Pharmacological Research - Natural Products 10 (2026) 100470

Contents lists available at ScienceDirect

Pharmacological Research - Natural Products

journal homepage: www.sciencedirect.com/journal/prenap

Determination of antidiabetic property and liver-renal protective potential of aqueous extract of *Annona reticulata* Linn. leaves and its synergistic action with glibenclamide in alloxan-induced diabetes using animal model

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ARTICLE INFO

Keywords:
Annona reticulata L.
Antidiabetic property
Aqueous extract
Liver-Renal protective activities
Synergistic action

ABSTRACT

Through this study, the antidiabetic activities of *Annona reticulata* L. (*Annonaceae*) leaves, as claimed by certain tribal communities, were investigated. The anti-diabetic and liver-renal protective properties of aqueous leaf extract and its combination with the standard drug glibenclamide were tested in rats with alloxan-induced diabetes at doses of 200 and 400 mg/kg body weight; both dosages were found to possess significant dose-dependent antidiabetic activity. After 21 days of treatment, standard medication glibenclamide (5 mg/kg) exhibited fasting blood glucose level of 5.91 ± 0.09 mmol/L, while aqueous leaf fraction (400 mg/kg); combination of extract (200 mg/kg) and glibenclamide (2.5 mg/kg) demonstrated fasting blood glucose levels of 5.13 ± 0.12 mmol/L and 5.21 ± 0.07 mmol/L. Additionally, the diabetic rats in the extract treated group (400 mg/kg dosage) and the combination group demonstrated 8.67 % and 6.19 % of body weight loss, respectively, which were less than the value observed from the group of rats treated with glibenclamide alone (12.31 %). Furthermore, the extract and its combination with glibenclamide were found to have beneficial effects on the liver and kidney ($p < 0.001$) and aided in the maintenance of healthy lipid profile even when diabetes was present; these effects were comparable to those of standard drug. Moreover, GC-MS analytical data aided in identification of potential components present in the test fraction that may be crucial in the achievement of these bioactivities.

Introduction

Several metabolic illnesses together are referred to as diabetes mellitus. Known as the hidden epidemic of the twenty-first century, diabetes is the biggest public health issue. Diabetes is a long-term condition that gradually damages a variety of body organs. The symptoms appear years after the disease first appears and advances gradually [1,2]. It is caused by insufficient production of insulin by the pancreas, either inherited, acquired, or ineffectively produced. In addition, insufficient insulin hormone secretion, a lack of target cell response to insulin, or a combination of these factors may be the reason [3]. It is not uncommon for modern oral hypoglycemic drugs to cause adverse effects. Therefore, it is imperative to switch to different indigenous plant and herbal compositions and use alternative medicine. There has been promise in using traditional medicines to treat diabetes [4]. Even though medicinal plants have been used for centuries to treat illnesses, it will take time for contemporary medicine to accept and employ them [5–7]. Type 2 diabetes can be effectively treated using a variety of common herbs and spices that are said to have blood glucose-lowering properties. Furthermore, the number of people using these natural chemicals to manage their illness has increased as a result of several pharmacological studies on the antidiabetic properties of medicinal plants [8,9]. In the past, diabetes and its consequences were treated with traditional herbal treatments prior to the development of insulin and other blood glucose-lowering medications [10,11]. Also, it has been demonstrated that over 1200 medicinal herbs have antidiabetic properties to far such as *Juglans regia*, *Cinnamomum verum*, *Lamium amplexicaule*, *Trigonella monpeliaca*, *Arctium lappa*, *Urtica dioica* etc [12]. Additionally, the three types of liver ailments that continue to be a global health concern are cirrhosis, hepatosis and acute or chronic hepatitis. While, the preferred treatments for liver illnesses are contentious, inadequate and may have detrimental side effects [13]. The liver-protective properties of herbal

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https://doi.org/10.1016/j.prenap.2025.100470
Received 29 April 2025; Received in revised form 12 December 2025; Accepted 17 December 2025
Available online 18 December 2025

medicine formulations used in traditional medicinal systems have long been known. Numerous plants have huge potentials for hepatoprotection in addition to other advantages. Additionally, many edible herbs have also been approved because of their potential to prevent and treat liver issues. These plants have proven their hepatoprotective action in a number of ways [14]. According to current research, hundreds of plants can be used to treat a variety of liver conditions [15]. Moreover, herbal extracts and other natural products may be quite helpful in the intoxicated liver's healing processes. Reliable scientific data gathered from medicinal plant research indicates that plants like *Silybum marianum*, *Glycyrrhiza glabra*, *Phyllanthus* species, and *Picrorhizakurroa* have been used extensively and frequently successfully to treat liver disorders. These plants work by acting through their antioxidant-related potentials [16–19]. In traditional medical systems around the world, renal failure has been treated with a variety of plants. Although there aren't many treatments for acute renal failure, ethnomedical plants can assist prevent the need for dialysis by treating the causes and effects of renal failure as well as lowering the numerous negative effects of dialysis [20]. In addition, research shows that artificial nephroprotective drugs have negative side effects. It is also notable that, the metabolic activation of highly reactive free radicals from a variety of environmental toxicants and clinically helpful medications, such as gentamicin and acetaminophen can result in severe renal toxicity [21]. Additionally, it is now known that nephrotoxicity from drugs used as treatments for other conditions, increases the likelihood of developing acute and chronic kidney disorders. Therefore, alternative methods of treating these conditions have been investigated in an effort to avoid the side effects of medication. Moreover, *Abelmoschus esculentus*, *Achyranthes aspera*, *Bambusa nutans*, *Cassia fistula*, *Digitalis purpurea* etc plants have been utilized as sources of nephroprotective agents [22,23]. Moreover, by combining gas chromatography and mass spectrometry, determination of substances in a sample can be conducted [24]. Plant extracts are commonly subjected to GC-MS analysis in order to detect and measure volatile and semi-volatile organic components. By comparing the plant's chemical profile with an existing library of chemicals, this method produces a comprehensive chemical profile of the plant, which aids in the identification of plant constituents and acknowledgement of biological roles of plant-derived compounds [25,26]. Since *Annona reticulata* L. has been utilized in ethnomedicine to treat a wide range of ailments. The plant is claimed to possess antipyretic, anthelmintic, antihyperglycemic, antiulcer, antinociceptive, analgesic, anti-inflammatory, antiproliferative, anticancer, antioxidant, antimicrobial, wound healing and antimarking activities [27]. Therefore, this historically significant herb was selected for assessment of its antidiabetic, and liver-renal protective potentials via this investigation.

Materials and methods

Preparation of plant extract

The plant part (leaves) of *Annona reticulata* L. was collected from Savar, Dhaka. After that, the leaves were identified by Dr. Khandakar Kamrul Islam (Senior Scientific Officer, Bangladesh National Herbarium). Sample was deposited in Bangladesh National Herbarium (Voucher number: DACB 114865). Dried plant material (538 g) was placed in dark-colored flasks with water (1.76 L). 24 h later, filtration of infusions was conducted by utilizing filter paper. The process was repeated 48 h later. Combined supernatant was vacuum-dried in rotary evaporator (40°C). When it was established that the plant materials had been exhausted, the extraction procedure was deemed finished. The plant material was extracted, dried, and then immersed in one litre of purified water. Over the course of seven days, the plant components were shaken and agitated periodically in a sealed container filled with water. A brand-new cotton bed filter was utilised. The filtrates were dried at 40 ± 2°C for production of sticky crude fraction. Following extraction, the material was properly labelled and kept in sterile sample containers at 4°C [28].

GC-MS analysis of the extract from Annona reticulata L. leaves

Shimadzu GCMS-TQ8040 was utilized to conduct GC-MS analysis. Helium (carrier gas) allowed for the successful separation and detection of the analytes. In accordance with GC parameters, column oven was kept at 50°C, ramped up to 200°C and lastly reached 300°C. The hold times were 1, 2, and 7 min, respectively. The injection was carried out at 250°C in splitless mode with a sampling duration of one minute, flow control mode set to pressure at 53.5 kPa, and total flow of 11 mL/min. Moreover, 230°C and 250°C were temperatures of ion source and contact under MS conditions. This technique provides excellent sensitivity and specificity for identifying and quantifying volatile and semi-volatile compounds in complex plant extracts by comparing them to library compounds [29].

In-vivo studies

Experimental animals

Swiss albino mice (25–30 g, male) and Wister albino rats (120–230 g, male) were used. Test animals were gathered from the department's animal house. Aqueous leaf fraction from *Annona reticulata* L. was studied pharmacologically in the Jahangirnagar University Pharmacology Laboratory. Polypropylene cages were used to house the animals with appropriate laboratory settings, including proper dark-light cycle, relative humidity (RH 55 ± 5 %), and temperature of 25 ± 2°C. The rats and mice were fed pelletised mouse feed from ICDDR, B., and they had unrestricted access to water. Every action involving the handling of animals was conducted in compliance with the ARRIVE guidelines and the rules imposed by Jahangirnagar University's animal ethics committee [Ref No: BBEC, JU/M 2024/11 (137)].

Assay of in-vivo acute toxicity study

Acute toxicity study was performed for determining the safety profile of plant extract for human ingestion. Guidelines given by Harizal et al. was followed in this work. In 2001, OECD established 423 guidelines for investigation of in vivo acute toxicity studies. Three groups of albino mice, each consisting of five mice were taken. Before plant extract was given, the animals were kept starved through the night with plenty of water. In order to find the optimal dosage, the experimental animals were then administered *Annona reticulata* L. aqueous leaf extract at increasingly greater concentrations, such as 1, 2 and 4 g/kg of body weight, via an intragastric tube. Each animal was then closely observed for any unusual movements that would indicate any kind of delayed toxicity. Following administration, each animal was evaluated separately for 14 days, paying special attention to the first four hours and then each day after that [30].

Evaluation of in-vivo anti-diabetic potential and protective effect on the liver and kidney

Anti-diabetic potential of aqueous leaf fraction from *Annona reticulata* L. and its combination with the standard medication glibenclamide were evaluated in this work using rats having Alloxan-induced diabetes. Glibenclamide was used as standard drug in this study because of its well-known mechanism of action, proven effects, availability, cost-effectiveness, FDA-approved status and wide-spread use in humans. Effectiveness of anti-diabetic potential was assessed by contrasting the anti-diabetic responses of animals given test samples (*Annona reticulata* L. leaf extract and extract's combination with glibenclamide) with animals given distilled water (negative control) and standard medication, glibenclamide (positive control). The extract and standard medication combination was examined to depict the treatment outcomes of the combination and to compare the results of the combination with the results of the extract and the medicine alone. The purpose of this was to see if the combination could reduce the side effects of traditional

treatment. To assess the test extract's and combinations' protective effects on the liver and kidney, testing of a number of serum biochemical parameters were also conducted because diabetes gradually destroys these organs. The influence on the kidney, liver, and pancreas was demonstrated through histopathological evaluation.

Induction of diabetes

After 18 h of fasting, 40 rats received an intraperitoneal injection of 150 mg/kg alloxan.5 % dextrose saline solution was used to dissolve the alloxan. After the injection, they were allowed unlimited access to food and water, and to avoid hypoglycemia shock, they were given 5 % glucose solution to drink throughout the night. The onset of diabetes was confirmed 48 h after the alloxan injection. For the investigation, rats with FBG values above 200 mg/dL were selected [31–34].

Treatment protocol

Rats were divided into six groups of five. Each rat was weighed and assigned a tail number. Group I was composed of 5 normal control rats. The remaining groups consist of five rats that have diabetes caused by alloxan. As normal controls, the animals in Group I were administered distilled water (10 mL/kg); alloxan-induced diabetic rats of Group II received the same treatment. Glibenclamide 5 mg/kg, the standard drug, was given orally to Group III diabetic rats. Furthermore, 200 and 400 mg/kg dosages of aqueous leaf fraction were given by oral route to animals in Group IV and V that had diabetes caused by alloxan. Diabetic rats of Group VI were given combination of 2.5 mg/kg glibenclamide and 200 mg/kg of aqueous fraction orally. For a total of 21 days, all animals in all groups received treatments in accordance with the experimental schedule. On days 1, 7, 14, and 21 (one hour after administration of medication), blood samples were collected via end tail vein cutting method. Then, blood glucose levels were measured using a one-touch electronic glucometer. Additionally, the rats' body weight was meticulously noted on days 1, 7, 14, and 21 [31,32] and percentage of body weight changes was calculated using the following formula:

Percentage (%) of Changes in Body Weight = $\{(B_F - B_I) \div B_I\} \times 100$

Here, B_F means Final body weight and B_I means Initial body weight.

Evaluation of anti-diabetic activity and protective effect on liver & kidney:

- **Assessment of Biochemical parameters:** The rats were sacrificed after 21 days of therapy via cervical dislocation (rats were anaesthetized by intraperitoneal administration of ketamin hydrochloride before performing cervical dislocation). By utilizing microcapillary technique, blood was drawn and serum was then extracted. The rodents had fasted overnight. Using a commercial kit, biochemical parameters of serum triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol, creatinine, blood urea nitrogen, ALT, AST, bilirubin, and random blood glucose level were estimated [32].
- **Histopathological Assessment:** Following the animals' sacrifice, the pancreas, liver, and kidney of each rat were taken out and placed in a 10 % formalin solution. After that, Section (5 μ thick) were cut. Then staining was conducted utilizing haematoxylin and eosin for histological investigation [34].

SPSS version-27 for Windows along with one-way ANOVA were utilized for carrying out statistical analysis. $p < 0.05$ was deemed significant, and mean ± SD was used to display all results. (Figs.1-4)

Results and discussion

GC-MS analysis report of aqueous extract of Annona reticulata L. leaves

78 components were found in aqueous fraction from *Annona reticulata* L. leaves, according to the library search report. Among these compounds, Cysteine was detected in the aqueous extract, which was reported to possess antidiabetic, renal-protective and hypolipidemic potentials [35,36]. Furthermore, 1,3-dioxolane-4-methanol was present that possessed hepatoprotective property as per report [37]. In our study, in the aqueous leaf extract, the concentrations of Cysteine and 1, 3-dioxolane-4-methanol were 4 μg/mL and 1 μg/mL.

In-vivo studies

In-vivo evaluation of acute toxicity

The experimental animals received doses of 1, 2 and 4 g/kg of aqueous leaf fraction from *Annona reticulata* L. No mortality, morbidity, or abnormal behavioural changes was found after total of 14 days of monitoring, indicating that the extract was well tolerated. Shivanna et al. also validated that there is no indication of acute or delayed toxicity in experimental animals when using extracts from *Annona reticulata* L. Therefore, it can be considered safe to give the extract to experimental animals [38].

Evaluation of in-vivo anti-diabetic potential and protective effect on the liver and kidney

Rats having alloxan-induced diabetes were treated for 21 days in order to assess the test samples' anti-diabetic properties. Since a tool for measurement of the test samples' anti-diabetic activity can be blood glucose level, it was checked at regular intervals. Normal range of FBG for rats was 4.4–4.9 mmol/L [39]. For normal control group, FBG level was 4.93 ± 0.16 mmol/L, 5.03 ± 0.16 mmol/L, 4.92 ± 0.06 mmol/L and 4.84 ± 0.15 mmol/L on day 0, 7, 14 and 21, respectively. As these rats were normal, they exhibited FBG values which were in normal range. Observed FBG levels for diabetic control group were 8.77

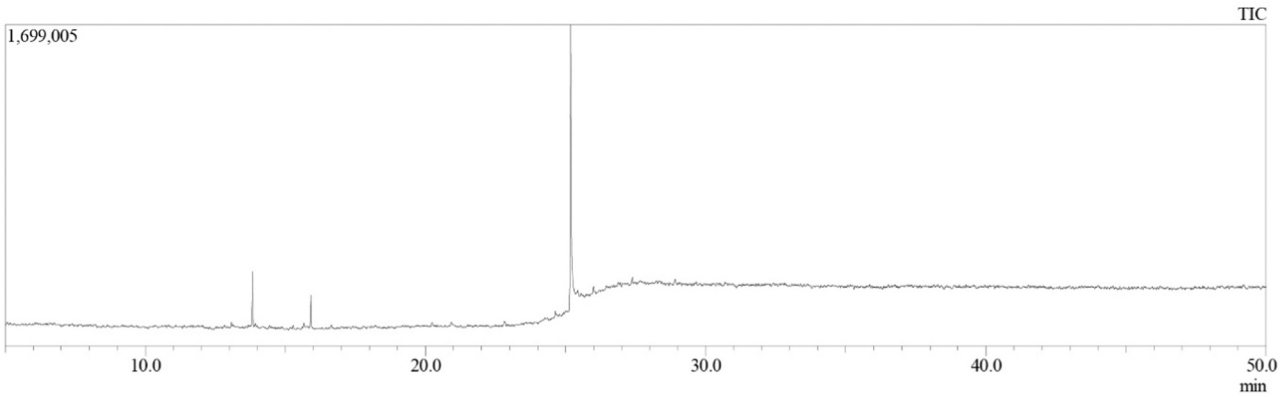

Fig. 1. Total ionic chromatogram of *Annona reticulata*'s crude aqueous extract.

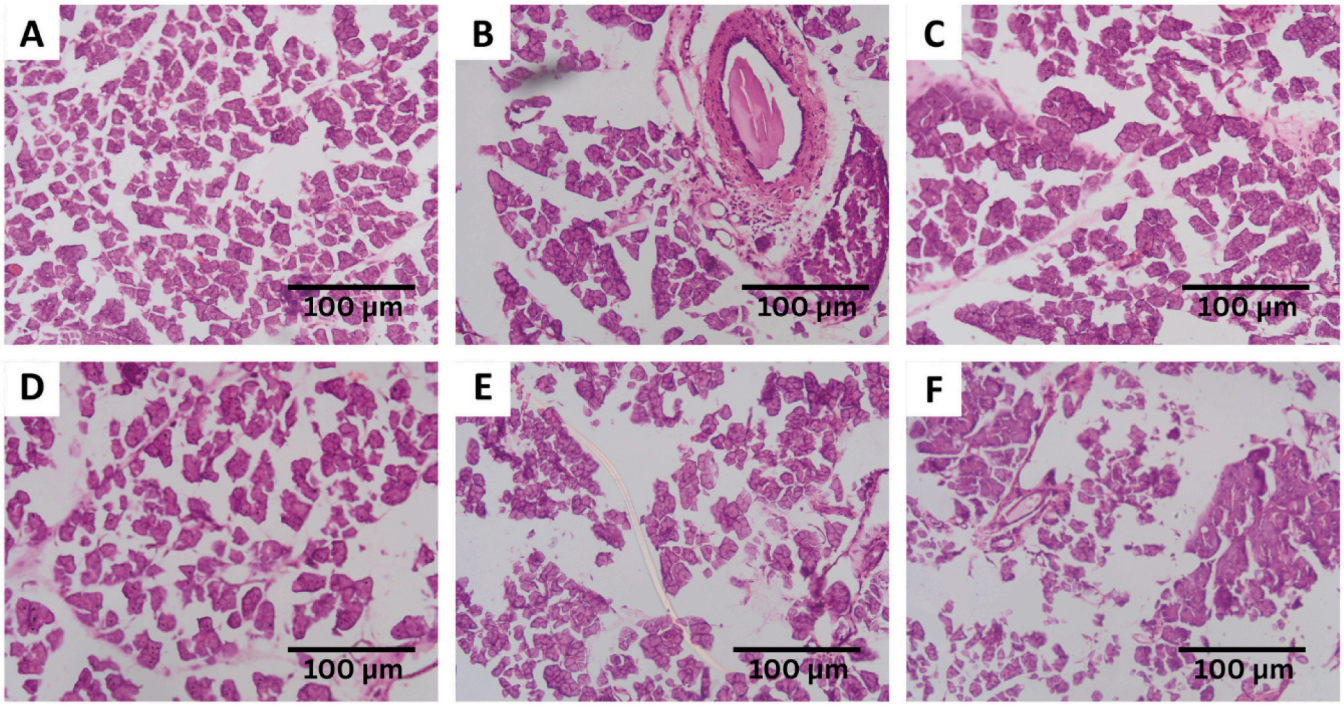

Fig. 2. H&E-stained histopathological sections demonstrating pancreatic morphology in control and treated rats (scale bar: 100 µm). A. Normal control rat showing normal pancreatic tissue. B. Alloxan induced diabetic rat exhibiting significant decrease in number of ß-cells. C. Diabetic rat treated with glibenclamide demonstrating partial restoration of structure of islet of Langerhans. D. Diabetic rat that received 200 mg/kg dose of aqueous extract showing regeneration of pancreatic tissue. E. Diabetic rat treated with 400 mg/kg dose of aqueous extract depicting restoration of pancreatic morphology. F. Diabetic rat that was given combination of aqueous extract and glibenclamide showing restoration of ß-cell population.

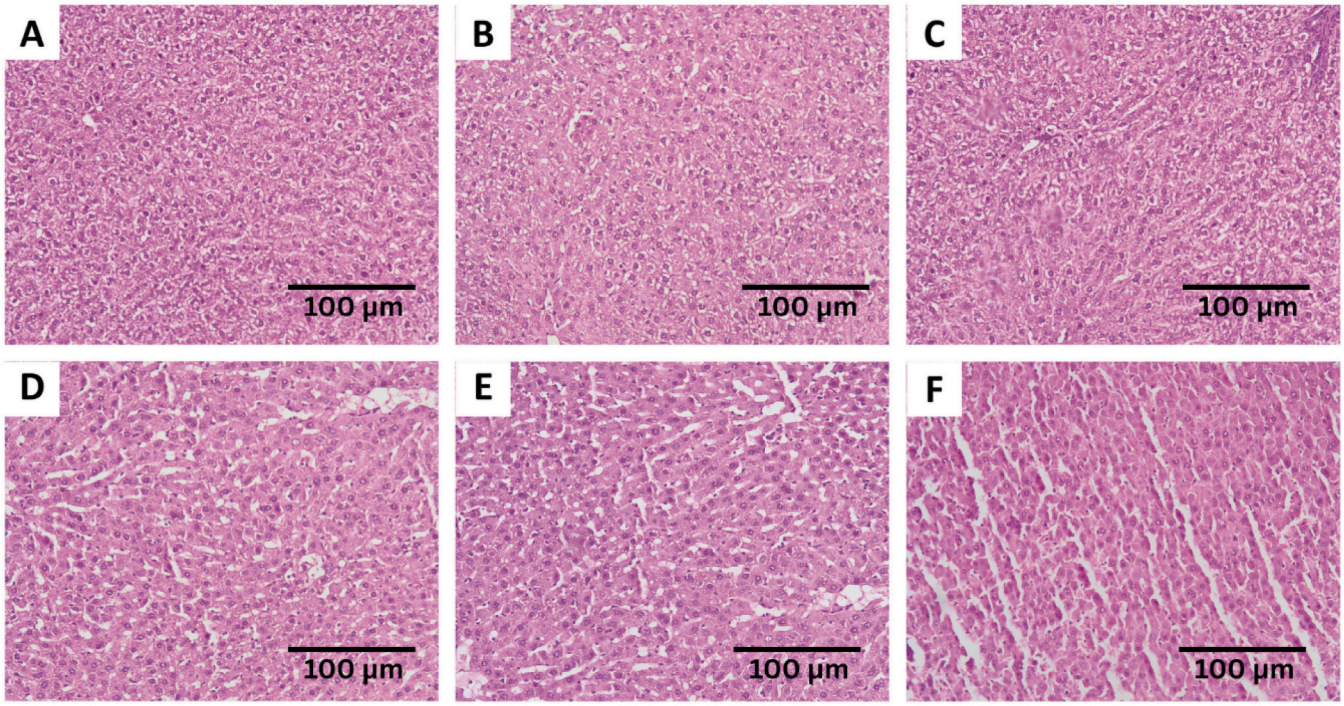

Fig. 3. H&E-stained histopathological sections demonstrating hepatic morphology in control and treated rats (scale bar: 100 µm). A. Normal rat showing normal hepatocytes. B. Alloxan induced diabetic rat exhibiting hepatocellular necrosis. C. Diabetic rat treated with glibenclamide depicting partial restoration of hepatic architecture. D. Diabetic rat that was given 200 mg/kg dose of aqueous extract demonstrating improvement in hepatocyte morphology. E. Diabetic rat that received 400 mg/kg dose of aqueous extract showing regeneration of hepatocyte. F. Diabetic rat that was administered combination of aqueous extract and glibenclamide illustrating significant restoration of hepatic structure.

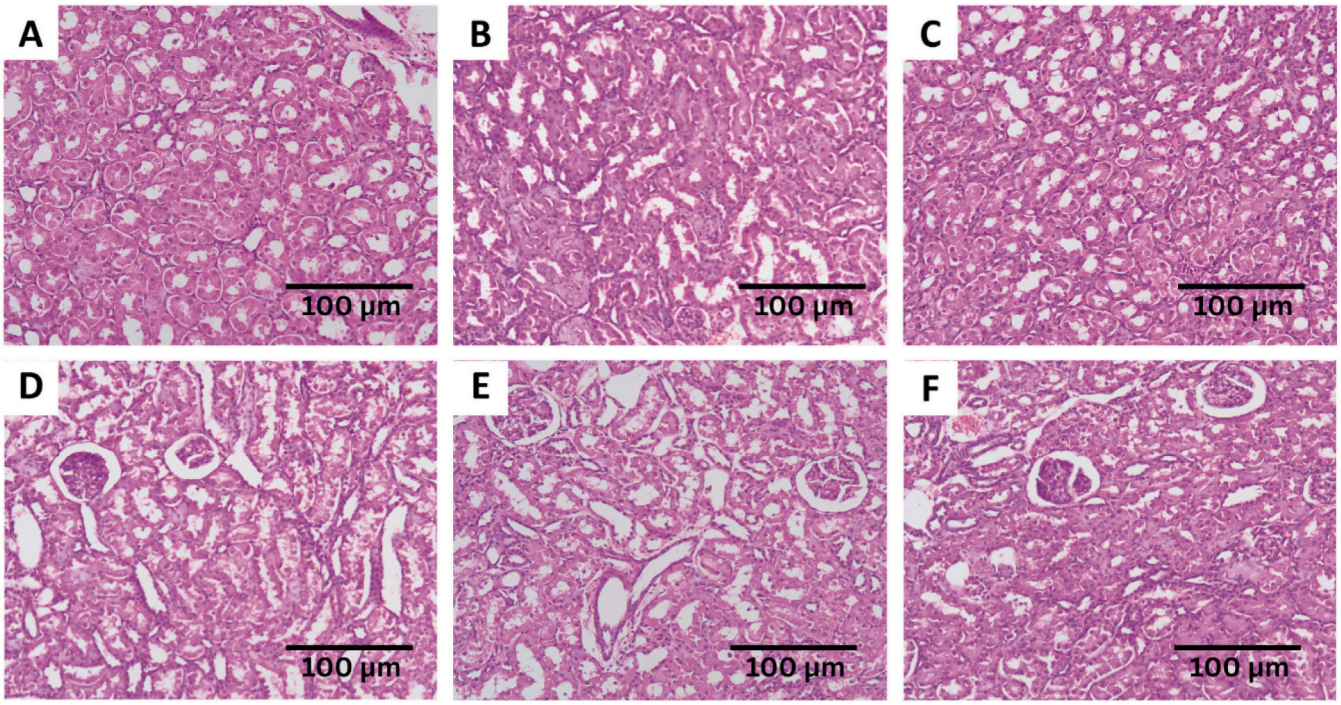

Fig. 4. H&E-stained histopathological sections demonstrating renal morphology in control and treated rats (scale bar: 100 µm). A. Normal rat demonstrating normal structure of glomeruli. B. Alloxan induced diabetic rat showing damaged glomeruli and glomerular sclerosis. C. Diabetic rat treated with glibenclamide depicting improved glomerular structure. D. Diabetic rat that was given 200 mg/kg of aqueous extract illustrating reduction in glomerular hypertrophy. E. Diabetic rat that was administered 400 mg/kg of aqueous extract demonstrating restoration of glomerular architecture. F. Diabetic rat that received combination of glibenclamide and aqueous extract exhibiting regeneration of glomerular morphology.

± 0.21 mmol/L, 8.83 ± 0.17 mmol/L, 8.78 ± 0.13 mmol/L and 8.90 ± 0.05 mmol/L on day 0, 7, 14 and 21, in due order; these values are much higher than normal range. Standard group that was given glibenclamide during the course of treatment, showed decreasing value of blood glucose level over the course of treatment; the values are 8.28 ± 0.16 mmol/L, 7.00 ± 0.07 mmol/L ($p < 0.001$), 6.42 ± 0.15 mmol/L ($p < 0.001$) and 5.91 ± 0.09 mmol/L ($p < 0.001$) on day 0, 7, 14 and 21. But these values are still higher than the normal range. The highest activity was found from aqueous leaf fraction at dosage of 400 mg/kg with FBG levels of 8.89 ± 0.12 mmol/L, 6.99 ± 0.06 mmol/L ($p < 0.001$), 5.98 ± 0.04 mmol/L ($p < 0.001$) and 5.13 ± 0.12 mmol/L ($p < 0.001$) on day 0, 7, 14 and 21. Moreover, aqueous leaf fraction at 200 mg/kg dosage also performed well, exhibiting FBG levels of 8.62 ± 0.25 mmol/L, 7.01 ± 0.07 mmol/L ($p < 0.001$), 6.03 ± 0.04 mmol/L ($p < 0.001$) and 5.42 ± 0.08 mmol/L ($p < 0.001$) on day 0, 7, 14 and 21. Additionally, the combination of aqueous fraction with glibenclamide also showed promising results, with FBG levels of 8.55 ± 0.18 mmol/L, 6.98 ± 0.05 mmol/L ($p < 0.001$), 6.02 ± 0.04 mmol/L ($p < 0.001$) and 5.21 ± 0.07 mmol/L ($p < 0.001$) on day 0, 7, 14 and 21, in due order. It can be seen that, on day 0 the blood glucose level is very high for all the experimental animals regardless of their group; this is because from this day the treatments were started and the measurements were done before the initiation of treatments. After that, gradually the blood glucose level decreased (Table 1). Test Extract and its combination with Glibenclamide produced duration-dependent activity because their effects are closely linked to how long they remain in the body at therapeutically active concentration. Also, a clear dose-dependent increase in anti-diabetic activity can be seen for the test extract. In the following order, the anti-diabetic activity of the test samples decreased:

AEAR 400>AEAR+Glibenclamide> AEAR 200> Standard

Here, AEAR = Aqueous fraction of *Annona reticulata* L.

Table 1
Effect of aqueous extract and its combination with glibenclamide on the FBG level (mmol/L) following 21 days treatment.

Group	Day 0	Day 7	Day 14	Day 21
Normal Control	4.93 ± 0.16	5.03 ± 0.16	4.92 ± 0.06	4.84 ± 0.15
Diabetic Control	8.77 ± 0.21	8.83 ± 0.17	8.78 ± 0.13	8.90 ± 0.05
Standard	8.28 ± 0.16	7.00 ± 0.07***	6.42 ± 0.15***	5.91 ± 0.09***
AEAR 200	8.62 ± 0.25	7.01 ± 0.07***	6.03 ± 0.04***	5.42 ± 0.08***
AEAR 400	8.89 ± 0.12	6.99 ± 0.06***	5.98 ± 0.04***	5.13 ± 0.12***
AEAR+Glibenclamide	8.55 ± 0.18	6.98 ± 0.05***	6.02 ± 0.04***	5.21 ± 0.07***

*$p < 0.05$ (Significance); **$p < 0.01$ (Highly significance); ***$p < 0.001$ (Very highly significance) against control; Standard is Glibenclamide.

Because diabetes is a metabolic disease, the changes in body weight was also closely observed during treatment. To find out if there was an abnormal change in body weight, the measurement of body weight was done at regular intervals [31–33]. On day 0, the normal control group weighed 110.17 ± 1.58 g, and on day 21, their final weight was 144.17 ± 2.08 g. This group demonstrated 30.86 % increase in body weight since they were healthy, while decrease in body weight was found in diabetic rats; their initial weight was 177.17 ± 4.35 g on day 0 and their final weight was 157.17 ± 5.94 g on day 21; thus demonstrated 11.29 % decrease in body weight. Following glibenclamide treatment, the standard group's body weight decreased as well, from 177.33 ± 17.81 g to 155.50 ± 13.61 g (12.31 % reduction). The groups that received aqueous extract and its combination with glibenclamide treatment also showed progressive reduction in body weight over time. The groups that were administered 200 and 400 mg/kg of aqueous fraction showed

initial body weight measurements of 137.50 ± 3.02 g, 157.67 ± 3.07 g, and final body weight measurements of 124.67 ± 2.39 g, 144.00 ± 2.10 g. Therefore, 200 mg/kg dosage of test extract demonstrated 9.33 % reduction in body weight; while 400 mg/kg dosage exhibited 8.67 % decrease in body weight. The aqueous extract and glibenclamide combination showed body weight of 153.50 ± 16.71 g on the first day and resulted in body weight of 144.00 ± 16.63 g on the last day; 6.19 % reduction was observed (Table 2). It is notable that, the body weight reduction percentages were greatly decreased in aqueous leaf extract treated groups in both 200 and 400 mg/kg dosages. However, the greatest decrease in the body weight reduction percentage was observed in the combination group.

As diabetes is a metabolic disorder, and liver is the major organ of metabolism; diabetes can have detrimental effect on liver. For that reason, several liver markers were tested after the treatment period to assess the protective effect of the test samples on liver. So, serum was collected from the blood of test animals after dissection and the serum levels of some important liver markers were measured. The normal ranges of serum ALT, AST and Bilirubin for rats are 6–45 IU/L, 6–30 IU/L and 3–3.2 μmol/L, respectively [40]. ALT, AST and Bilirubin levels for normal control group were 17.31 ± 2.55 IU/L, 10.99 ± 1.05 IU/L and 1.97 ± 0.33 μmol/L. Whereas, diabetic control group demonstrated serum ALT, AST and Bilirubin levels of 24.60 ± 1.80 IU/L, 29.83 ± 1.74 IU/L and 5.44 ± 0.61 μmol/L; these values are much higher than the normal range. In contrast, the standard group that received glibenclamide exhibited good effect on the liver markers with three liver markers values of 13.32 ± 2.43 IU/L ($p < 0.001$), 11.14 ± 1.15 IU/L ($p < 0.001$) and 3.10 ± 0.10 μmol/L ($p < 0.001$). The aqueous extract showed very good control over the liver markers both alone and in combination with glibenclamide. At 400 mg/kg dosage, aqueous extract exhibited ALT, AST and Bilirubin levels of 13.82 ± 2.11 IU/L ($p < 0.001$), 10.94 ± 0.30 IU/L ($p < 0.001$) and 3.07 ± 0.10 μmol/L ($p < 0.001$); in case of 200 mg/kg dosage, it showed levels of three liver markers of 15.60 ± 1.92 IU/L ($p < 0.001$), 10.85 ± 0.68 IU/L ($p < 0.001$) and 3.01 ± 0.63 μmol/L ($p < 0.001$); lastly, in combination with glibenclamide the aqueous extract demonstrated the values of three liver markers of 16.09 ± 2.15 IU/L ($p < 0.001$), 10.92 ± 0.76 IU/L ($p < 0.001$) and 3.15 ± 0.18 μmol/L ($p < 0.001$) (Table 3). Hepatocellular stress brought on by insulin resistance or hyperglycemia frequently results in increased ALT in diabetic rat models. In addition, AST can also increase in early diabetes. In order to determine the intensity of liver damage, a test called the AST/ALT ratio, sometimes called the De Ritis ratio is performed, in which the concentration of aspartate transaminase (AST) is divided by the concentration of alanine transaminase (ALT). A ratio of less than one is considered normal, however a ratio of more than one may indicate chronic liver injury [41]. From Table 3 it is notable that, the rats of normal control group demonstrated AST:ALT ratio value of 0.63 ± 0.06; which increased significantly in the diabetic control group. The rats of diabetic control group showed AST:ALT ratio value of 1.21 ± 0.09; which is higher than 1; that indicates to chronic liver damage. However, the standard group of rats that were treated with glibenclamide exhibited AST:ALT ratio of 0.84 ± 0.07 ($p < 0.001$); that indicates towards the hepatoprotective effect of glibenclamide. Aqueous leaf extract at both 200 and 400 mg/kg of dosage demonstrated significant hepatoprotective potential with observed AST:ALT ratio of 0.71 ± 0.08 ($p < 0.001$) and 0.79 ± 0.06 ($p < 0.001$). Additionally, in combination with glibenclamide aqueous extract showed the highest hepatoprotective property with AST:ALT ratio of 0.69 ± 0.05 ($p < 0.001$). Therefore, the outcomes indicate that these treatments prevented severe liver damage that could happen from diabetes. Hence, it can be said that the extract has the potential to protect liver to certain extent from the damage caused by diabetes.

Here, AEAR = Aqueous extract of *Annona reticulata* L.

The primary role of the kidney is to eliminate waste and excess fluid from the body. Being a metabolic disease, diabetes can damage the kidney in various pathways, in most cases it damages the kidney's blood arteries. So, two key kidney markers were examined following the course of treatment for understanding whether the test sample can have beneficial effect on kidneys even in the presence of diabetes. Rats typically have blood urea nitrogen (BUN) level between 0.4 and 0.8 mg/dL and serum creatinine level between 14 and 22 mg/dL [42]. For normal control group obtained BUN and serum creatinine levels were 13.87 ± 2.57 mg/dL and 0.72 ± 0.09 mg/dL while, for diabetic control group these values were much higher if compared to normal range, which were 32.93 ± 3.74 mg/dL and 1.61 ± 0.35 mg/dL. Both serum creatinine and BUN levels in the glibenclamide-treated standard group were found to be in normal range, measuring 0.73 ± 0.05 mg/dL ($p < 0.001$) and 13.49 ± 3.64 mg/dL ($p < 0.001$). Even when diabetes is present, the aqueous extract can still benefit the kidneys because it demonstrated good control over renal markers when used alone and in combination with glibenclamide. 400 mg/kg of aqueous fraction demonstrated serum creatinine and BUN levels of 0.72 ± 0.08 mg/dL ($p < 0.001$), and 16.29 ± 1.00 mg/dL ($p < 0.001$); while for 200 mg/kg dosage these values were 0.75 ± 0.08 mg/dL ($p < 0.001$), and 15.33 ± 1.14 mg/dL ($p < 0.001$). Furthermore, 0.74 ± 0.06 mg/dL and 17.58 ± 3.02 mg/dL ($p < 0.001$) were obtained serum creatinine and BUN levels when combination of glibenclamide and aqueous extract was given (Table 4).

Here, AEAR = Aqueous extract of *Annona reticulata* L.

Diabetic dyslipidaemia, a term used to describe lipid abnormalities in patients with diabetes, is commonly defined by elevated levels of small dense LDL particles, low HDL, high triglycerides (TG), and high

Table 2
Effect of aqueous extract and its combination with glibenclamide on body weights following 21 days treatment.

Group	Initial BW (g) (Mean±SEM)	Final BW (g) (Mean±SEM)	Percentage (%) of Body Weight Changes
Normal Control	110.17 ± 1.58	144.17 ± 2.08	+ 30.86 %
Diabetic Control	177.17 ± 4.35	157.17 ± 5.94	–11.29 %
Standard	177.33 ± 17.81	155.50 ± 13.61	–12.31 %
AEAR 200	137.50 ± 3.02	124.67 ± 2.39	–9.33 %
AEAR 400	157.67 ± 3.07	144.00 ± 2.10	–8.67 %
AEAR+Glibenclamide	153.50 ± 16.71	144.00 ± 16.63	–6.19 %

Here, AEAR = Aqueous extract of *Annona reticulata* L.
Standard is Glibenclamide; BW means body weight.
(+) sign indicates increase in body weight and (–) sign indicates decrease in body weight.

Table 3
Effect of aqueous extract and its combination with Glibenclamide on ALT, AST and Bilirubin levels following 21 days treatment.

Group	ALT (IU/L)	AST(IU/L)	AST:ALT Ratio	Bilirubin (μmol/L)
Normal Control	17.31 ± 2.55	10.99 ± 1.05	0.63 ± 0.06	1.97 ± 0.33
Diabetic Control	24.60 ± 1.80	29.83 ± 1.74	1.21 ± 0.09	5.44 ± 0.61
Standard	13.32 ± 2.43***	11.14 ± 1.15***	0.84 ± 0.07***	3.10 ± 0.10***
AEAR 200	15.60 ± 1.92***	10.85 ± 0.68***	0.71 ± 0.08***	3.01 ± 0.63***
AEAR 400	13.82 ± 2.11***	10.94 ± 0.30***	0.79 ± 0.06***	3.07 ± 0.10***
AEAR+Glibenclamide	16.09 ± 2.15***	10.92 ± 0.76***	0.69 ± 0.05***	3.15 ± 0.18***

*$p < 0.05$ (Significance); **$p < 0.01$ (Highly significance); ***$p < 0.001$ (Very highly significance) against control; Standard is Glibenclamide.

Table 4
Effect of aqueous extract and its combination with glibenclamide on creatinine and BUN levels following 21 days treatment.

Group	Creatinine (mg/dL)	BUN (mg/dL)
Normal Control	0.72 ± 0.09	13.87 ± 2.57
Diabetic Control	1.61 ± 0.35	32.93 ± 3.74
Standard	0.73 ± 0.05***	13.49 ± 3.64***
AEAR 200	0.75 ± 0.08***	15.33 ± 1.14***
AEAR 400	0.72 ± 0.08***	16.29 ± 1.00***
AEAR+Glibenclamide	0.74 ± 0.06***	17.58 ± 3.02***

*p < 0.05 (Significance); **p < 0.01 (Highly significance); ***p < 0.001 (Very highly significance) against control; Standard is Glibenclamide.

total cholesterol (TC) [43]. So, the overall lipid profile can be affected by diabetes. Therefore, four important markers of lipid profile in serum were evaluated to assess if the test samples can maintain the lipid profile in diabetic rats or not. The normal ranges for serum TC, TG, LDL and HDL are 10–54 mg/dL, 26–145 mg/dL, 40–50 mg/dL and 40–50 mg/dL, respectively [44]. For normal control group, the serum levels of TC, TG, LDL and HDL were 52.67 ± 3.90 mg/dL, 63.75 ± 5.63 mg/dL, 44.33 ± 4.50 mg/dL and 40.45 ± 2.83 mg/dL; while for diabetic control group, the values were 99.82 ± 44.26 mg/dL, 83.72 ± 2.49 mg/dL, 67.50 ± 7.15 mg/dL and 25.22 ± 4.43 mg/dL, in due order. Therefore, for the diabetic control group the values for TC, LDL were found to be above the usual range while, the case for HDL measurement was opposite; so, a clear disruption in the lipid profile can be seen in this group. For glibenclamide treated standard group, the values obtained for TC, TG, LDL and HDL were 74.58 ± 7.95 mg/dL ($p < 0.001$), 60.07 ± 8.04 mg/dL ($p < 0.001$), 40.17 ± 4.62 mg/dL ($p < 0.001$) and 45.25 ± 4.81 mg/dL ($p < 0.001$). However, standard group exhibited almost all the values within the normal range except higher TC value. The aqueous extract produced good impact on the lipid profile at both test doses; with serum levels of 83.43 ± 7.78 mg/dL ($p < 0.001$), 63.37 ± 2.88 mg/dL ($p < 0.001$), 49.50 ± 3.51 mg/dL ($p < 0.001$), 41.60 ± 3.11 mg/dL ($p < 0.001$) and 82.78 ± 2.26 mg/dL ($p < 0.001$), 61.83 ± 1.38 mg/dL ($p < 0.001$), 43.50 ± 2.59 mg/dL ($p < 0.001$), 42.43 ± 3.20 mg/dL ($p < 0.001$); for TC, TG, LDL and HDL, in due order. Additionally, combination of aqueous extract and glibenclamide showed serum levels of 77.53 ± 4.43 mg/dL ($p < 0.001$), 62.50 ± 2.76 mg/dL ($p < 0.001$), 44.33 ± 3.62 mg/dL ($p < 0.001$) and 42.67 ± 2.42 mg/dL ($p < 0.001$) for TC, TG, LDL and HDL, respectively (Table 5).All the groups that received test samples showed all values in the normal range except for TC values which were higher than the normal range. So, the extract from *Annona reticulata* L. and its combination provided promising results (in diabetic rats) in the maintenance of lipid profile.

Here, AEAR = Aqueous extract of *Annona reticulata* L.

Table 5
Effect of aqueous extract and its combination with glibenclamide on lipid profile level following 21 days treatment.

Group	TC (mg/dL)	TG (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)
Normal Control	82.67 ± 3.90	63.75 ± 5.63	44.33 ± 4.50	40.45 ± 2.83
Diabetic Control	99.82 ± 44.26	83.72 ± 2.49	67.50 ± 7.15	25.22 ± 4.43
Standard	74.58 ± 7.95***	60.07 ± 8.04***	40.17 ± 4.62***	45.25 ± 4.81***
AEAR 200	83.43 ± 7.78***	63.37 ± 2.88***	49.50 ± 3.51***	41.60 ± 3.11***
AEAR 400	82.78 ± 2.26***	61.83 ± 1.38***	43.50 ± 2.59***	42.43 ± 3.20***
AEAR+Glibenclamide	77.53 ± 4.43***	62.50 ± 2.76***	44.33 ± 3.62***	42.67 ± 2.42***

*p < 0.05 (Significance); **p < 0.01 (Highly significance); ***p < 0.001 (Very highly significance) against control; Standard is Glibenclamide.

Histopathological evaluation of pancreas, liver and kidney of diabetic rats

Following sacrifice, the pancreas, liver, and kidney tissues were removed from each rat and preserved for 24–48 h in 10 % neutral buffered formalin. Graded ethanol was utilized to dehydrate the fixed tissues, which were then cleaned in xylene and embedded in paraffin wax. A rotary microtome was used to segment paraffin blocks at a thickness of 5 μm. Following standard procedures, three non-consecutive sections of each organ were produced for each animal and stained with hematoxylin and eosin (H&E). Five randomly chosen, non-overlapping fields from each section were analyzed for histological evaluation using a light microscope set to 20 × objective magnification.

Impact of aqueous leaf extract and its combination with glibenclamide on the pancreatic histopathological profile in rats in normal condition, rats that were untreated with alloxan-induced diabetes, and rats that were treated with alloxan-induced diabetes (original magnification ×20). (A) Section of normal control rat's pancreas demonstrates normal islet of Langerhans. In this segment islets are found to be well-defined, with ß-cells, embedded within acinar tissue. (B) The structure of islet of Langerhans is found to be damaged, shrunken and disrupted in the pancreatic region of alloxan-induced diabetic rat. Degeneration and reduction of ß-cells within the islets can be observed. The segment demonstrated swelling and altered morphology of acinar cells. Inflammation can also be seen in the pancreatic tissue, contributing to ß-cell damage. (C) A pancreatic section from diabetic rats given glibenclamide shows an increase in beta cells and pancreatic islet of Langerhans that is nearly normal. The segment demonstrated restoration of normal islet morphology and improvement in the structure in diabetic rats. (D) Pancreatic segment of rat having alloxan-induced diabetes that administered aqueous fraction at 200 mg/kg demonstrating improvement in the morphology of islets and regeneration of pancreatic ß-cells**.** (E) Pancreatic slice obtained from diabetic rat that was given 400 mg/kg of aqueous fraction illustrating better structural features of islet of Langerhans. The outcome indicated, administration of the extract at 400 mg/kg dose provided protection to the islets from degeneration, improvement of cell proliferation and aided in the restoration of pancreatic architecture. (F) Segment of pancreas taken from diabetic rat that was administered combination of aqueous extract and glibenclamide depicting improvement in structure of islet of Langerhans. The segment demonstrated regeneration of islets, restoration of ß-cell population, lessened cytoplasmic damage.

The effects of aqueous leaf fraction and its combination with glibenclamide on the histopathological profiling of the liver in rat that was in normal condition and rats having untreated and treated alloxan-induced diabetes have been studied (magnification ×20). (A) Liver segment of normal rat displays normal hepatocytes with a central vein. The segment showed arrangement of hepatocytes in a regular pattern around the central vein. Also, the section demonstrated clear cytoplasm of hepatocytes, round nuclei which are centrally located. (B) Rat liver section that were given alloxan (no treatment) shows central vein and hepatocytes that are disorganised and destroyed. The segment exhibited hepatocellular necrosis with vacuolated cytoplasm of hepatocytes indicating cellular degeneration. Infiltration of mononuclear inflammatory cell in the portal areas can also be seen. Accumulation of lipid in hepatocytes, sinusoidal dilatation and congestion are also observed. (C) Liver section treated with glibenclamide demonstrates restoration in normal hepatic architecture, reduction in the severity of cellular damage and lipid accumulation. The section also demonstrates mild hepatocyte vacuolization (less than diabetic control), near normal sinusoidal pattern, reduction in necrosis and inflammation. (D) Hepatic segment obtained from diabetic rat that was given 200 mg/kg of aqueous fraction illustrating marked improvement in liver histology, reduced fat accumulation, very low inflammatory infiltration, improved hepatocyte morphology. (E) Liver slice taken from diabetic rat which administered 400 mg/kg of aqueous fraction demonstrating regeneration of hepatocyte, restoration of normal lobular structure, reduction of cellular damage and fatty changes, decrease in inflammatory infiltration, fewer

necrotic cells, reduction in sinusoidal congestion. (F) Hepatic section of diabetic rat that was given combination of aqueous fraction and glibenclamide depicting near normal hepatocytes and hepatic lobules, minimal vacuolization or inflammatory cells, no visible fibrosis or congestion.

Impact of aqueous leaf extract and its combination with Glibenclamide on kidney histopathological profile in rats having normal physiology, untreated alloxan-induced diabetes, and treated alloxan-induced diabetes (original magnification ×20). (A) Kidney slice of normal rat depicts normal structure of glomeruli along with Bowman's capsule and intact capillary loops. Also, Proximal and distal convoluted tubules are found to have distinct, regular lumens and normal epithelial cells. Interstitial spaces are observed to be in normal state with no cellular infiltration. (B) A kidney section of an alloxan-induced diabetic rat (no therapy) shows immune cell infiltration and damaged glomeruli with baseline fat deposition. The section demonstrated thickening of glomerular basement membrane, mesangial expansion, abnormalities in glomerular capillary and glomerular sclerosis. Tubular epithelial cell damage and deposition of eosinophilic material in the tubular interstitium are also found. Infiltration of inflammatory cells, scarring and thickening of the interstitial tissue are also observed. (C) Rat kidney section which was treated with glibenclamide demonstrating preserved tubule and glomerular structure. The section depicted reduction in glomerular hypertrophy and mesangial expansion, decrease in tubular degeneration and vascular wall thickening, no inflammatory infiltration, improvement in capillary perfusion and reduced congestion. (D) Renal segment obtained from diabetic rat that was given 200 mg/kg of aqueous fraction illustrating mild reduction in glomerular hypertrophy, less vacuolar degeneration in tubules, decreased mesangial expansion and mild improvement in vascular congestion. (E) Renal slice taken from diabetic rat that administered 400 mg/kg of aqueous fraction demonstrating restored glomerular architecture, marked decrease in glomerular basement membrane thickening, minimal mesangial expansion, near-normal tubular epithelial structure and no inflammatory cells in interstitium. (F) Diabetic rat's kidney section treated with combination of glibenclamide and aqueous extract demonstrates reduction in glomerular hypertrophy, preservation of glomerular basement membrane thickness, normal architecture of capillary loop and decrease in mesangial expansion. The section also depicts restoration of tubular architecture, reduction in tubular epithelial degeneration, minimal infiltration of inflammatory cells and arteriolar wall thickening.

According to Ighodaro et al., the mechanism by which alloxan causes diabetes is mainly the partial degradation of pancreatic β cells, which reduces the quantity and quality of insulin that these cells produce [45]. The aqueous extract demonstrated good antidiabetic benefits in male rats with alloxan-induced diabetes, both when used alone and in combination with glibenclamide. However, the antidiabetic property of the aqueous extract was not investigated in female rats because only male rats were employed as experimental animals in this study; which is a limitation of our investigation. Furthermore, Cysteine was detected in the aqueous extract, per the GC-MS analytical data. By enhancing insulin sensitivity, decreasing oxidative stress, and possibly influencing insulin secretion, the compound produces significant antidiabetic effects [35,36]. Cysteine may increase the synthesis of glutathione and adiponectin, which would improve the effects of insulin on glucose metabolism in cells such as adipocytes [46]. The potent antioxidant glutathione, which shields cells from damage brought on by reactive oxygen species (ROS), is derived from cysteine. The activity of pancreatic beta-cells may be hampered by elevated oxidative stress, which is frequently linked to type 2 diabetes. In order to combat this, cysteine supplements can strengthen antioxidant defenses [47]. The complicated effects of cysteine on insulin secretion can be altered by a number of variables, such as the presence of hydrogen sulphide or other chemicals [48]. Supplementing with cysteine has been demonstrated to increase insulin sensitivity in diabetic animal models [46]. The antioxidant and anti-inflammatory potentials of cysteine can help guard against a number of diabetes-related complications, including kidney damage, nerve damage, and vascular inflammation. Research has demonstrated that in diabetic animal models, cysteine supplementation can lower levels of inflammatory markers. It is also possible to combine cysteine with other antidiabetic medications, such as metformin. Combining the two may help manage diabetes more effectively by focusing on various pathways [49]. Furthermore, 1,3-dioxolane-4-methanol was present in the aqueous fraction. The presence of this hepatoprotective compound may be the reason for its hepatoprotective effectiveness both alone and when combined with glibenclamide [37]. 1,3-Dioxolane-4-methanol's hepatoprotective action is probably caused by its capacity to lessen inflammation and oxidative stress in the liver, as well as its capacity to shield liver cells from harm. In particular, it can assist in lowering excessive levels of liver enzymes such as ALT, AST, and ALP that are indicative of liver damage. By boosting glutathione and decreasing lipid peroxidation, it may help raise the liver's antioxidant levels. The compound 1,3-dioxolane-4-methanol has antioxidant properties. It has the ability to scavenge free radicals and lessen oxidative stress, which is a primary cause of liver damage. To further shield liver cells, it can prevent lipid peroxidation, a process in which free radicals break down fats. Additionally, it may raise the liver's levels of glutathione, an essential antioxidant that aids in the liver's ability to neutralize toxic substances. Moreover, 1,3-dioxolane-4-methanol can lessen liver inflammation by shielding liver cells from oxidative stress. Through the reduction of inflammation and oxidative stress, 1,3-dioxolane-4-methanol can support the self-healing and normal function of liver cells. To further protect liver tissue, it might also aid in halting the liver cells' apoptosis [50–52]. In addition, GC-MS analysis revealed that the aqueous extract contained cysteine. The extract may have kidney-protective effect because of the substance's renal-protective activity [35]. Due to its antioxidant qualities, capacity to promote glutathione synthesis, impact on mitochondrial function and redox balance, activation of Nrf2, and general modulation of signalling pathways involved in kidney cell survival and function, cysteine has a variety of protective effects on the kidneys [53]. An essential constituent of glutathione (GSH), a potent antioxidant that combats dangerous free radicals and reactive oxygen species (ROS), is cysteine. Furthermore, cysteine directly scavenges free radicals, shielding kidney cells from the oxidative harm that these reactive molecules might cause. Moreover, oxidative stress plays a significant role in kidney injury. The antioxidant potentials of cysteine aid in lowering oxidative stress and preventing or lessening renal damage [54,55]. Additionally, cysteine can safeguard mitochondrial function, which is frequently altered in kidney illness. For mitochondria to function properly, the correct balance of oxidation and reduction (redox state) must be maintained, and cysteine aids in this maintenance. Thus, cysteine aids in preventing mitochondrial damage, a major contributing factor to kidney cell death, by maintaining mitochondrial activity and redox balance [56]. Nrf2 is a transcription factor that is essential for cellular defense against inflammation and oxidative stress and can be activated by cysteine. Kidney disease is frequently caused by inflammation, which cysteine can help lessen by affecting Nrf2 and other pathways [57]. Furthermore, cysteine has the ability to affect a number of signaling pathways that are important for kidney cell survival and function, which may improve kidney health. Cysteine can also lessen the harm that results from ischemia-reperfusion injury, which is when blood flow to the kidneys is diminished and then restored (Jo, 2011). In addition, cysteine can prevent kidney damage brought on by a variety of toxins and medications [58]. As cysteine was present in the aqueous extract and this chemical possesses hypolipidemic potential, it may assist regulate lipids and cholesterol in diabetic rats [35]. Mostly because of its antioxidant potentials and function in glutathione formation, cysteine can help decrease cholesterol. An antioxidant that contains cysteine, glutathione, aids in lowering lipid oxidation, and cysteine itself can scavenge free radicals directly and prevent the oxidation of LDL cholesterol. Cysteine also affects triglyceride levels, fat mass, and food consumption, all of which have an impact on lipid

metabolism [59,60]. Some researchers have investigated and confirmed that extracts from *Annona reticulata* L. contain antidiabetic potentials [61–63]. Additionally, some researches evaluated the hypolipidemic potential of a number of *Annona reticulata* L. extracts [61,62]. Outcomes obtained from current investigation consistent with those of other researchers. This study, however, evaluated for the first time the protective roles of aqueous leaf fraction from *Annona reticulata* L. on kidney and liver.

Conclusion

Rats with diabetes treated with *Annona reticulata* L. leaf extract alone and in combination with glibenclamide had notable antidiabetic effects in addition to liver-renal protective effects. Additionally, it was discovered that these treatments effectively maintained the lipid profile in diabetic rats. Thus, the study's findings point to the plant extract from *Annona reticulata* L. having anti-diabetic, anti-hyperlipidemic, and liver-renal protective properties. According to the GC-MS analysis report, several compounds having considerable antidiabetic, liver-renal protecting, and anti-hyperlipidemic activities were identified in the extract. For aforementioned reasons, aqueous leaf fraction from *Annona reticulata* L. could serve as an option to treat diabetes. Furthermore, separating the bioactive substances from the extract and testing them in both in vitro and in vivo investigations could aid in clarifying the mode of actions that produced various bioactivities and could open the door to a firm conclusion regarding the present results.

CRediT authorship contribution statement

Tasnia Binte Bari Kabbo: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Pritesh Ranjan Dash:** Writing – review & editing, Supervision, Formal analysis, Data curation. **Md. Sohel Rana:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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