doi.org/10.36721/PJPS.2026.39.8.229.1

Aqueous-ethanol extract of *Acacia ampliceps* Maslin mitigates hyperglycemia and oxidative stress in streptozotocin-induced diabetic rats

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Abstract: **Background**: Diabetes mellitus is a major global health issue with a significant and increasing prevalence. Despite advancements in diabetes mellitus treatment, its occurrence and associated mortality rates remain high. **Objectives**: This study investigated the potential antidiabetic effects of Acacia ampliceps Maslin and validated its traditional use in folk medicine. **Methods**: Streptozotocin was used to induce type 2 diabetes mellitus in male Sprague-Dawley rats. After diabetes induction, oral doses of Acacia ampliceps leaf extract at various concentrations, such as 125 mg/kg, 250 mg/kg and 500 mg/kg, as well as glibenclamide, were administered for a duration of 35 days. Subsequently, antioxidant and histopathological assessments, blood samples and tissue specimens were taken. **Results**: EAA underwent a series of in-vitro analyses, including phytochemical screening, antioxidant activity testing and evaluation of its inhibitory effects on α-amylase. It is well known that acarbose inhibits α-amylase, thereby slowing the breakdown of carbohydrates and leading to a subsequent decline in blood glucose levels. EAA demonstrated α-amylase inhibitory activity, but its potency was lower than that of acarbose (IC_{50}: 53.15 vs 11.98 µg/mL). EAA decreased malondialdehyde levels while superoxide dismutase, glutathione and catalase levels increased. During the gas chromatography-mass spectrometry analysis of EAA, potential antidiabetic phytoconstituents were also identified. **Conclusion**: The results indicate that EAA contains pharmacologically active compounds with antioxidant and α-amylase-inhibitory properties, making it effective in treating STZ-induced T2DM.

Keywords: *Acacia ampliceps* extract; Catalase; Diabetes mellitus; DPPH; Gas chromatography-mass spectrometry; Glutathione; Malondialdehyde; Oral glucose tolerance test; Streptozotocin; Superoxide dismutase; Total phenols contents; Type 2 diabetes mellitus; Total flavonoids contents

Submitted on 12-03-2025 – Revised on 11-10-2025 – Accepted on 19-10-2025

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder marked by sustained elevation in blood sugar levels (Balaji *et al*., 2019; Kumar *et al*., 2020; Mukhtar *et al*., 2020). It can result from various factors, including decreased insulin production and insulin resistance (Marusic *et al*., 2021; Ohishi, 2018). In 2015, the International Diabetes Federation (IDF) reported that around 415 million people aged 20 to 79 were affected by DM (Cho *et al*., 2018; Zheng *et al*., 2018; Saeedi *et al*., 2019). Prolonged elevated blood sugar, coupled with other metabolic imbalances, can cause harm to multiple organs, resulting in severe and potentially fatal health complications (Zhou *et al*., 2021). Among these complications, microvascular and macrovascular adversities are the most notable (Holt *et al*., 2021). They significantly elevate the risk of cardiovascular diseases, with estimates suggesting a two to four-fold increase (Cole and Florez, 2020).

Various classes of oral hypoglycemic drugs are utilized to manage diabetes. These classes include biguanides, sulfonylureas, glucosidase inhibitors, non-sulfonylurea secretagogues and thiazolidinediones each offering distinct ways to manage hyperglycemia (Balkhi *et al.,* 2019; Brunerova *et al.,* 2018; Li and Yang, 2019; Padhi *et al.,* 2020; Sekar *et al.,* 2019). Despite the progress in oral antidiabetic medications, challenges persist (Muller *et al.,* 2022; Vinogradov *et al.,* 2019). Drugs of these classes may lead to drawbacks such as drug resistance, adverse reactions and harmfulness. For example, sulfonylureas forfeit efficiency in a significant percentage of patients over time and these medications cannot effectively manage hyperlipidemia (Kalra and Khandewal, 2018; Lupoli *et al.,* 2020; Napoli *et al.,* 2020). As a result, the pursuit of novel antidiabetic therapies, particularly those derived from natural sources, continues to address these limitations. This is why the search for more advanced antidiabetic therapies derived from natural sources continues (Admassu *et al.,* 2018; Alam *et al.,* 2018).

Numerous naturally occurring chemical compounds and substances are present in plants, collectively known as

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phytochemicals. Some of these phytochemicals have been associated with potential hypoglycemic effects (Ardalani *et al.,* 2021; Gandhi *et al.,* 2020; Aba and Asuzu, 2018; Ramirez-Alarcon *et al.,* 2021; Saleem *et al.,* 2019). Traditional herbal remedies known for their potent anti-diabetic properties offer a promising avenue without the burden of adverse effects. These botanical sources harbor a wealth of bioactive compounds, including flavonoids, alkaloids, phenols and tannins which prepare a multifaceted approach to enhance the functionality of pancreatic beta cells (Balkrishna *et al.,* 2021; Garg *et al.,* 2023; Guo *et al.,* 2020; Liu and Chen, 2024; Ullah *et al.,* 2023; Yang *et al.,* 2023).

This enhancement is achieved by either upregulating insulin production or attenuating glucose absorption in the intestinal tract (Akhter and Barabutis, 2021). Various plants showed the positive results for diabetes such as *Aegle marmelos* (Manandhar *et al.,* 2018), *C. sativum* (Wei *et al.,* 2019), *Zingiber officinale* (Akullo *et al.,* 2022), *Syzygium cumini* (Chhikara *et al.,* 2018), *Gymnema sylvestre* (Sekar *et al.,* 2019), *Phyllanthus emblica* (Ahmad *et al*., 2021), *Cinnamomum verum* (Rashmi and Himanshu, 2021), *Momordica charantia* (Samadov, 2022), *Ocimum tenuiflorum* (Piras *et al.,* 2018), *Coccinia grandis* (Senthamaraikannan and Kathiresan, 2018). In this study, Acacia *ampliceps* was investigated as a potential therapeutic option for managing diabetes and its antioxidant properties were validated. Moreover, i*n-vitro* alpha-amylase inhibitory activity was examined. Furthermore, Gas Chromatography was used to confirm the existence of glycosides, flavonoids and phenolic compounds. An acute toxicity study, along with biochemical analysis and histological examination, was conducted to determine its safety profile.

MATERIALS AND METHODS

Materials

The reagents, such as methanol, ascorbic acid, DPPH and ethanol, were procured from Sigma Aldrich (Germany). The finest analytical grade chemicals and reagents were used in this research. Additionally, freshly prepared deionized water obtained from the research laboratory at the University of Lahore was used *Acacia. ampliceps* leaves were gathered from plants in March from the region of Bahawalpur, Punjab, Pakistan. Professor Dr. Zaheer-ul-din, Department of Botany, Government College University, Lahore, certified the taxonomic identity of the plant to confirm its legitimacy. For future use, a voucher specimen designated GC Herb Bot 3445 was placed in the Department of Botany's herbarium. Subsequently, the freshly harvested plant material was shade-dried at room temperature (25-27°C). Once dried, it was finely powdered, accurately weighed and securely stored in airtight containers.

Methods

Preparation of an aqueous ethanol extract from plant leaves

Fresh leaves (a maximum of 5kg) were collected and ground into a fine powder, yielding 2 kg. The process began by immersing the plant powder obtained after grinding into an amber glass bottle. To this, a solvent mixture consisting of ethanol (90%) and deionized water (10%) was added in sufficient quantity to completely saturate the plant material. Subsequently, one liter of the same solvent mixture was added in the same ratio. This mixture was allowed to macerate for 7 days and then filtered three times. The filtrate was collected and the residual plant material (marc) was preserved after the maceration process. A rotary evaporator (RV 10B S99, IKA) at 40°C was used to concentrate the resulting filtrate until a stable weight was achieved. The final filtrate was then collected and stored in a desiccator. Following this, the sample was defatted using ethanol. The ethanolic extract was transferred to a petri dish, its weight was measured and the yield was calculated. This defatted ethanolic extract was kept at 4°C for examination.

Qualitative phytochemical evaluation

Total phenolic content

The total phenolic content (TPC) in various EAA extracts was quantified using the outlined methodology. (Awah *et al*., 2012), with minor adjustments. In particular, distilled water (9 mL) was integrated into aliquots of the plant extract (1 mL). After that, the mixture was rapidly stirred for 5 minutes while containing 1 mL of the Folin-Ciocalteu (FC) reagent. Then, 10 mL of a 7% sodium carbonate solution was mixed with the combination and the total volume was adjusted with additional distilled water to make the volume 25 mL. The same procedural steps were applied to standard gallic acid solutions at concentrations of 10, 20, 40, 80 and 100 µg/mL as well as blank solution where the plant extract was replaced with distilled water. All these solutions, comprising samples, standards and blanks, were allowed to incubate in a darkened environment at room temperature for a period of 1.5 hours. A UV-visible spectrophotometer (Shimadzu, Japan) was used to measure the absorbance of each solution at 750 nm after incubation. Following that, a calibration curve prepared using standard gallic acid solutions was used to quantify phenolic compound levels in the plant extract. The results were expressed in milligrams of gallic acid per gram of plant extract, indicating the total phenolic content (Khan *et al*., 2021).

Total flavonoid content

Several modifications of the aluminum chloride colorimetric method were adopted to determine the total flavonoid content (TFC) in EAA. A stock solution was prepared by dissolving 4 mg of the plant extract precisely in 4 mL of methanol. 200 µL was taken from the stock solutions for each extract and combined with 100 µL of

each solution containing aluminum chloride and 1 M potassium acetate. 4.6 mL of distilled water was added to dilute the resulting mixture. The same procedure was used to create serial dilutions of standard quercetin, which was originally synthesized at a 1 mg/mL concentration and diluted to achieve concentrations of 10, 20, 40, 80 and 100 µg/mL. Both quercetin standard and sample solutions were incubated for 30 minutes at room temperature. After the incubation period, the absorbance of all solutions was measured at 415 nm using a UV-visible spectrophotometer. The quercetin calibration curve was utilized to determine the flavonoid concentration. The findings were mentioned as mg of quercetin per gram of extract, indicating quercetin equivalent (Zafar *et al*., 2021).

In-vitro antioxidant activity using DPPH free radical scavenging method

The potential of EAA for free radical scavenging was assessed using the DPPH assay, with ascorbic acid as the standard (Batool *et al*., 2019). To prepare a stock solution, 4 mg of DPPH was dissolved in 100 mL of ethanol. After stirring, the mixture was left to stand at room temperature for 30 minutes in complete darkness. A standard ascorbic acid solution was used as the positive control, whereas a combination of 2 mL of DPPH solution, 1 mL of EAA and 1 mL of methanol at different concentrations (0, 20, 40, 60, 80, 100 µg/mL) was subsequently incubated for 30 minutes. At 517 nm, the absorbance of each solution was measured against a blank methanol sample. By using the following equation, the plant extract's antioxidant capacity was determined as a percentage of DPPH inhibition:

$$\text{Percentage Inhibition} = \frac{(A_0 - A_1)}{A_0} \text{ X } 10 \text{ (Formula 1)}$$

Where:

A_0 is a representation of the negative control's absorbance.

A_1 stands for the absorbance of the sample or the standard.

Comparing the inhibition rate with extract concentrations allowed researchers to calculate the IC_{50}.

Phosphomolybdate assay

The total antioxidant capacity of EAA was determined using the phosphomolybdate method, with ascorbic acid as the reference standard. To conduct this assessment, 3 mL of a freshly prepared phosphomolybdate reagent (comprising sodium phosphate at 28 mM and sulfuric acid at 0.6 M) was combined with ammonium molybdate at 4 mM and added to 300 microliters of EAA. The mixture was heated at 95°C and incubated for 90 minutes. Following the incubation period, the absorbance was measured using a UV spectrophotometer set at 765 nm abb (Batool *et al.,* 2019). The aforementioned formula was used to identify antioxidant capacity and represented as picograms of ascorbic acid equivalents per milliliter.

Nitric oxide scavenging assay

Using a nitric oxide scavenging experiment and ascorbic acid used as a reference standard, the antioxidant capacity of EAA was assured. In this test, 100 mL of 10 mM sodium nitroprusside and 100 µL of EAA were mixed and incubated for 3 hours. After incubation, we determined the antioxidant capacity as ascorbic acid equivalents per milliliter using a UV spectrophotometer to measure absorbance at 546 nm. Utilizing the previously mentioned formula, the percentage suppression of nitric oxide radical production was calculated. (Khan *et al*., 2020).

In-vitro α-amylase inhibitory assay

Acarbose was used as the reference. The α-amylase inhibitory assay was performed to assess the hypoglycemic activity of EAA. 1 mL of α-amylase solution, 2 mL of 0.02 M phosphate buffer and 1 mL of various EAA solutions (varying from 30.00 to 1000 µg/mL) were mixed. The solution mixture was incubated for 30 minutes at 37°C. After the initial 15 minutes of incubation, 1 mL of a 0.5 percent starch solution was added to the mixture. 1 mL of 3,5-dinitrosalicylic acid reagent was introduced and the mixture was subsequently heated in a water bath at 90°C for 8 minutes. The absorbance of the resulting solution was measured at 540 nm using a UV spectrophotometer. The α-amylase activity was calculated with the previously mentioned formula. This assay provided insights into the potential α-amylase inhibitory activity of EAA with acarbose serving as the standard for comparison (Bahman Nickavara and Gholamreza Amin, 2010).

GC-MS analysis of EAA

The analysis was conducted applying the Agilent Technologies 6890 N network GC system, coupled with an Agilent Series 5975 Inert XL mass selective detector and a 7683 B series Agilent Series injector was used to analyze EAA utilizing GC-MS. A DB35-ms 122-3832 column was used to find plant constituents. By comparing mass spectra at specific retention times with library data, it was possible to identify different EAA components. This enabled us to identify the chemical components of the material (Yadegari *et al*., 2019).

Experimental animal

A total of 30 eight-week-old male Sprague-Dawley rats (180 to 220g) were obtained from the Post Graduate Medical Institute's (PGMI) animal housing facility and kept in a pathogen-free environment. The animals were randomly divided into six groups, with five rats in each group. The rats were housed in large polyacrylic cages labeled with their respective group names. The housing facility's environmental controls maintained an average temperature range of 20°C to 30°C and a humidity level of 60 to 70%. The rats were kept on a 12-hour light-dark cycle and exposed to temperatures ranging from 16°C to 25°C. All rats were given unrestricted access to a standard diet and ad libitum. This diet had the following nutritional composition: 58% carbohydrates, 24% protein, 5.7% fat, 6% fiber and 8% ash. Furthermore, the rats had continuous access to clean water throughout the entire study period.

The Institute Animal Ethics Committee of the University of Lahore approved and oversaw this study (IREC-2018-46), which was conducted in accordance with high ethical standards (Saleem *et al.,* 2019). All experiments involving animals adhered to the animal welfare standards outlined by the Organization for Economic Cooperation and Development.

Acute toxicity study

The rats were randomly divided into five groups, each with six rats. (n = 6). Prior to the experiment, the rats underwent an overnight fast while having unlimited access to water. Following the fasting period, EAA was orally administered to the rats through gastric gavage at various concentrations, including 100, 500, 2000 and 5000 mg/kg. Then, over the course of a 24-hour period, the animals were carefully monitored for any alterations in behavior or indications of toxicity. Following the observation period, the rats that received doses of 2000 and 5000 mg/kg were euthanized, and their liver, kidney and pancreas tissues were carefully isolated for subsequent histopathological analysis. This enabled a comprehensive evaluation of potential histological abnormalities in these vital organs (Kpemissi *et al.,* 2020).

Oral glucose tolerance test

Five groups of rats were formed with each group (n = 5) from the overall rat population. Group I was designated as the negative control and received saline, Group II was labeled as the positive control and received 100 mg/kg of glibenclamide, Group III received 125 mg/kg of EAA orally, Group IV received 250 mg/kg of EAA, and Group V received 500 mg/kg of EAA orally. Five minutes after the rats received each treatment, they were administered of 2 g/kg glucose load. Following the administration of glucose, blood samples were taken at predetermined intervals i.e., 0, 30, 60, 120 and 180 minutes from the tail vein. An On-Call EZ II glucometer (ACON Lab Inc., US) was used to measure blood sugar levels at these predetermined times. To calculate the area under the curve (AUC), use equation (2) for blood glucose levels, aiming to assess the hypoglycemic potential of EAA. This measurement was allowed for comprehensive assessment of the effects of EAA on blood glucose regulation over the specified time period (Khan *et al.,* 2020).

$$\text{Percentage hyperglycemia Inhibition} = \frac{(AUC_0 - AUC_1)}{AUC_0} \; X\; 100$$

Where AUC0 denotes the control group and AUC1 represents the treatment group.

Evaluation of anti-diabetic activity of EAA

Induction of type II diabetes

T2DM was developed in male Sprague-Dawley rats following a 12-hour fast. A dose of 55 mg/kg STZ was dissolved in a freshly prepared 0.5 mL solution of 0.01M citrate buffer (pH 4.5) and was given as the first dosage of this induction after nicotinamide (110 mg/kg) was given a single dose in 0.5 mL of normal saline. Post-STZ administration, the rats were provided with a 5% dextrose solution overnight to prevent hypoglycemic shock. Blood glucose levels were measured using a glucometer. Rats were categorized as diabetic and included in the trial if their blood sugar levels exceeded 250 mg/dL within 72 hours of STZ treatment (Sahlan *et al.,* 2019).

Design experimental

The experimental animals were randomly divided into six distinct groups (Table 1), with each group consisting five rats (n = 5).

Antihyperglycemic activity

On days 0, 7, 14, 21, 28 and 35 after induction of T2DM, fasting blood glucose levels were measured. On these specified days, the consumption of food and water intake by the rats was documented. The animals were humanely euthanized on the 35th day and samples of blood and tissue were taken for further analysis (Khan *et al.,* 2020).

Antioxidant assays

The experimental animals' pancreas, kidney and liver were assessed for oxidative stress markers using the following techniques (Giribabu *et al.,* 2020).

Tissue preparation

At a 1:10 (w/v) ratio, tissues were suspended in phosphate-buffered saline (pH 7.4). The supernatants were collected from resultant homogenates by centrifugation (Jeto tech, Korea) for 15 minutes at 6000 rpm for additional analysis, which included measuring the antioxidant level of SOD, GSH, MDA, CAT and other compounds.

Malondialdehyde (MDA)

By employing the double-heating procedure, the MDA concentration was determined. A centrifuge tube containing 0.5 mL of supernatant and 2.5 mL of 10% trichloroacetic acid (TCA) was filled with the mixture, heated for 15 minutes, and then centrifuged at 1000 rpm for 10 minutes. Next, a 15-minute boil in a water bath was performed with 1 mL of 0.67% thiobarbituric acid (TBA) solution, to which 2 mL of supernatant from each tube was added. The formula below was used to calculate the MDA amount present in the tissue samples and its absorbance was recorded at 532 nm.

Concentration of MDA = OD532 x 100 x TV / (molar extinction coefficient) x dWt x AV

dWt represents the dissected tissue weight; AV is the volume of the aliquot and TV is the solution volume.

Glutathione (GSH)

1 mL of homogenized tissue and 1 mL of TCA (10%) were combined to test GSH levels. To measure absorbance at 412 nm, the mixture was combined with DNTB reagent (0.5 mL), and after precipitation, phosphate buffer (4 mL) was added. The results were estimated as follows: nm of reduced GSH / mg protein.

GSH level = Y - 0.00314 / 0.0314 x DF / T x VU
Where, Y is the value of the tissue homogenate tissue absorbance, DF stands for the dilution factor, T is the homogenized tissue and VU is an aliquot volume.

Catalase (CAT)
1 ml of homogenized tissue and hydrogen peroxide were mixed with 1.95ml of 50 mM phosphate buffer (pH 7.0) to determine the CAT activity. CAT activity was quantified as the unit/mg of proteins at a wavelength of 240 nm. For the CAT activity, the following formula was used:

CAT activity = S x 6.0.D / E x vol. of sample (mL) x mg of protein
Where E is the extinction coefficient (0.071 mmol cm^{-1}), 6.0.D is the change in absorbance per minute, and protein levels were determined with the Lowry method by a standard curve derived from various BSA concentrations.

Superoxide dismutase (SOD)
The xanthine oxidase method was utilized to determine the total SOD activity in the tissue homogenate, and kits were recommended. The protocol was followed. These tests enabled the demonstration of oxidative stress indicators in targeted tissues.

The following formula was used to measure SOD activity:
SOD = A0- A1⁄ A0 + 50% × system volume/sample volume × dilution factor
A0 is the absorbance in the absence of SOD.
A1 is the absorbance in the presence of SOD.
One unit of enzymatic activity is defined as 50% inhibition in the system. One unit of SOD activity is defined as A1 = 0.5 A0.

Biochemical examination
Blood samples were obtained and centrifuged for 15 minutes at 1500 rpm to perform biochemical analysis. Serum was separated using this technique and various parameters were measured, such as total cholesterol (TC), triglycerides (TG), high-density lipoproteins (HDL), low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL), as well as aspartate aminotransferase (AST), alkaline phosphatase (ALP) and Alanine aminotransferase (ALT). In this investigation, standard kits were employed, and measurements were taken in Hamburg, Germany, using a Roche Cobas C311 chemical analyzer (Al Khazraji *et al.,* 2020).

Histopathology
Tissue samples were initially prepared for histopathology procedures. These samples underwent several procedures, including washing, alcohol dehydration, xylene cleaning and final embedding in paraffin blocks. Then, 4-5 mm slices were painstakingly cut by using a rotary microtome. Hematoxylin and eosin were used to stain these sections, allowing for histological analysis. A light microscope was used for histological analysis to detect potential indicators of toxicity. The fact that the pathologist overseeing this evaluation was unaware of the treatment groups is notable. The severity of tissue damage was evaluated using a standardized scoring system with grades ranging from 0 to 4. These grades were assigned based on specific criteria that encompass various factors. These included inflammatory response, glomerular thickness and tubular degradation in kidney injury. In the case of hepatic alterations, the criteria considered were inflammation, necrosis, fibrosis and steatosis. Lastly, for pancreatic injuries, the assessment criteria encompassed inflammation, acinar cell degeneration and β-cell degeneration (Sekar *et al.,* 2019). The grading system was as follows: <1% = 0; 1-25% = 1; 26-50% = 2; 51-75% = 3; and >75% = 4.

Statistical analysis
Findings of the statistical study was expressed as mean ± SD (standard deviation). GraphPad Prism 6.0 software was used to conduct Tukey's multiple-comparison tests and a one-way analysis of variance (ANOVA) on the collected data. Preset thresholds for statistical significance were set at * ≤ 0.05, ** ≤ 0.01 and *** ≤ 0.001.

RESULTS

Phytochemical analysis and percentage yield of EAA
EAA had a yield of about 3.82%. Phytochemical examination indicated the presence of important bioactive components, including proteins, fixed oils, carbohydrates, phenolic compounds, flavonoids, cardiac glycosides and saponins.

Total phenolic content
Using varying amounts of gallic acid from a stock solution of 100 ug/ml, a calibration curve was created to quantify the phenolic content of EAA. Using the formula y = 0.0021x + 0.0553, R2 = 0.9812, the concentration of the phenolic compounds was determined and represented in micrograms of gallic acid equivalent (ug/g). It was discovered that EAA's TPC was 168.56 mg/g.

Total flavonoid content
Concentration of quercetin, a well-known flavonoid of the flavonol family, was used in varied amounts to build the flavonoid calibration curve. The extract's TFC was given as milligrams equivalent per gram of sample. Using the linear equation y = 0.004x-0.037 and an R2 of 0.973, the AlCl3 technique was used to calculate the extract's TFC, which came out to be around 103.41 mg/g.

EAA exhibited robust in-vitro antioxidant activity
Analysis showed that throughout the concentration range of 20 to 100 µg/mL of EAA, the scavenging abilities against phosphomolybdate, DPPH and nitric oxide consistently increase. EAA's IC_{50} in the DPPH assay was

roughly 26.13 g/mL, which was quite similar to the IC_{50} of the reference ascorbic acid (IC_{50} = 26.52 g/mL). The IC_{50} values for EAA were also 36.05 g/mL and 52.13 g/mL in the phosphomolybdate and nitric oxide tests, respectively. Notably, these outcomes closely matched those of ascorbic acid (Fig. 1).

EAA inhibited the α-amylase enzyme

Similar to EAA, acarbose also produced a clear, concentration-dependent inhibition of α-amylase activity (Table 2). The IC_{50} values were 11.98 µg/mL for acarbose and 53.15 µg/mL for EAA, indicating that although the extract was less potent, it exhibited a comparable inhibitory profile. Acarbose is well recognized for delaying carbohydrate breakdown by inhibiting glycosidase and α-amylase, thereby lowering postprandial glucose levels. In contrast, EAA required a higher concentration to achieve similar effects, yet its consistent inhibition across replicates (with very low variability, ±0.001–0.003) supports the reliability of these IC_{50} estimates. Taken together, these *in-vitro* findings suggest that EAA, though weaker than acarbose, still contains bioactive constituents with promising antidiabetic potential.

Acute toxicity study

Rats receiving dosages of up to 5000 mg/kg of EAA therapy did not have any cases of fatality throughout the acute toxicity investigation. It's important to note that the highest dose of EAA, 5000 mg/kg, was the only one at which acinar and β-cell degeneration and substantial inflammation were specifically seen. Similar to the negative control (NC) group, the liver samples from the EAA (5000 mg/kg)-treated groups showed modest inflammation and fibrosis. Unlike the control group (NC), kidney samples from the subjects treated with EAA exhibited normal renal features, including the renal capsule, nephrons and tubules (Fig. 2).

Oral glucose tolerance assay

Normoglycemic rats underwent an oral glucose tolerance test, and glucose concentrations were plotted against time to calculate the area under the curve (AUC). To determine the hyperglycemic inhibition rate, mean peak glucose levels (Cmax) for each experimental group were compared with those of the control group. It's interesting to note that for every measured EAA concentration, the glucose AUC and Cmax dropped considerably. This decline was nearly identical to that of glibenclamide, with fig. 3 showing the maximum effect at 500 mg/kg.

STZ-induced diabetic rats with antidiabetic activity

Compared with NC, STZ injection significantly increased blood glucose levels ($p < 0.001$). Conversely, STZ-induced increases in blood glucose levels decrease in a dose-dependent manner with increasing amounts of EAA. The injection of 500 mg/kg EAA reduced the blood glucose level to 140 mg/dL by day 35, similar to the 125 mg/dL observed in the positive control (PC) group (Fig. 4).

EAA treatment reduced MDA and increased GSH, CAT and SOD levels, showing its potential as an antioxidant

In comparison to NC (kidney: 0.9119 ± 0.005, liver: 0.8121 ± 0.0013) and pancreatic: 0.152 ± 0.003), the study indicated that DC considerably ($p < 0.05$, $p < 0.01$) increased MDA concentrations in the kidney (1.663 ± 0.004), liver (1.349 ± 0.003) and pancreas (0.323 ± 0.002). A reduction in STZ-induced MDA levels that is dose-dependent was seen with EAA treatment, peaking at 500 mg/kg (Fig. 5). Furthermore, the DC group demonstrated a notable ($p < 0.05$, $p < 0.01$) induce GSH levels in the kidney (31.37± 0.931), liver (41.37± 0.929) and pancreas (21.37± 0.929) 5sa compared to NC. The STZ-induced GSH levels were recovered by the EAA therapy with a maximal rise seen at 500 mg/kg (Fig. 6). Moreover, diabetic rats CAT activity also dramatically decreased to 33.78 ± 0.012, 41.78 ± 0.012, 22.36 ± 0.012 mg/gm in the kidney, liver and pancreas. The largest effect was seen at 500 mg/kg for the EAA treatment's induction of CAT levels 53.27 ± 0.018, 62.45 ± 0.020 and 39.36 ± 0.020 units/mg (Fig. 7). Like this, the DC group's SOD activity declined in comparison to the NC group. SOD levels were recovered by EAA administration. The maximum effect was seen at 500 mg/kg for the EAA treatment's induction of SOD levels 57.37 ± 0.016, 69.4 ± 0.021 and 45.38 ± 0.021 units/mg. (Fig. 8).

Effects of EAA treatment on biochemical parameters

Insulin resistance-induced dyslipidemia severely impacted biochemical markers. When STZ was administered, ALT, AST and ALP levels increased; however, EAA therapy returned these enzyme levels to normal ($p< 0.001$). Similarly, the management of EAA resulted in a markedly lower level ($p < 0.001$). Furthermore, serum TG, TC, LDL and VLDL levels increased remarkably ($p <0.001$) in response to STZ-induced T2DM, while HDL levels decreased. When compared to DC, EAA treatment significantly ($p <0.001$) reduced LDL, TC and TG levels while simultaneously raising HDL levels (Fig. 9).

Histopathological results show EAA protects the pancreas, liver and kidneys from STZ-induced damage

The NC group's pancreas underwent histological examination, which showed normal Langerhans islets and healthy beta cells that produce insulin. The DC group, on the other hand, exhibited deteriorating alterations, the formation of vacuoles in the pancreatic islets and minor variations in acinar cell structure. The administration of EAA protected the pancreas from deterioration, restoring the pancreatic tissue to a healthy state with normal Langerhans islets. In the DC group, liver histological results revealed mild to moderate hepatic damage, including hepatocyte degeneration.

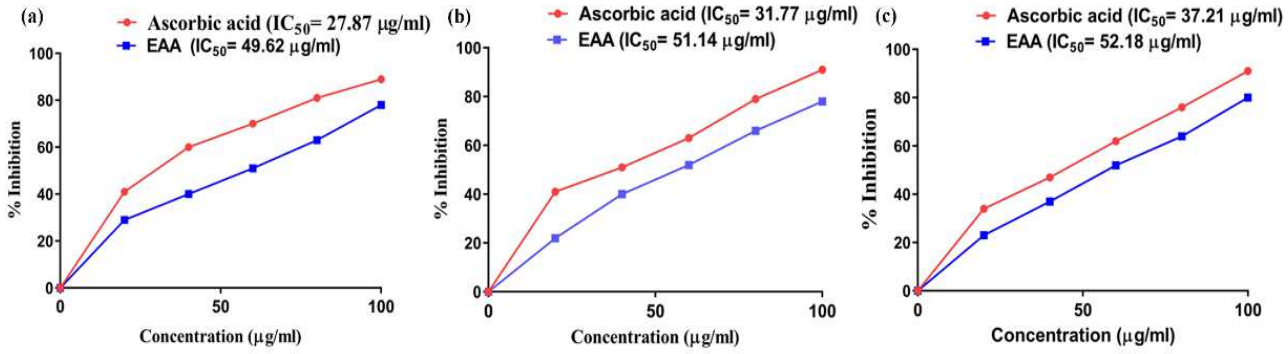

Fig. 1: Antioxidant impact of EAA *in-vitro*. The three assays are (a) DPPH; (b) Phosphomolybdate; (c) Nitric oxide scavenging. With IC50 values close to those of ascorbic acid, EAA demonstrated potent antioxidant activity.

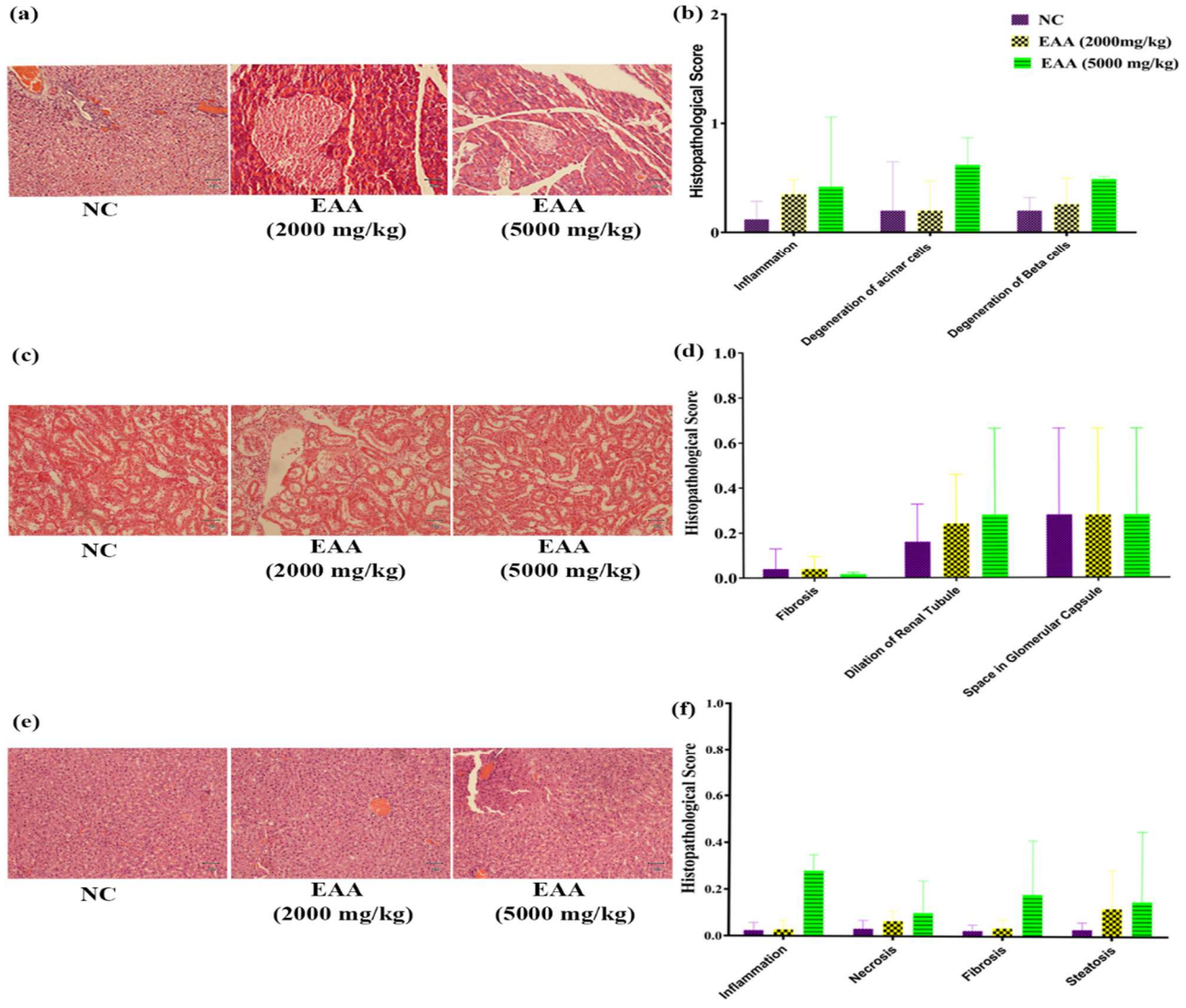

Fig. 2: Acute poisoning revealed broad EAA of safety margin. Histopathological analysis and tissue slice scoring in a semiquantitative manner. Pathological alterations were noted at a higher EAA dosage of 5000 mg/kg. The liver in (a, b) has some necrosis and moderate inflammation. Comparing the kidneys of (c, d) to NC (negative control), no significant alterations were seen. (e, f) Pancreas with somewhat elevated levels of inflammation and β-cell and acinar degeneration.

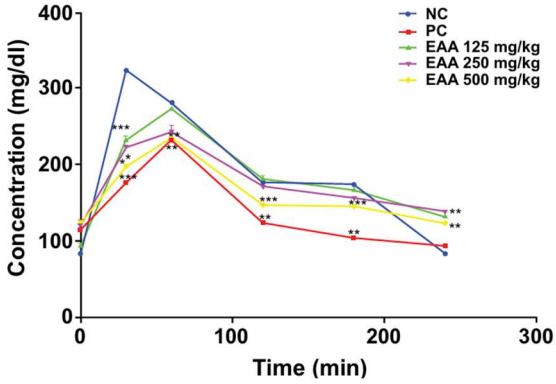

Fig. 3: EAA's hypoglycemic effects were demonstrated by OGTT. When compared to NC, EAA revealed a dose-dependent decrease in blood glucose; at 100 mg/kg, this drop was resembled to glibenclamide. PC stands for positive control, NC for negative control. Two-way ANOVA with multiple comparisons using Bonferroni, n = 5, ** ≤0.01, *** ≤0.001.

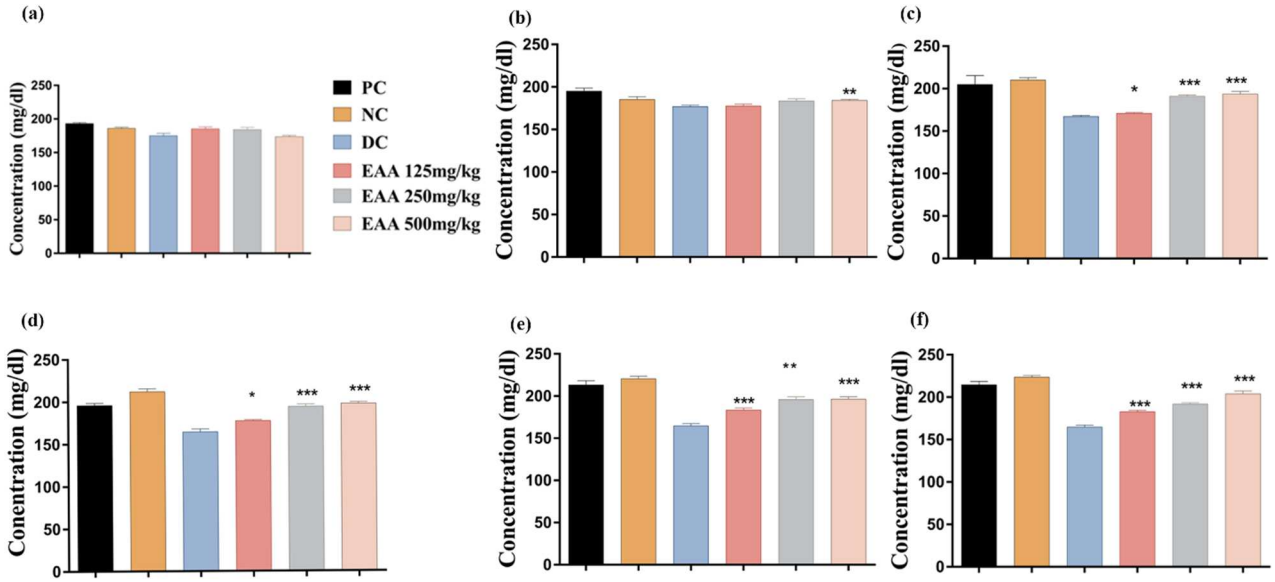

Fig. 4: Impact of *Acacia ampliceps* (EAA) ethanolic extract on blood glucose levels in streptozotocin (STZ)-induced diabetic rats at various times: (a) Day 0; (b) Day 7; (c) Day 14; (d) Day 21; (e) Day 28; (f) Day 35. The animals were split into four groups: EAA-treated (125, 250 and 500 mg/kg), diabetic control (DC), negative control (NC), and positive control (PC). Mean ± SEM (n = 5) is used to express the data. Two-way ANOVA and the Bonferroni multiple comparison test was used for statistical analysis. $p < 0.05$, $p \leq 0.01$, and $p \leq 0.001$ in relation to the diabetes control group.

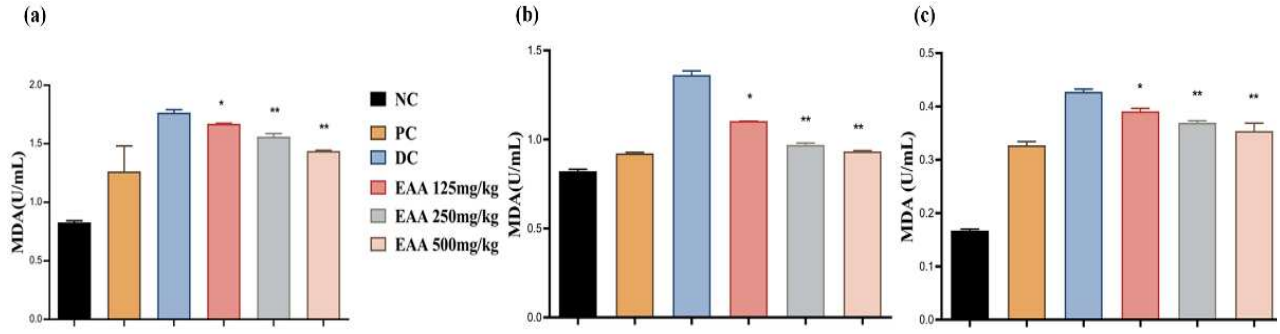

Fig. 5: MDA levels caused by EAA in rats given STZ-induced diabetes. Compared with DC, EAA showed a considerable drop in MDA levels. (a) Kidney; (b) Liver; (c) Pancreas. DC stands for diabetic control, NC for negative control, and PC for positive control. Multiple comparison test (Tukey) follows a one-way ANOVA; n = 5; * ≤ 0.05, ** ≤ 0.01.

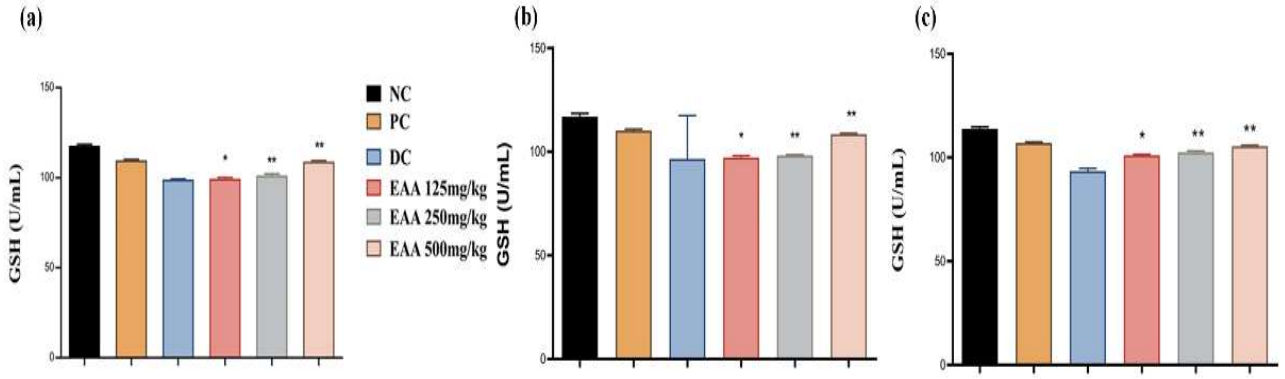

Fig. 6: GSH levels produced by EAA in diabetic rats caused by STZ. Compared with DC, EAA elevates GSH levels in a dose-dependent manner. (a) Kidneys; (b) Liver; (c) Pancreas. DC is diabetic control, NC is negative control and PC is positive control. One-way ANOVA and Tukey's test for multiple comparisons were performed; n = 5; * ≤ 0.05, ** ≤ 0.01.

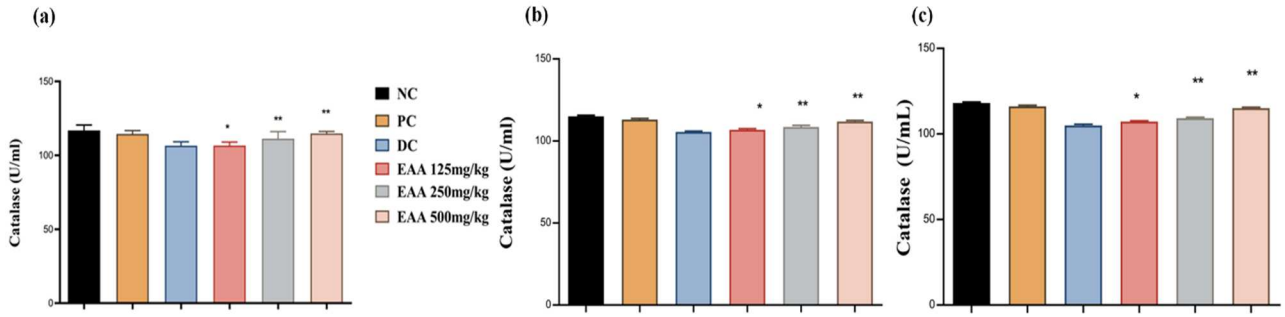

Fig. 7: CAT levels in diabetic rats produced by STZ were induced by EAA. CAT levels are higher according to EAA than DC. (a) Kidneys; (b) Liver; (c) Pancreas. DC is diabetic control, NC is negative control and PC is positive control. One-way ANOVA, followed by a test of multiple comparisons using Tukey, n = 5, * ≤ 0.05, ** ≤ 0.01.

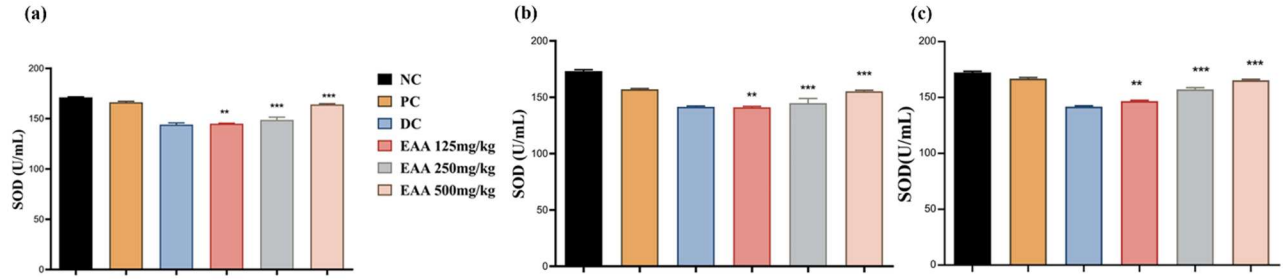

Fig. 8: SOD levels raised by EAA in diabetic rats receiving STZ. SOD levels in EAA were much higher than in DC. (a) Kidneys; (b) Liver; (c) Pancreas. DC stands for diabetic control, NC for negative control and PC for positive control. Tukey's multiple comparison test follows a one-way ANOVA; ** ≤ 0.01, *** ≤ 0.001.

After EAA therapy, only a few hepatocytes exhibited necrosis, leading to a significant reduction in these pathological alterations. The NC group showed healthy renal nephron, capsule and tubules in kidney histology. As a result of inflammation in the gaps between renal tubules and coagulative necrosis were detected in the proximal and distal convoluted tubules within the cortical region, the DC group showed signs of tubule-interstitial nephritis. Bowman's capsule and normal glomeruli in the EAA-treated group suggested that STZ-induced pathological alterations had been prevented (Fig. 10).

GC-MS analysis of extracted Acacia ampliceps

The possible antidiabetic chemicals in EAA were discovered by using Gas Chromatography-Mass Spectrometry (Fig. 11). The primary constituents identified within EAA included 1,2,4-triazole, 1-methylhydrazine-1-(5-hexenyl) ($C_2H_3N_3$), Carbamic acid, 4-[N-nitro] amino-(2-hydroxyethyl) ($C_7H_{16}N_2$), ethyl- nitrosopropyl ester ($C_6H_{12}N_2O_3$), Propylene glycol ($C_3H_8O_2$), Thiazolidine-4-one, 2-hydroxymethylimino ($C_4H_6N_2O_2S$) and (S)-2-hydroxypropanoic acid ($C_3H_6O_3$).

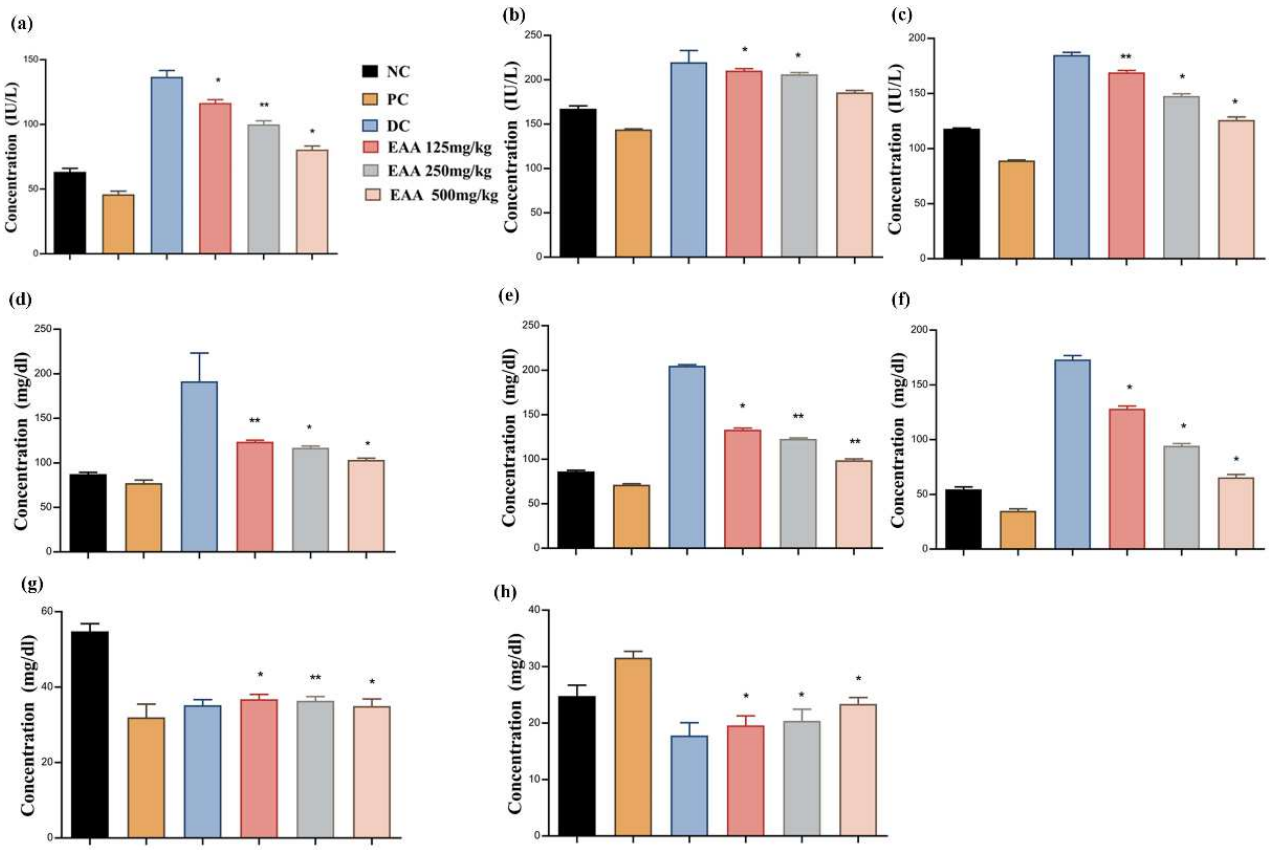

Fig. 9: (a) ALT; (b) AST; (c) ALP; (d) TG; (e) TC; (f) LDL; (g) VLDL; (h) HDL. DC had high ALT, AST and ALP levels, however, EAA brought these enzymes back to normal. EAA markedly reduced TC, TG, LDL, and VLDL and raised HDL. Levels in comparison to DC. PC stands for positive control, NC for negative control, and DC for diabetic control. Tukey's multiple comparison test and One-way ANOVA, *≤ 0.05 **≤ 0.01, *** ≤ 0.001.

Some compounds belonging to the thiazolidine, hydrazine, carbamic acid and triazole classes have been shown in previous studies to inhibit α-amylase and to reduce blood glucose *in-vitro*/*in-vivo* models, suggesting potential antidiabetic effects (Abdellatif *et al.,* 2019; Marina *et al.,* 2019). Consequently, the idea that the existence of these compounds might account for the stated antidiabetic benefits of EAA seems tenable.

DISCUSSION

T2DM is rapidly becoming a global health concern, with projections indicating that it will affect around 693 million adults by 2045 (Alkahtani *et al.,* 2024; Kyrou *et al.,* 2020; Wu *et al.,* 2020). The positive effects of EAA in countering STZ-induced T2DM were examined to identify a more effective alternative to antihyperglycemic therapies. STZ-induced T2DM has been employed as a valuable animal model for diabetes research (Hedya). Reactive oxygen species (ROS) are overproduced in this model because it exclusively targets pancreatic beta cells, leading to the onset of diabetic symptoms such as hyperlipidemia and hyperglycemia. The Study revealed that EAA exhibited significant antihyperglycemic effects in diabetic rats when compared to their healthy counterparts. These effects were accompanied by substantial enhancement in body weight and regulation of serum lipid levels. Normal rats showed significantly greater weight gain compared to all other groups. Rats treated with STZ exhibited increased food and water intake, alongwith weight loss. This study supports the idea that reduced food and water intake is associated with improved diabetes management, likely due to shorter intestinal transit time and prolonged gastric emptying time. Additionally, EAA demonstrated a remarkable safety profile, as evidenced by the absence of toxicity even at doses up to 2000 mg/kg in experimental rats. This indicates that the bioactive substances found in the extract have a very large margin of safety (Choi and Kim, 2024; Park *et al.,* 2025).

It's also important to note that EAA showed significantly higher TPC and TFC levels than A. capillus-veneris L. Differences between plant species or the choice of extraction solvent may account for these variations in phenolic and flavonoid content. EAA considerably reduced blood glucose levels when compared to the disease control group. The potential of EAA to reduce hyperglycemia may be due to its high phenolic and flavonoid content.

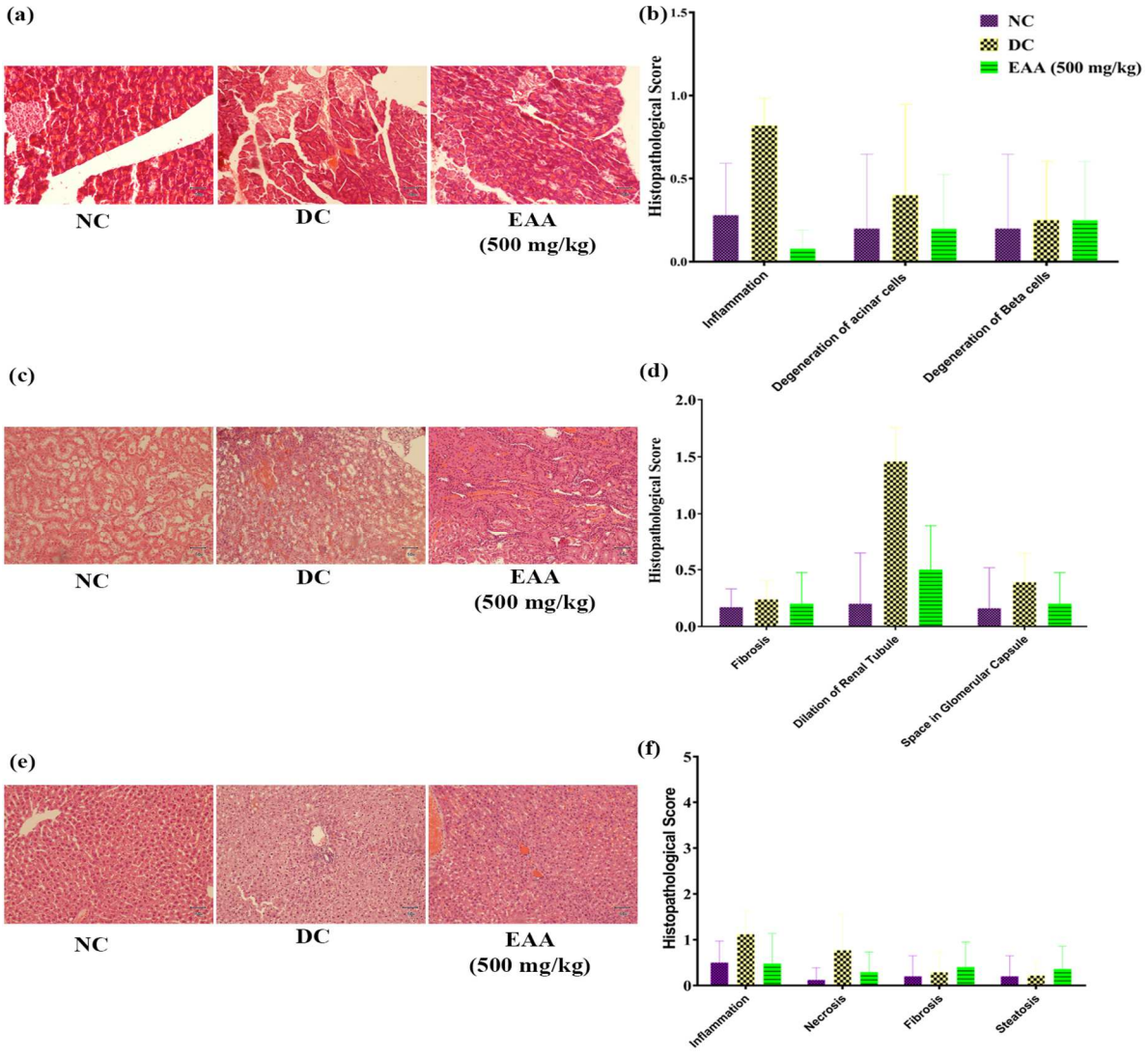

Fig. 10: Histological analysis demonstrated that EAA prevented tissue damage brought on by STZ. (a and b) Pancreas: NC revealed typical Langerhans islets; on the other hand, the DC group showed considerable acinar cell necrosis in addition to significant degenerative changes and inflammation in the Langerhans islets. Treatment stops acinar cell deterioration and inflammation. Negative control is NC, and disease control is DC; (c and d) Kidney: Nephrons, tubules and renal capsule were all normal in the NC group. DC showed tubule interstitial nephritis and mild coagulated necrosis in the cortical region of the proximal tubules. Following EAA treatment, the basement membranes, Bowman's capsule and the Bowman space were all intact; (e and f) Liver: DC moderated hepatocyte deterioration in the peripheral zone, whereas NC showed normal hepatocytes in the sinusoidal space and hepatic plates. EAA treatment results are nearly normal.

Managing blood glucose levels is an essential strategy for mitigating most complications associated with T2DM. Blocking enzymes that hydrolyze carbohydrates, such as α-amylase and α-glycosidase, is a beneficial approach, as it helps manage the hyperglycemia associated with T2DM. By inhibiting these enzymes, the absorption and digestion of carbohydrates are delayed, resulting in reduced postprandial (after-meal) plasma glucose levels. It was demonstrated that EAA effectively inhibited α-amylase in a dose-dependent way, highlighting its significant potential in managing hyperglycemia (Balu *et al.,* 2019; Bashary *et al*., 2020; Liu *et al.,* 2020; Proença *et al.,* 2022). EAA produced reliable inhibition of α-amylase, consistent across replicates. Although less potent than the reference inhibitor, this activity highlights the pharmacological relevance of plant-derived compounds as complementary agents for glycemic regulation. Further evidence for this came from the OGTT results, which revealed a reduced area under the glucose concentration curve which indicated low blood glucose levels brought on by EAA. Peak glucose levels have been shown to fluctuate, which may be related to changes in the absorption process, which are strongly related to the expression of the sodium-glucose transporter (SGLT-1) being downregulated.

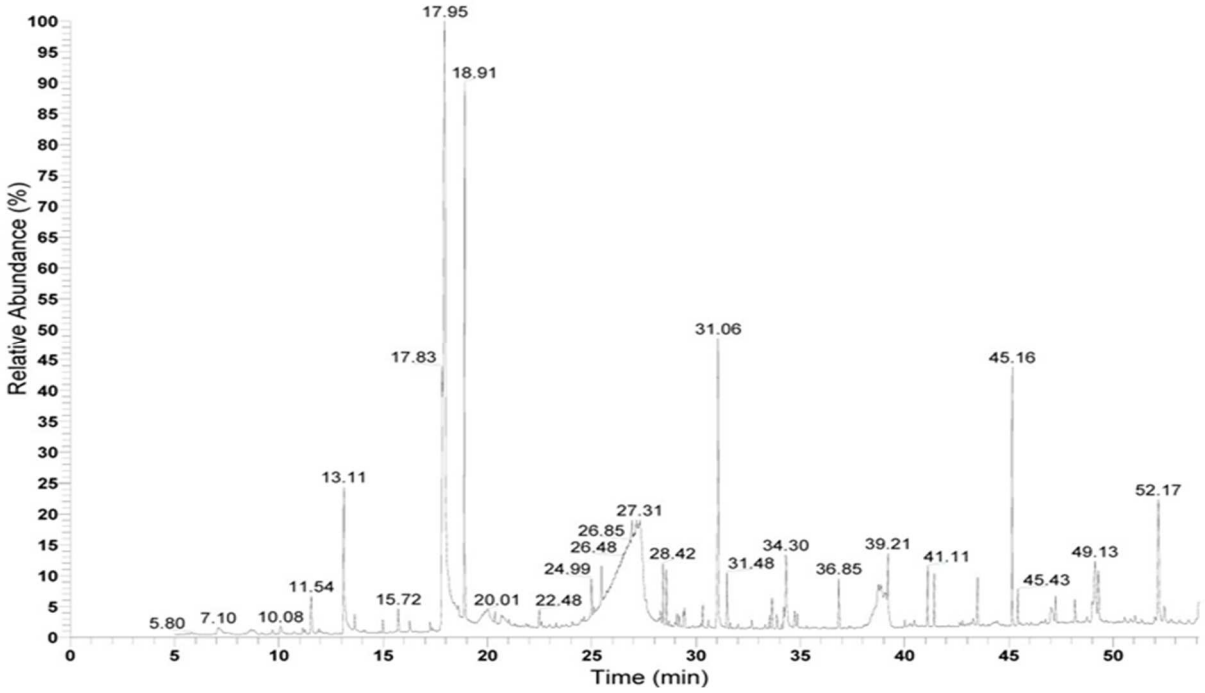

Fig. 11: GCMS Analysis

Table 1: Experimental design

Groups	Dosage	Description
Group I	Negative control	Normal rats that received daily oral administration of normal saline for a period of 35 days.
Group II	Positive control	Glibenclamide (100 mg/kg) were administered orally once daily (OD) for 35 days to diabetic rats. Induced by STZ.
Group III	Disease control	STZ-induced diabetic rats that received daily oral doses of normal saline for a duration of 35 days
Group IV	EAA - 125 mg/kg	STZ-induced diabetic rats were given to oral administration of the plant extract at a dose of 125 mg/kg OD for 35 days.
Group V	EAA - 250 mg/kg	STZ-induced diabetic rats given oral doses of 250 mg/kg of the plant extract OD for 35 days.
Group VI	EAA - 500 mg/kg	Rats with STZ-induced diabetes were given 500 mg/kg of plant extract orally OD for 35 days.

Table 2: The percentage of α-amylase inhibition at varying EAA concentrations.

Concentration (µg/mL)	Inhibition of acarbose [n(%)±SD]	IC_{50} value (µg/mL)	Inhibition of EAA [n(%)±SD]	IC_{50} value (µg/mL)
20	63.06±0.002	11.98	14.35±0.002	53.15
40	73.96±0.001		35.04±0.001	
60	78.71±0.002		53.36±0.003	
80	86.73±0.002		72.77±0.001	
100	91.88±0.001		81.68±0.002	

Abbreviations: Streptozotocin STZ; OD, once daily; IC_{50} half-maximal inhibitory concentration; SEM, standard error of the mean; µg/mL, micrograms per milliliter µg/mL.

These findings suggest that EAA's antihyperglycemic activity may involve mechanisms beyond just inhibiting carbohydrate-hydrolyzing enzymes. It is possible that EAA enhances glucose utilization, contributing to its potent antihyperglycemic effects. The STZ-induced diabetic rats in the DC group had hyperglycemia, most likely as a result of the drug's cytotoxic action on the islets of Langerhans. EAA's superior antihyperglycemic performance may also be attributed to improved glucose utilization in these animals.

T2DM is often associated with diabetic nephropathy, a condition that can lead to renal morbidity and mortality. Diabetic nephropathy is characterized by pathological changes, including glomerular sclerosis and fibrosis, that contribute to the development of proteinuria. The reduced insulin levels in diabetic individuals can lead to reduced energy availability and increased protein breakdown, ultimately contributing to glomerular dysfunction. In a study, EAA therapy resulted in reduced urea and creatinine levels. These findings are supported by previously published studies in which *Acacia nilotica* extract provided not only an antihyperglycemic effect but also ameliorated diabetic complications. The normalization of urea and creatinine levels observed in the present study further supports these findings (Omara *et al.,* 2012).

Our histological analysis, which showed that EAA treatment successfully prevented tubular degradation and glomerular thickening, thereby preserving renal function, confirmed these improvements in renal parameters (Arroyave Ospina *et al.,* 2021; Chen *et al.,* 2020; Smirne *et al.,* 2022). Similarly, *A. nilotica* extract also normalized tubular lesions and glomerular morphology, corroborating the findings of the current study.

In addition, these diabetic rats receiving EAA had considerably higher levels of SOD, CAT and GSH while concurrently having lower MDA concentrations in the pancreas, liver and kidney. Consuming antioxidants can considerably reduce the risk of free radical-induced disorders like T2DM. As the body's initial defense against free radicals, antioxidant enzymes such as SOD, CAT and GSH play a defensive role (Ighodaro and Akinloye, 2018; Jain *et al.,* 2025). An increase in insulin resistance or an impairment of insulin secretion, both of which contribute to the onset and progression of T2DM, can result from excessive free radical levels. Therefore, administering antioxidants can help counteract these harmful effects and potentially alleviate the condition. A substantial body of research has underscored the potential role of free radicals and decreased antioxidant levels in the initiation and progression of T2DM. Phenolic compounds, for instance, have garnered attention for their rapid neutralization of lipid-free radicals, indicating their potent antioxidant properties. Additionally, it is known that this phenolic composite forms a lactose complex with α-amylase (via nucleophilic groups), thereby blocking it. In addition to phenolic compounds, flavonoids, another class of antioxidants, have demonstrated remarkable antioxidant properties. Flavonoids are known not only to combat oxidative stress but also to enhance insulin release, contributing to their antihyperglycemic effects. These findings collectively suggest that antioxidants, particularly phenolic compounds and flavonoids found in natural sources such as EAA, have the potential to ameliorate oxidative stress and help manage T2DM. Considering the available data and observations, it is plausible to hypothesize that the phenolic and flavonoid content of EAA may help reduce the oxidative stress induced by STZ and the onset of T2DM. The study's findings align with previous research indicating a positive correlation between flavonoids extracted from A. capillus-veneris L. hydroalcoholic extract and increased serum insulin levels, ultimately leading to antidiabetic effects in rat models. This suggests that the antioxidant properties of phenolic compounds and flavonoids, alongwith their potential to modulate insulin secretion and sensitivity, may collectively contribute to EAA's therapeutic effects in managing T2DM (Farooq *et al.,* 2020).

EAA therapy considerably decreased the ALP, ALT and AST levels. STZ administration is associated with oxidative stress. The increased oxidative stress within the endoplasmic reticulum is associated with increased release of free fatty acids in the liver, which may contribute to hepatic damage and elevated liver enzyme concentrations in the current study. Administration of EAA normalized liver enzyme levels, possibly by overcoming the aforementioned mechanism. The results are consistent with a previous study in which another species of the same genus, Acacia, showed decreased liver enzyme levels upon doxorubicin administration (Sathya and Sidduraju, 2013). It was reported that *Acacia auriculiformis* bark and empty pod extracts exhibited promising effects and provided evidence of improvement in hepatic injury. Individuals with T2DM face an enhanced risk of coronary heart disease due to disruptions in their lipid metabolism. Elevated levels of LDL, TC and triglycerides, alongwith lower concentrations of HDL are common characteristics of individuals with T2DM. Managing these lipid abnormalities is crucial for effectively controlling T2DM and mitigating the risk of diabetes-related complications. In the current investigation, the administration of EAA had a significant normalizing effect on lipid levels, underscoring its potential to treat dyslipidemia associated with T2DM. The results are consistent with the earlier investigation, in which administration of A. capillus-veneris L hydro-alcoholic extract decreased TC and LDL concentrations, but had no effect on TG and HDL levels (Amssayef and Eddouks, 2020; Dos Santos *et al.,* 2023; Mansouri *et al.,* 2023).

Additionally, the histological analysis showed that pancreatic islets and acinar cells of diabetic rats both exhibited degenerative alterations. However, treatment with EA (Amssayef and Eddouks, 2020) effectively prevented STZ-induced pancreatic damage and consequently improved glucose regulation. It's crucial to remember that STZ is known for its detrimental effects on hepatic cells and their mitochondria, due to the generation of free radicals. It has been reported previously that STZ disrupts the hepatic architecture, leading to the formation of vacuoles in hepatocytes, vascular congestion and cellular infiltration. The current study corroborated

previous findings, showing that STZ treatment caused similar pathological alterations in the liver, whereas EAA exhibited protective effects against T2DM-induced kidney and liver damage (Mittal *et al.,* 2024; Titova *et al.,* 2020; Waataja *et al.,* 2023; Zhu *et al.,* 2024). Nevertheless, more research is needed to elucidate the exact mechanisms underlying these effects and to confirm EAA's potential as an antidiabetic agent.

CONCLUSION

The research suggests that EAA contains biologically active substances that can help reduce hyperglycemia and oxidative stress in STZ-induced T2DM rats. Further research can be conducted to isolate and identify the specific active components of EAA responsible for its anti-diabetic effect. These studies would help elucidate the precise mechanisms through which these compounds operate. This study has the potential to open avenues for developing innovative therapies or supplements to manage T2DM and its associated complications.

Acknowledgments
The authors would like to extend their sincere gratitude and appreciation to Dr. Sajid Nawaz for his words of encouragement, support and assistance in completing this work.

Authors' contributions
F.K: Carried out the investigation. H.M.Z: Data on antioxidants and histopathology were examined; H.M.B and B.A: Wrote the original draft of the work; A.S: planned and oversaw the project and revised it in its final version. Every author took part in the manuscript's revision

Funding
There was no funding.

Data availability statement
The datasets generated during and /or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval
All animal experiments complied with institutional and national guidelines for animal care and were conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) of the University of Lahore's Faculty of Pharmacy's Institutional Research Ethics Committee (Approval number: IREC-2018-46). Efforts were made to minimize animal suffering and all procedures were designed to ensure the humane treatment of animals used in the study. This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

Conflict of interest
The authors declare no conflict of interest.

Supplementary data
https://www.pjps.pk/uploads/2026/06/SUP1780405805.pdf

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