treated diabetic group	1.50±0.069[c]	223.620±6.81[c]	5.34±0.21[c]
10 mg IMHME treated diabetic group	1.0±0.059[d]	169.453±8.54[d]	4.12±0.18[d]
20 mg IMHME treated diabetic group	0.9±0.044[d]	165.535±6.51[d]	4.06±0.20[d]
30 mg IMHME treated diabetic group	0.8±0.056[d]	150.651±5.59[d]	3.92±0.14[d]

Note: Data were presented as Mean ± SEM (n = 6), ANOVA followed by multiple comparisons of Students' two-tail 't' test. Values with different superscripts (a, b, c, and d) within each column differ significantly from each other, ($p<0.05$).

3.DISCUSSION

Ipomoea mauritiana (Jacq.) has a wide range of medicinal values [12,13] and is used as a reputed ethnomedicine for diabetes management in Bangladesh [12-13]. The tuber paste of *Ipomoea mauritiana (Jacq.)* was consumed twice a day to control diabetes as folk medicine [14]. To find out its antidiabetic mode of action, the present investigation has been executed on diabetic model animals. Experimental rats were given STZ injection, which works specifically on pancreatic β cells to induce type 1 diabetes. The alkylating properties of STZ alter biological macromolecules in β cells, causing DNA damage and β cell destruction. Thus, for glucose metabolism, β cells were unable to synthesize and release the requisite amount of insulin [15]. The experimental finding demonstrated that STZ induces diabetes by damaging pancreatic β cells, which decreases islets' diameter and cell density in diabetic rats. Also, hepatocyte degeneration, acinar cell enlargement, and the islet of β-cell destruction in pancreatic tissues are all caused by STZ [16]. The resulting diminution in the level of serum insulin impaired glucose oxidation and increased FBG level. The plant extract supplementation recovered the islet's size and cell density by generating β cells from hepatic stem cells [17]. This was also in the same line with our past observations of other plant extracts and the present histological study of pancreatic islets [18]. Besides this, the therapeutic effect of the extract may activate voltage-dependent channels and increase intracellular Ca^{++} concentration, which helps insulin exocytosis by β cells and increases serum insulin level in different dose-treated diabetic groups [19]. In another way, it may be stated that the remedial effect of IMHME acts genetically for mRNA expression of insulin receptor substrate (IRS-1) and glucose transporter 4 (GLUD-4) in muscles and adipose tissues to normalize hyperglycemia, parallel with previous studies using separate phytomolecules [20].

Deviation in the carbohydrate metabolic enzyme activities also caused impairment of glucose utilization [21]. The major enzymes in the metabolism of glucose are hexokinase and glucose-6-phosphatase. Significant diminution in hexokinase activity and elevation in the activity of glucose-6-phosphatase in diabetic animals may be due to increased ATP to Mg^{2+} ratio by low insulin in diabetes that impaired glucose transport and phosphorylation [22]. The activation of glucose-6-phosphate translocase and inadequate secretion of insulin results in a significant increase in glucose-6-phosphatase activities in diabetes [23]. This plant extract may activate the adenosine monophosphate-activated protein kinase pathway, which may restore insulin signaling molecules for increasing insulin-mediated glucose uptake in the liver and muscle. The increasing activity of the hexokinase enzyme and diminution in glucose-6-phosphatase activity in extract-treated groups may normalize the blood glucose level [24]. The recent work on the effect of *Borassus flabellifer* palm sprouts on diabetic rats supports this justification [25].

Induction of diabetes causes oxidative damage in the hepatic and renal tissues by excess generation of reactive oxygen species (ROS), supported by decreasing antioxidative enzyme activities of catalase and SOD and increasing lipid peroxidation end product, i.e., TBARS levels in diabetic rats [26-27]. The plant extract recovered oxidative damage by elevating the activities of antioxidant enzymes and interfering with the generation of free radicals and the end product of lipid peroxidation, i.e., TBARS [28]. The oxidative stress management by the said plant extract is an alternative possible way for diabetes management, supported by other plant extract studies [29].

Transaminase enzyme activities are considered as general toxicity sensors due to oxidative stress related to diabetes. The activities of these enzymes were elevated, as noted by others [30]. The plant extract resulted in the correction of both these enzymes, which may be due to the protection of cellular organelles damage concerned with transaminase sub-cellular secretion as proposed by others [31].

Urea, creatinine, and albumin are primary metabolites derived from the cellular metabolism of proteins, which are reabsorbed during the filtration of urine and reused for body function. Serum levels of creatinine, urea, and albumin are also important and sensitive indicators of renal toxicity [32]. In diabetic rats, the high level of these sensors indicated that protein breakdown due to insulin resistance causes glomerular dysfunction and diabetic nephropathy, another complication of diabetes [33]. Diabetes-induced renal toxicity has been reduced by this extract, which indicates the management of diabetes-induced nephropathy and related complications. The therapeutic effect of IMHME triggers glucose utilization and subsequently minimizes protein breakdown, which decreases creatinine, urea, and albumin levels. The study of *Hordeum vulgare Linn* effects on diabetic rats supports this mechanism [34].

The dose-dependent therapeutic effects of IMHME may not reach a saturation level of interaction with the target receptors at a lower dose (5 mg), resulting in a sub-maximal impact. Despite increasing the dose from 10 to 30 mg, 10 mg executed the maximal effects due to the full receptor occupancy. Therefore, at 20 and 30 mg, no further elevated therapeutic recovery was observed, possibly due to the targeted receptors being spared because agonists show their maximal effects [35]. Thus, a 10 mg dose ensures maximum effect without increasing any toxic effects on metabolism [36].

Besides the alternative antidiabetic herbal drug development from IMHME, it may be used as a parallel treatment regimen at the sub-therapeutic level along with synthetic anti-glycemic agents, which may delay drug resistance, improve life efficiency and the DALYs of individuals. To establish a novel antihyperglycemic drug, clinical administration and detailed investigation regarding its mode of action will be needed.

4. CONCLUSION

It concluded that in a dose-dependent study of IMHME, the 10 mg dose has the maximum efficacy for managing diabetes and its associated oxidative stress. Further investigation is in the process of identifying the responsible phytomolecules in IMHME to validate its antidiabetic and antioxidant capabilities. The findings of this experiment will pave the way for the development of innovative and sustainable approaches to address the complex interplay of hyperglycemia and oxidative stress in diabetes.

5. MATERIALS AND METHODS

5.1. Collection of plant material

Taxonomist of the Botany and Forestry Department, Vidyasagar University (West Midnapore, West Bengal) verified the *Ipomoea mauritiana (Jacq.)* root tuber collected from the West Midnapore tribal area. The voucher referral no. was *I, mauritiana*/VU/Bio/16/23. The root tubers were cleaned, peeled off the outer layer, then chopped into small pieces, and kept at room temperature (25ºC) for seven days for air drying. The dried pieces were grinded in powder form.

5.2. Preparation of hydro-methanol (60:40) extract of *Ipomoea mauritiana* (*Jacq.*) (IMHME)

Initially, a suspension was prepared by mixing 50 gram of *Ipomoea mauritiana* (*Jacq.*) root tuber powder at a volume of 1 litre with distilled water (DW) and methanol at a ratio of 3:2. The mixture was stirred intermittently for 48 hours. At the end of 2 days, the mixture underwent filtration using a cotton filter. The final filtration was done by Whatman filter paper, and then the filtrate was lyophilized into powder form and subsequently stored in the refrigerator at 8ºC for future use purposes.

5.3. Chemicals

Streptozotocin (STZ), adenosine triphosphate (ATP), nicotinamide adenine dinucleotide phosphate (NADP), and 2-[4-(2-Hydroxyethyl)1-piperazinyl] ethane sulfonic acid (HEPES) were supplied by Sisco Research Laboratories Pvt. Ltd. (SRL), India.

5.4. Animal selection and care

Thirty-six fully matured normoglycemic male albino rats with a BW range of 120 ± 10 g were housed in a light-maintained (12 hours dark and 12 hours light), well-ventilated, temperature-controlled room for acclimatization. Rats were given a sufficient amount of *ad libitum* water and food. Animal care was adopted

as per the protocol given by the Institutional Ethics Committee (IEC) and complies with the regulations of the Committee for the Control and Supervision of Experiments on Animals (CCSEA). Ethical permission no. for this experiment was 3/8/VU/IAEC/Dt.11.04.23.

5.5. Development of diabetes mellitus

The experiment was initiated with a single intramuscular injection of STZ at the dose of 4 mg/100 g of BW in a citrate buffer of 100 mM (pH of 4.5) for induction of diabetes in experimental animals. After 12 hours of fasting, blood glucose level was noted on 8th day of STZ injection. The range of 300–350 mg/dl was finalized as a diabetic state for this experiment.

5.6. Grouping and Treatment

The study consisted of five groups, and each group comprised six rats. Treatment by gavage was conducted under 12 hours of fasting conditions. After treatment, no food was provided to rats for next 1hour.

5.6.1. Group 1: Vehicle-treated control (VT control)

Rats having fasting blood glucose level at normal range underwent DW through gavage at a dose of 0.5 ml/100 g of BW/day for 28 days.

5.6.2. Group 2: Vehicle-treated diabetic (VT diabetic)

Diabetic rats were subjected to gavage of DW at a dose of 0.5 ml/100 g of BW/day for 28 days

5.6.3. Group 3: 5 mg IMHME treated diabetic group

Treatment of IMHME was performed at the dose of 5 mg/0.5ml DW/100 g BW/day to diabetic rats for 28 days.

5.6.4. Group 4: 10 mg IMHME treated diabetic group

Treatment of IMHME at the dose of 10 mg/0.5ml DW/100 g/ BW/day for 28 days was conducted on diabetic rats.

5.6.5. Group 5: 20 mg IMHME treated diabetic group

Diabetic rats were subjected to gavage with IMHME at the dose of 20 mg/0.5ml DW/100 g BW/day for 28 days.

5.6.6. Group 6: 30 mg IMHME treated diabetic group

The diabetic rats were delivered IMHME at the dose of 30 mg/0.5ml DW/100 g BW/day for 28 days.

5.7. Sacrifice of experimental animals

Rats were sacrificed after 12 hours of fasting in an anesthetic condition on the 29th day following cervical dislocation. Blood was obtained from the dorsal aorta of each animal. The serum sample was prepared from blood by centrifugation at 5000 rpm for 5 minutes and was used to measure serum insulin. The pancreas, skeletal muscle, kidney, and liver were collected from the animals, cleansed with normal saline separately, and then refrigerated for subsequent biochemical and histological assessment.

5.8. Fasting blood glucose level measurement

One drop of blood from the tail vein was touched to a glucometer strip to test the FBG level in all rats at seven days intervals over the following twenty-eight days of the experiment [37].

5.9. Serum insulin level measurement

The ELISA kit was used to examine serum insulin level, and the value was expressed in ng/dl (Ray Biotech, Norcross, GA 30092, USA) [38].

5.10. Carbohydrate metabolic enzymes assessment in skeletal muscle and liver

Hexokinase activity in skeletal muscle and liver tissue were assessed by the spectrophotometric method. The tissues were homogenized in phosphate buffer saline (PBS) and centrifuged at 10,000 rpm. Hexokinase activity was measured by the preparation of an assay mixture (glucose, $MgCl_2$, thioglycerol, and HEPES buffer), NADP, ATP, glucose-6-phosphate, and tissue supernatant, were added into a cuvette for spectrophotometric analysis. Optical density (OD) was recorded at the 340 nm wavelength [39]. The glucose-6-phosphatase activity was assessed by adding 0.1 M maleic acid buffer and 0.01 ml of glucose-6-phosphate

solution in a calibrated tube. Tissue supernatant was added and incubated for 15 minutes at 37ºC. The stopped solution (10% TCA) and DW were added and centrifuged. The OD was recorded at 340 nm [40].

5.11. Assessment of antioxidant enzyme activities in hepatic and renal tissue

Activities of antioxidant enzymes were determined by homogenizing the tissue in PBS buffer and centrifuging at 10,000 rpm. A mixture of tissue supernatant, DW, and H_2O_2 were prepared to measure catalase activity by recoding OD at 240 nm in a spectrophotometer [41]. For the evaluation of SOD activity, a spectrophotometer cuvette was filled with tissue supernatant and pyrogallol. The OD was noted at 420 nm [42].

5.12. Estimation of thiobarbituric acid reactive substances (TBARS)

Tissue-homogenate was used to measure the TBARS levels. Homogenate tissue at a volume of 0.5 ml, 2 ml of TBA-TCA, and 9% NaCl of 0.5 ml were mixed and centrifuged for 5 minutes. The OD was noted in the spectrophotometer at 535 nm [43].

5.13. Histological study of the pancreas

The collected pancreas tissue samples of all the animals all the animals were preserved for 12 hours in Bouin's fixative. Following a standard protocol for tissue dehydration, the tissue was processed for paraffin-embedding and then sectioned (5 µm) using the LEICA Semiauto microtome. The tissue sections were deparaffinized and then stained with hematoxylin-eosin. The histological analysis of stained tissue was performed under the microscope at a magnification of 400X [44].

5.14. Toxicity biomarkers study

Activities of GOT and GPT in hepatic tissue were determined using a standard protocol for assessing general toxicity [45].

The acute toxicity was assessed at the dose applied by noting immediate mortality, behavioral abnormalities, anorexia, drowsiness, etc. [46].

Serum creatinine, urea, and albumin levels were determined by diagnostic kits (Meril Diagnostics, India) for the assessment of subchronic cum chronic toxicity of the said extract [47].

5.16. Statistical analysis

The data were shown as Mean ± SEM (n = 6). Analysis of variance (ANOVA) was used for statistical analysis, followed by multiple comparisons of Students' two-tail 't' tests. A significant difference was determined at the level of $p<0.05$ [48].

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