

Aqueous Ethanolic Extract of *Adiantum incisum* Forssk. Protects against Type 2 Diabetes Mellitus via Attenuation of α -Amylase and Oxidative Stress

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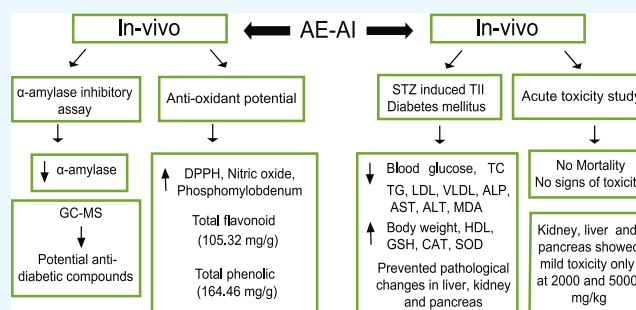
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ABSTRACT: *Purpose:* This study was designed to investigate the antidiabetic effects of the aqueous ethanolic extract of *Adiantum incisum* Forssk. whole plant (AE-AI) in order to validate the folkloric claim. *Methods:* Streptozotocin (STZ) was used to induce type 2 diabetes mellitus (TII DM) in male Sprague–Dawley rats. STZ-induced diabetic rats were later treated orally with either AE-AI (125, 250, and 500 mg/kg) or glibenclamide for 35 days. Blood glucose levels were measured weekly and on day 35, animals were sacrificed, and blood samples and tissues were harvested for subsequent antioxidant and histopathological analyses. AE-AI was also analyzed *in vitro* for phytochemical, antioxidant, and α -amylase inhibitory assays. *Results:* The phytochemical screening of AE-AI confirmed the presence of essential bioactive compounds like cardiac glycosides, flavonoids, phenolic compounds, saponins, and fixed oils. AE-AI demonstrated abundant amounts of total phenolic and flavonoid contents and displayed prominent antioxidant activity as assessed via DPPH, phosphomolybdate, and nitric oxide scavenging assays. AE-AI treatment also showed α -amylase inhibitory activity comparable to acarbose. In addition, AE-AI treatment exhibited a wide margin of safety in rats and dose-dependently reduced STZ-induced blood glucose levels. Moreover, AE-AI increased the levels of GSH, SOD, catalase, and reduced MDA, and therefore prevented pathological effects of STZ on the kidney, liver, and pancreas. The blood glucose regulatory effect and antioxidant activity of AE-AI also aided in normalizing TII DM-mediated dyslipidemias. GC-MS analysis also demonstrated several potential antidiabetic phytoconstituents in AE-AI. *Conclusion:* These findings reveal that AE-AI possesses certain pharmacologically active compounds that can effectively treat STZ-induced TII DM owing to its antioxidant and α -amylase inhibitory potentials.



INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder that results from hyperglycemia, insulin deficiency, and reduced insulin utilization. The syndrome is usually associated with disrupted metabolism of carbohydrates, proteins, and lipids. These metabolic disturbances cause several micro and macro complications such as heart attack, stroke, kidney failure, leg amputation, vision loss, and nerve damage.¹ DM has different types, which include type 1 (TI DM) and type II (TII DM). In TI DM, insulin production is compromised owing to the destruction of β -cells, which results in reduction of circulating insulin levels. TII DM is the most common form of DM, which affects about 90–95% of diabetic patients and is normally characterized by insulin resistance in peripheral tissues and reduced insulin responsiveness.² Other forms of DM include pregnancy-induced diabetes, maturity-onset diabetes in young individuals, and latent autoimmune diabetes in adults.³

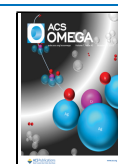
TII DM is a serious threat to the health-care system of developed and underdeveloped countries as it is considered the third major cause of death.⁴ Frequent urination, lethargy,

increased thirst, weight loss, sweating, and increased hunger are the indicators of TII DM.⁵ Oxidative stress is considered as one of the major contributors of TII DM.⁶ Endogenous antioxidants are the major source of defense in the human body and any disturbance in this defense mechanism usually leads to oxidative stress due to production of free radicals and reactive oxygen species (ROS).⁷ Enhanced ROS levels play a key role in the development and progression of TII DM.⁸ Another detrimental factor is insulin deficiency, resulting in reduced glucose cellular uptake from blood.⁹ This disarray in a number of regulatory processes involving insulin deficiency renders diabetic patients also vulnerable to hyperlipidemia.¹⁰

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A large number of hypoglycemic agents are currently available in the market for the management of TII DM. These glucose-lowering entities possess different mode of actions and selection is based on the prevalence of disease, age, sex, body mass index, and other factors.^{11,12} Most of these therapies are associated with adverse effects such as weight gain, hypoglycemia, hypersensitivity, and gastrointestinal disturbances and in severe cases, liver and heart failure.¹³ Hence, there is a need to find new alternatives, and scientists are now evaluating herbal and complementary medications that have interestingly shown an improvement in glucose homeostasis. Studies have reported that several herbal constituents such as flavonoids, phenolic compounds, terpenoids, alkaloids, glycosides, and coumarins exert a beneficial effect on carbohydrate regulation.¹⁴ The traditional system of herbal medicines has offered some promising alternate therapies for TII DM.¹⁵ Extracts of medicinal plants offer a wide diversity of chemical constituents, which can lead to the discovery of novel antidiabetic agents.¹⁵ Most of the plant extracts have not been scientifically validated with standard and appropriate models of TII DM. Therefore, it is worth appraising and scrutinizing the efficacy of plant extracts against hyperglycemia and dyslipidemia in animal models of TII DM.

In this study, we have assessed the antihyperglycemic and antioxidant activities of *Adiantum incisum* extract in streptozotocin-induced diabetic rats. *A. incisum* Forssk. is an evergreen fern belonging to the Adiantaceae family, which is commonly found in the mountaineous range of the Kashmir and Murree region of Pakistan and is widespread all over the world. The traditional local names of *A. incisum* are Mayursikha, Sumble, Hansraj, and Raja hans.¹⁶ The fronds powder and juice of *A. incisum* have been used traditionally to treat TII DM; however, there is no scientific data to validate its folkloric claim.^{17–19} Another member of its family, *Adiantum capillus-veneris* L., has been used commercially in multiherbal formulations to treat a wide variety of ailments.²⁰ Based on the traditional use of *A. incisum* and the therapeutic potential of its family member, it was hypothesized that the plant extract of *A. incisum* would be beneficial for treating TII DM in rats.

MATERIALS AND METHODS

Chemicals. Streptozotocin (STZ), α -amylase, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, acarbose, and nicotinamide were purchased from Sigma-Aldrich (St. Louis, Missouri). Standard kits were purchased from Biolabs (Boston) and Vivantis Technologies (Selangor Darul Ehsan, Malaysia). All other chemicals and reagents used in the study were of standard analytical grade.

Plant Material. BT-harvested *A. incisum* whole plant was obtained from Mirpur, Azad Kashmir, Pakistan in March 2019. Taxonomic identification was performed by Dr Zaheer-Uddin, Department of Botany, Government College University, Lahore (GCUL) using a binocular microscope and comparing the specimens in GCUL herbarium. The voucher specimen (GC Herb Bot 3444) has been deposited in the University herbarium.

Preparation of Aqueous Ethanolic Whole Plant Extract. The whole plant of *A. incisum* was washed with tap water to get rid of the adhering dust and foreign particles. The plant material was shade dried and sliced into small pieces. The dried plant material was powdered and macerated in aqueous ethanolic mixture [deionized water 20/ethanol 80] for 7 days.

The mixture was filtered with Whatman no. 1 filter paper and the filtrate was concentrated under reduced pressure in a rotary evaporator at 40 °C. The aqueous ethanolic extract of *A. incisum* (AE-AI) was dried in an oven at 40 °C and stored at 4 °C for further use.

Qualitative Phytochemical Analysis. AE-AI was analyzed for the presence of phytochemical constituents like alkaloids, glycosides, phenolics, flavonoids, saponins, carbohydrates, proteins, and fixed oils using standard procedures.^{21–23}

Total Phenolic Content. The total phenolic content (TPC) of AE-AI was assessed by Folin-Ciocalteu's (FC) method with slight modifications. Briefly, AE-AI was diluted at different concentrations (0, 20, 40, 60, 80, and 100 $\mu\text{g/mL}$) with methanol. Subsequently, 9 mL of distilled water and 1 mL of FC reagent were added to the dilutions and mixed for 5 min. Ten mL of Na_2CO_3 (7%) and 4 mL of distilled water were later added and incubated for 90 min at 37 °C. The absorbance was determined by a UV spectrophotometer at 760 nm. The linear regression model was used to determine TPC using the standard calibration curve of gallic acid.²⁴

Total Flavonoid Content. The total flavonoid content (TFC) of AE-AI (0, 20, 40, 60, 80, and 100 $\mu\text{g/mL}$) was determined by the aluminum chloride colorimetric method with slight modifications. The procedure involved the addition of extract dilutions (200 μL), AlCl_3 (100 μL), $\text{CH}_3\text{CO}_2\text{K}$ solution (1 M; 100 μL), and distilled water (4.6 mL) followed by incubation at 37 °C for 45 min. A similar procedure was performed for quercetin and the absorbance of the samples was determined by a UV spectrophotometer at 415 nm. TPC was estimated using a quercetin calibration curve²⁵ and the results were expressed as per the quercetin equivalent.

Antioxidant Analyses. DPPH Assay. The free radical scavenging potential of AE-AI was evaluated by DPPH assay using ascorbic acid as the standard.²⁶ Briefly, 4 mg of DPPH was dissolved in 100 mL of ethanol for the preparation of stock solution. The procedure involved addition of freshly prepared DPPH (2 mL), methanol (1 mL), and AE-AI (1 mL) at different concentrations (0, 20, 40, 60, 80, and 100 $\mu\text{g/mL}$) followed by incubation for 30 min. Absorbance was measured at 517 nm using a UV spectrophotometer and radical scavenging activity was measured using the following formula:

percentage inhibition of AE – AI

$$= \left[\frac{\text{absorbance}(\text{control}) - \text{absorbance}(\text{sample})}{\text{absorbance}(\text{control})} \right] \times 100$$

Phosphomolybdate Assay. The total antioxidant capacity (TAC) of AE-AI was estimated by phosphomolybdate assay using ascorbic acid as the standard. Three milliliters of freshly prepared phosphomolybdate reagent was added to 300 μL of AE-AI and incubated for 90 min at 95 °C. The absorbance was recorded by a UV spectrophotometer at 765 nm.^{27,28} The antioxidant capacity was measured by the above-mentioned formula and reported as μg of ascorbic acid equivalents (AAE) per mL.

Nitric Oxide Scavenging Assay. The antioxidant potential of AE-AI was also evaluated by nitric oxide scavenging assay using ascorbic acid as the standard. Hundred milliliters of sodium nitroprusside (10 mM) was added to 100 μL of AE-AI and incubated for 3 h. The absorbance was recorded by a UV spectrophotometer at 546 nm and the antioxidant capacity was reported as AAE per mL. The percentage inhibition of nitric

oxide radical formation was determined by the above-mentioned formula.

In Vitro α -Amylase Inhibitory Assay. The *in vitro* hypoglycemic activity of AE-AI was assessed by α -amylase inhibitory assay using acarbose as the standard. One milliliter of α -amylase solution and 2 mL of phosphate buffer (0.02 M) were added to 1 mL of AE-AI dilutions (31.05, 62.5, 125, 250, 500 to 1000 μ g/mL) and incubated at 37 °C for 30 min. Later, 1 mL of starch solution (0.5%) was added to the previous solution and incubated for 15 min. Then, 1 mL of 3,5-dinitrosalicylic acid reagent was added and heated at 90 °C in a water bath for 8 min. The absorbance was measured by a UV spectrophotometer at 540 nm and percentage inhibition was calculated by the above-mentioned formula.²⁹

GC-MS Analysis of AE-AI. AE-AI was subjected to GC-MS analysis using the Agilent Technologies 6890 N network GC system attached to an Agilent series 5975 inert XL mass selective detector and a 7683 B series Agilent series injector equipped with a DB35-ms 122–3832 column for detection of phytoconstituents. Components were identified by comparing their mass spectra at specific retention times using the library.

Experimental Animals. Colony-bred, adult male albino Sprague–Dawley rats (7 weeks old; 170–220 g) were purchased from the Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore. The selected animals were kept in spacious polypropylene cages under standard conditions. A standard diet (carbohydrate 58%, fat 5.7%, protein 24%, ash 8%, and fiber 6%) was given to all rats *ad libitum* with free access to tap water. All of the experiments were performed according to the OECD guidelines, and ethical approval (IREC-2020-09) of the experimental protocol was obtained from the Institutional Research Ethics Committee of the Faculty of Pharmacy, The University of Lahore.³⁰

Acute Toxicity Study. Rats were randomly assigned to 5 groups ($n = 6$ in each group) and fasted overnight with free access to water. A single dose of AE-AI was orally administered via gastric gavage at different concentrations (100, 500, 2000, and 5000 mg/kg) and the animals were carefully observed for behavioral and toxicological symptoms for 24 h. Later, animals were sacrificed and the liver, kidney, and pancreas of rats treated with 2000 and 5000 mg/kg were isolated for histopathological analysis.³¹

Oral Glucose Tolerance Test (OGTT). Rats were assigned to five groups and each group consisted of five animals ($n = 5$). Group I (negative control) was fed with normal saline and group II (positive control) was fed with 100 mg/kg glibenclamide. Groups III, IV, and V were fed orally with different doses of AE-AI, i.e., 125, 250, and 500 mg/kg, respectively. All treatments were given to the rats 5 min before loading glucose (2 g/kg). Blood samples were collected from tail vein after 0, 30, 60, 120, and 180 min of glucose administration and blood glucose levels were measured at the indicated time points using an Accu-Check glucometer (Roche, Switzerland).³² The hypoglycemic potential of AE-AI was determined by calculating the area under the curve (AUC).^{33,34}

% hyperglycemia inhibition factor

$$= \frac{\text{AUC}(\text{control group}) - \text{AUC}(\text{treatment group})}{\text{AUC}(\text{control group})} \times 100$$

Assessment of Antidiabetic Activity of AE-AI.

Induction of TII Diabetes. TII DM was induced in 12 h fasted Sprague–Dawley male rats by a single dose of nicotinamide (110 mg/kg) dissolved in 0.5 mL of normal saline followed by a single dose of streptozotocin (STZ; 55 mg/kg), freshly dissolved in 0.5 mL of citrate buffer (0.01 M; pH 4.5).³⁵ The rats were given 5% dextrose solution overnight after the STZ administration to avoid hypoglycemic shock.³⁶ The blood glucose levels were assessed by drawing blood from the tail vein by a glucometer. After 72 h of STZ administration, the rats having glucose levels exceeding 250 mg/dL were selected for the study and were considered diabetic.^{37–39}

Experimental Design. The experimental animals were randomly assigned into six groups, each containing five rats ($n = 5$). The division of groups was done in the following manner:

Group I, negative control (NC): normal rats that received normal saline by an oral gavage tube once daily for 35 days.

Group II, positive control (PC): STZ-induced diabetic rats that received glibenclamide (600 mg/kg) as a standard drug by an oral gavage tube once daily for 35 days.

Group III, diabetic control (DC): STZ-induced diabetic rats given normal saline by an oral gavage tube once daily for 35 days.

Group IV, AE-AI (125 mg/kg): STZ-induced diabetic rats that received 125 mg/kg plant extract by an oral gavage tube once daily for 35 days.

Group V, AE-AI (250 mg/kg): STZ-treated diabetic rats that received 250 mg/kg plant extract by an oral gavage tube once daily for 35 days.

Group VI, AE-AI (500 mg/kg): STZ-induced diabetic rats that received 500 mg/kg plant extract by an oral gavage tube once daily for 35 days.

Antihyperglycemic Activity. After induction of TII DM, the fasting blood glucose levels were measured at 0, 7, 14, 21, 28, and 35 days using a glucometer. Moreover, the body weights, food, and water intake of the rats were also assessed on these days.⁴⁰ On the 35th day, animals were sacrificed and their blood samples and tissues were harvested for subsequent analyses.

Antioxidant Assays. The markers of oxidative stress in the liver, kidney, and pancreas of the experimental animals were estimated. Briefly, tissues were homogenized in phosphate-buffered saline (1:10 w/v; pH = 7.4) and centrifuged at 6000 rpm for 15 min at 4 °C to obtain the supernatants for the estimation of malondialdehyde, glutathione, catalase, and superoxide dismutase levels.

Malondialdehyde (MDA). MDA concentration was assessed using the double heating method. The supernatant (0.5 mL) was added to 2.5 mL of trichloroacetic acid (TCA; 10% w/v) in a centrifuge tube. The tubes were boiled for 15 min and centrifuged for 10 min at 1000g. One milliliter of thiobarbituric acid (TBA) solution (0.67 per w/v) was added to 2 mL of supernatant from each centrifuge tube and boiled again for 15 min in a water bath. The absorbance was measured at 532 nm and the following formula was used to estimate the level of MDA in the tissue samples

concentration of MDA

$$= \text{OD}_{532} \times 100 \times \text{TV} / (\text{molar extict coefficient}) \times \text{dWt} \times \text{AV}$$

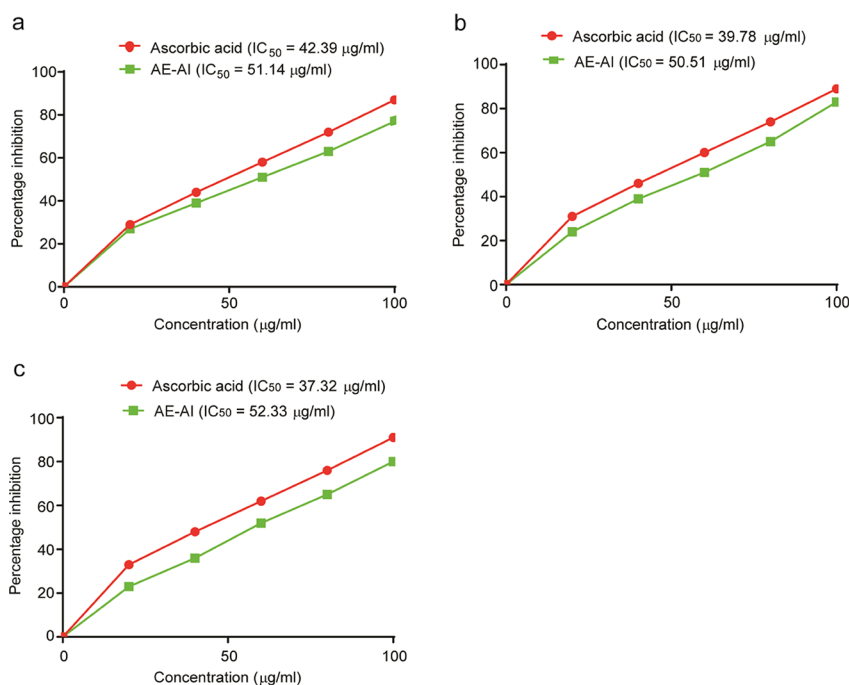


Figure 1. *In vitro* antioxidant effects of AE-AI. (a) DPPH assay; (b) phosphomolybdate assay; and (c) nitric oxide scavenging assay. AE-AI exhibited IC_{50} values comparable to ascorbic acid, demonstrating strong antioxidant activity.

where TV is the volume of the solution (4 mL), 1.56×10^5 is the molar extinction coefficient, dWt is the weight of the dissected tissue (1 g), and AV is the aliquot volume (1 mL).

Glutathione (GSH). One milliliter of tissue homogenates was mixed with 1 mL of 10% TCA. Following precipitation, 0.5 mL of DNTB reagent and 4 mL of phosphate buffer were added to the above mixture and absorbance was measured at 412 nm. The results were expressed as nM of reduced GSH per mg of protein.

$$\text{GSH level} = Y - 0.00314 \div 0.0314 \times \text{DF} / T \times \text{VU}$$

where Y is the absorbance at 412 nm of the tissue homogenate, DF is the dilution factor (1), T is the tissue homogenate (1 mL), and VU is the aliquot volume (1 mL).

Catalase (CAT). CAT activity was measured by adding 1.95 mL of phosphate buffer (pH = 7.0, 50 mM) to 1 mL of tissue homogenates and 1 mL of hydrogen peroxide. Absorbance was measured at 240 nm and CAT activity was expressed as unit/mg protein, where 1 CAT unit equals 1 M of hydrogen peroxide decomposed per minute at 25 °C.

The following formula was used to measure the CAT activity

$$\text{CAT activity} = \delta. \text{O. D} / E \times \text{vol. of sample (ml)} \\ \times \text{mg of protein}$$

where $\delta. \text{O.D}$ is the change in absorbance/minute and E is the extinction coefficient ($0.071 \text{ mmol cm}^{-1}$). The protein contents were estimated by using the Lowry method. The standard curve of the protein was obtained by plotting the graph at different concentrations of BSA.

Superoxide Dismutase (SOD). The total SOD activity in the tissue homogenate was determined using the xanthine oxidase method according to the standard kit protocol.

Biochemical Parameters. For biochemical analyses, blood samples were centrifuged at 1500g for 15 min at 4 °C. Serum was separated for the estimation of triglycerides (TG), total

cholesterol (TC), high-density lipoproteins (HDL), low-density lipoproteins (LDL), very low-density lipoproteins (VLDL), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and aspartate aminotransferase (AST). The analyses was performed using standard kits with a Roche Cobas C311 chemistry analyzer (Hamburg, Germany).

Histopathology. The tissue samples were washed, dehydrated with alcohol, cleared by xylene, and later paraffin blocks were prepared. Sections of 4–5 mm thickness were cut using a rotary microtome and stained with hematoxylin and eosin. Histological changes were observed under a light microscope for any signs of toxicity by random selection of the fields of view. The histological sections were examined by a pathologist who was blinded to the treatment groups. The damage to the tissues was scored according to a scoring system mentioned in Figure 9. The scoring system had a total of five grades according to the severity of the damage ranging from 0 to 4. The severity of the kidney injury included inflammation, glomerular thickening, and tubular degeneration. Hepatic alterations included inflammation, necrosis, fibrosis, and steatosis, whereas pancreatic injury included inflammation, degeneration of acinar cells, and degeneration of β -cells. Injuries were graded as follows for each criterion: <1% = 0; 1–25% = 1; 26–50% = 2; 51–75% = 3; and >75% = 4.⁴¹

Statistical Analysis. The results were expressed as mean $\pm$ SD and data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using Graph Pad Prism 6.0 software. IC_{50} values were calculated using the nonlinear regression model and a probability level of $p < 0.05$ was considered statistically significant. The following symbols were used to represent the level of significance: # ≤ 0.001 , ** ≤ 0.01 , * ≤ 0.05 .

RESULTS

Percentage Yield and Phytochemical Analysis of AE-AI. The percentage yield of AE-AI was around 3.64% and the

phytochemical screening confirmed the presence of essential bioactive compounds like cardiac glycosides, flavonoids, alkaloids, phenolic compounds, carbohydrates, proteins, saponins, and fixed oils.

Total Phenolic Content. In order to estimate the phenolic contents of AE-AI, a calibration curve was established using different concentrations of gallic acid from a stock solution of 100 $\mu\text{g/mL}$. The concentration of the phenolic compounds was then calculated using $y = 0.006x + 0.024$, $R^2 = 0.965$ and expressed in micrograms of gallic acid equivalent ($\mu\text{g/g}$). The TPC of AE-AI was found to be 164.46 mg/g .

Total Flavonoid Content. The flavonoid calibration curve was established using different concentrations of quercetin, a well-known flavonoid of the flavonol family. The TFC of the extract was determined by AlCl_3 method, using the linear equation $y = 0.004x - 0.039$, $R^2 = 0.976$, and was around 105.32 mg/g .

AE-AI Displayed Strong *In Vitro* Antioxidant Activity. The findings demonstrated that the scavenging activities of DPPH, phosphomolybdate, and nitric oxide increased proportionally from 20 to 100 $\mu\text{g/mL}$ of AE-AI. The IC_{50} of AE-AI by DPPH assay was around 51.14 $\mu\text{g/mL}$, which was comparable to the standard ascorbic acid ($\text{IC}_{50} = 42.39 \mu\text{g/mL}$). Similarly, the IC_{50} values of AE-AI by phosphomolybdate and nitric oxide assays were 50.51 and 52.33 $\mu\text{g/mL}$, respectively, and these results were also comparable to ascorbic acid (Figure 1a–c).

AE-AI Inhibited the α -Amylase Enzyme, Indicating Its Antidiabetic Potential. AE-AI, like acarbose, produced a dose-dependent inhibition of α -amylase and the IC_{50} of AE-AI (51.34 $\mu\text{g/mL}$) was also comparable to acarbose (6.179 $\mu\text{g/mL}$) (Table 1). Acarbose is a well-known α -glucosidase and α -

Table 1. Percentage Inhibition of α -Amylase at Different Concentrations of AE-AI

concentration ($\mu\text{g/mL}$)	% inhibition of acarbose	IC_{50} value ($\mu\text{g/mL}$)	% inhibition of AE-AI	IC_{50} value ($\mu\text{g/mL}$)
20	71.19 $\pm$ 0.003	6.179	29.47 $\pm$ 0.008	51.34
40	75.25 $\pm$ 0.003		40.56 $\pm$ 0.001	
60	79.21 $\pm$ 0.001		52.37 $\pm$ 0.007	
80	85.15 $\pm$ 0.003		61.24 $\pm$ 0.008	
100	92.08 $\pm$ 0.005		70.24 $\pm$ 0.006	

amylase inhibitor and reduces the level of glucose by delaying the digestion of carbohydrates. These *in vitro* findings clearly indicated that AE-AI might possess strong antidiabetic effects.

Acute Toxicity Study. No mortality in rats was observed up to 5000 mg/kg of AE-AI treatment. However, significant inflammation and degeneration of acinar and β -cells were observed only at 5000 mg/kg of AE-AI. Similarly, AE-AI (5000 mg/kg)-treated liver samples displayed mild inflammation and fibrosis when compared to the NC group. Kidney samples of AE-AI-treated groups on the other hand displayed a normal renal capsule, nephrons, and tubules in comparison to NC (Figure 2).

Oral Glucose Tolerance Assay. OGTT was performed on normoglycemic rats and area under the curve (AUC) was calculated by plotting the concentration of glucose against the time interval. Based on the mean of the highest glycemic values (C_{max}) of each experimental group, the hyperglycemic inhibition rate was determined by comparison with the mean peak value of the glucose group. All tested concentrations of

AE-AI demonstrated a significant reduction in glucose AUC and C_{max} , an effect practically comparable with the one observed with glibenclamide with the maximum effect observed at 500 mg/kg (Figure 3).

AE-AI Attenuated Blood Glucose Levels and Restored the Body Weights of STZ-Induced Diabetic Rats. STZ administration induced a significant increase ($p < 0.001$) in blood glucose levels when compared to NC. Treatment with different concentrations of AE-AI produced a dose-dependent reduction in STZ-induced hyperglycemia. At day 35, 500 mg/kg of AE-AI reduced the blood glucose concentration to 142 mg/dL , which was comparable to PC (126 mg/dL) (Figure 4). Moreover, STZ administration reduced the body weight of all of the animals; however, glibenclamide and AE-AI prevented weight loss over the period of 35 days (Figure S1).

AE-AI Treatment Induced GSH, CAT, and SOD Levels, While Reducing MDA, Indicating Its Antioxidant Potential. The findings revealed that DC exhibited a significant ($p < 0.001$) increase in MDA concentration in the kidney (1.669 ± 0.005), liver (1.352 ± 0.002), and pancreas (0.325 ± 0.003) with reference to NC (kidney: 0.9123 ± 0.001 , liver: 0.811 ± 0.0014 , and pancreas: 0.156 ± 0.003). AE-AI treatment reduced STZ-induced MDA levels in a dose-dependent fashion and the maximum reduction in MDA was observed at a dose of 500 mg/kg (Figure 5a–c).

In addition, the DC group exhibited a significant ($p < 0.001$) reduction in GSH concentration in the kidney (33.39 ± 0.932), liver (43.39 ± 0.932), and pancreas (23.39 ± 0.932) as compared to NC. AE-AI treatment dose-dependently restored the STZ-induced GSH levels, and a maximum increase in GSH levels (75.80 ± 0.444 , 85.80 ± 0.444 , and $65.80 \pm 0.44 \mu\text{mol/mg}$ in the kidney, liver, and pancreas, respectively) was observed at 500 mg/kg (Figure 6a–c).

The CAT activity in diabetic rats also declined to 34.85 ± 0.013 , 44.85 ± 0.013 , and $20.38 \pm 0.013 \text{ mg/gm}$ in the kidney, liver, and pancreas, respectively, when compared to the activities in NC. AE-AI treatment significantly induced STZ-induced CAT levels, with maximum effect observed at 500 mg/kg (55.29 ± 0.020 , 65.29 ± 0.020 , and $40.29 \pm 0.020 \text{ unit/mg}$) (Figure 7a–c).

Similarly, the SOD activity in the DC group declined when compared to NC. AE-AI treatment restored the SOD levels and displayed dose-dependent effects (Figure 8a–c).

Restoration of LFTs, RFTs, and Lipids to Normal Levels by AE-AI Treatment. Biochemical parameters are severely affected by dyslipidemia due to insulin resistance. In this study, we witnessed induction of ALT, AST, and ALP levels by STZ, while treatment with AE-AI restored the enzyme levels ($p < 0.001$) to normal. Similarly, treatment of the diabetic animals with AE-AI significantly ($p < 0.001$) decreased serum urea and creatinine concentrations (Table 2). Moreover, STZ-induced TII DM caused a significant ($p < 0.001$) elevation in the serum levels of TG ($132 \pm 1.52 \text{ mg/dL}$), TC ($205 \pm 2.42 \text{ mg/dL}$), LDL ($171 \pm 2.68 \text{ mg/dL}$), and VLDL (32.8 ± 0.58) but reduction of HDL ($16 \pm 0.447 \text{ mg/dL}$). AE-AI treatment remarkably ($p < 0.001$) decreased serum TG, TC, and LDL levels when compared to DC, and an increase in HDL levels was also observed (Table 2).

Histopathological Data Depicted Protective Effect of AE-AI against STZ-Induced Pancreatic, Hepatic, and Renal Damage. A histopathological study of pancreas revealed that the NC group had normal islets of Langerhans and insulin-producing β -cells. The DC group displayed

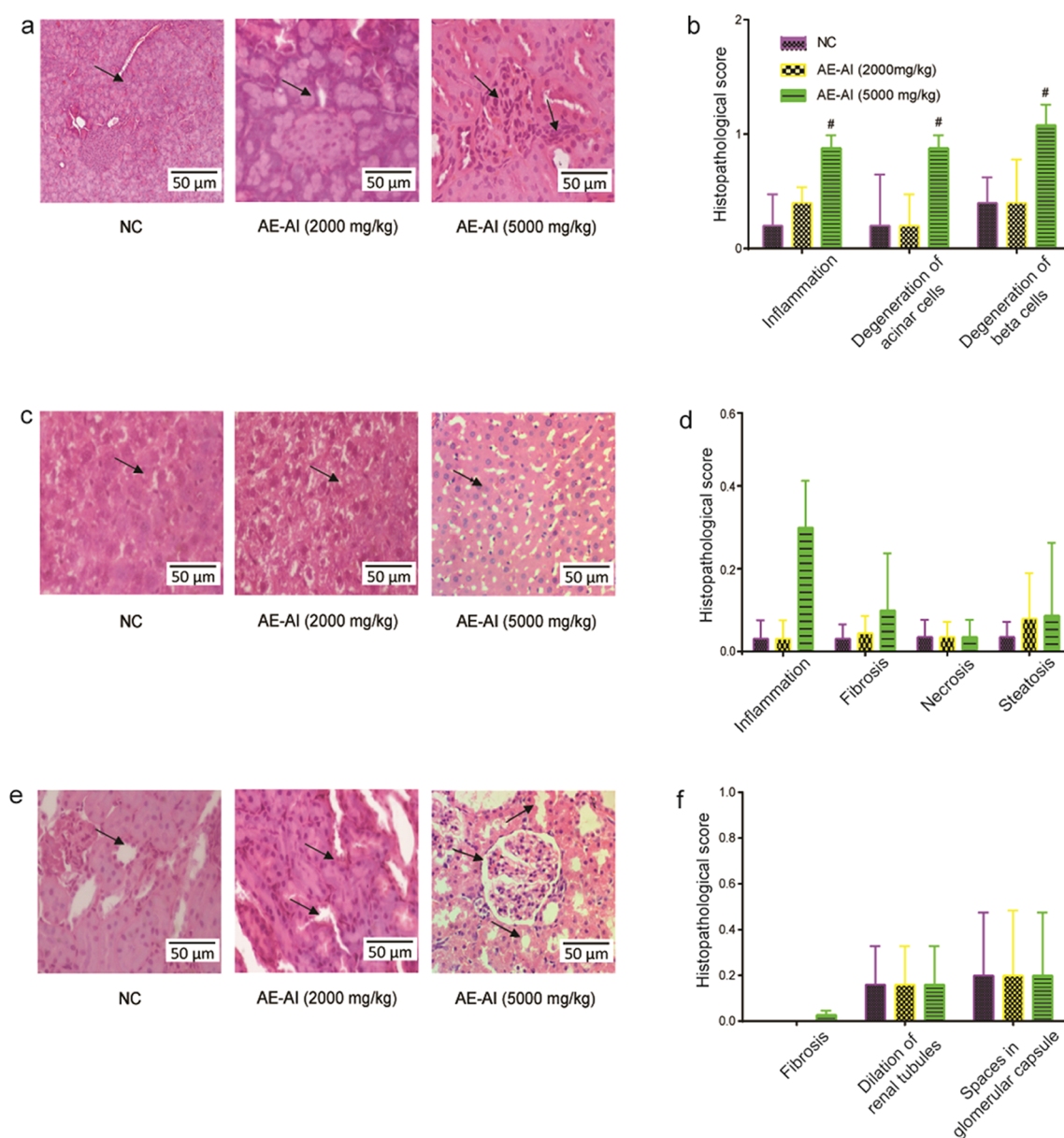


Figure 2. Acute toxicity study demonstrating a wide margin of safety for AE-AI. Histopathological examination and semiquantitative scoring of tissue sections. Pathological changes were witnessed at a higher dose (5000 mg/kg) of AE-AI. (a, b) Pancreas displaying a moderate degree of inflammation and degeneration of acinar and β -cells. (c, d) Liver showing mild inflammation and necrosis in a few regions. (e, f) Kidney not displaying any drastic changes when compared to NC (negative control). One-way ANOVA followed by Tukey's multiple comparison test, $n = 5$, $** \leq 0.01$.

degenerative changes and vacuole formation in islets of Langerhans along with mild necrosis of acinar cells. AE-AI treatment protected the pancreas against degenerative changes and the pancreatic tissue revealed normal islets of Langerhans. Similarly, liver histopathological data showed mild to moderate hepatic injury with degeneration of hepatocytes in the DC group. AE-AI remarkably reduced these pathological changes and only a few hepatocytes showed necrosis. The histopathological study of kidney showed that the NC group had a normal renal capsule, nephrons, and tubules. On the other hand, DC revealed a mild coagulative necrosis in proximal and distal convoluted tubules in the cortical area, and inflammation of the spaces between renal tubules, i.e., tubulo-interstitial nephritis. The AE-AI-treated group displayed a normal glomerulus with intact basement membranes, Bowman's

space, and Bowman's capsule. Based on these findings, it is clear that AE-AI significantly prevented STZ-induced pathological changes (Figure 9).

GC-MS Analysis Revealed Potential Antidiabetic Compounds in AE-AI. The major constituents of AE-AI included 1-(5-hexenyl)-1-methylhydrazine ($C_7H_{16}N_2$), 1,2,4-triazole, 4-[N-(2-hydroxyethyl)-N-nitro]amino ($C_4H_7N_5O_3$), carbamic acid, nitrosopropyl-ethyl ester ($C_6H_{12}N_2O_3$), thiazolidine-4-one, 2-hydroxymethylimino ($C_4H_6N_2O_2S$), propylene glycol ($C_3H_8O_2$), and (S)-2-hydroxypropanoic acid ($C_3H_6O_3$) (Figure S2). Studies have shown that certain derivatives of hydrazine, triazole, thiazolidine, and carbamic acid possess antidiabetic activity^{42–45} and based on previous studies, it could be assumed that the antidiabetic activity of

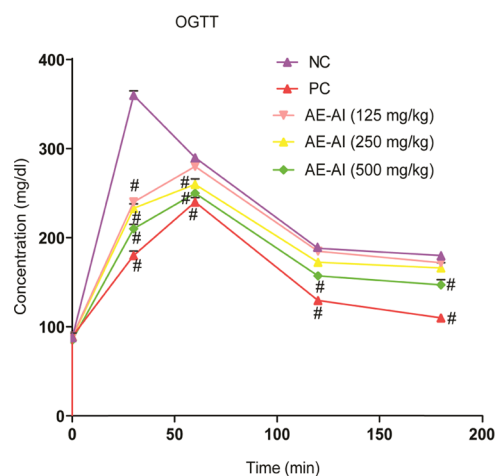


Figure 3. OGTT displayed hypoglycemic effects of AE-AI. AE-AI exhibited a dose-dependent decrease in blood glucose level as compared to NC, and at a dose of 500 mg/kg, this reduction was comparable to glibenclamide. NC = negative control, PC = positive control. Two-way ANOVA followed by Bonferroni's multiple comparison test, $n = 5$, $\# = <0.001$.

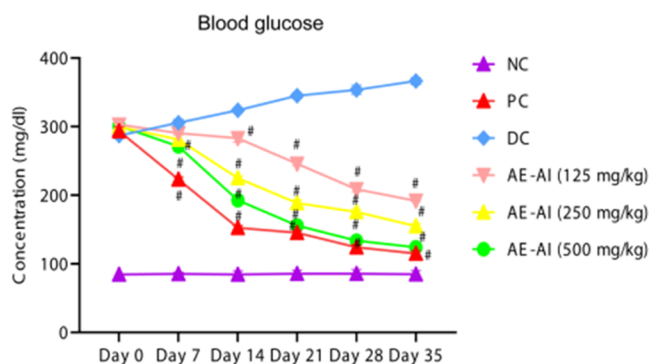


Figure 4. Antihyperglycemic effect of AE-AI in STZ-induced diabetic rats. AE-AI reduced STZ-induced blood glucose level in a dose-dependent fashion when compared to DC. NC = negative control, PC = positive control, DC = diabetic control. Two-way ANOVA followed by Bonferroni's multiple comparison test, $n = 5$, $\# \leq 0.001$.

AE-AI could be due to the presence of the above-mentioned compounds.

DISCUSSION

TII DM is one of the fastest-growing diseases worldwide, predicted to affect 693 million adults by 2045.⁴⁶ In this study, we evaluated the beneficial effects of AE-AI against STZ-induced TII DM in order to find a better alternative to antihyperglycemic therapy. STZ-induced TII DM has long been considered as an ideal diabetic model in animals. Generally, it induces diabetes by a mechanism involving its consumption by the pancreatic β -cells, which in turn produces excess ROS, leading to diabetic symptoms such as hyperlipidemia and hyperglycemia.^{47–50} The present study witnessed that AE-AI showed potent antihyperglycemic activity in diabetic rats compared to normal rats with significant improvement in body weight, serum lipids, and diabetic biomarkers associated with the pancreas, liver, and kidney. Moreover, AE-AI did not exhibit toxicities up to the dose of 2000 mg/kg in experimental rats, which indicated a high margin of safety of bioactive principles present in the extract.

Generally, most of the TII DM complications can be managed by control of blood glucose levels. Inhibition of carbohydrates hydrolyzing enzymes (α -amylase and α -glucosidase) is regarded as beneficial in controlling hyperglycemia associated with TII DM. Inhibition of these enzymes leads to delay in carbohydrates' digestion and absorption, resulting in postprandial reduction in plasma glucose levels.^{51,52} The present study revealed that AE-AI dose-dependently inhibited α -amylase, indicating its potent antihyperglycemic potential. OGTT activity reiterated a reduction in area under the curve, depicting a decrease in blood glucose levels by AE-AI. The variation in peak glucose level might be due to change in the absorption process, which is directly linked with the decreased expression of sodium glucose transporter (SGLT-1).⁵³ This suggests that a mechanism other than carbohydrate-hydrolyzing enzymes might also be involved. AE-AI treatment also demonstrated a dose-dependent decrease in glucose level in STZ-induced diabetic rats. The DC group showed hyperglycemia and hypoinsulinemia probably due to the cytotoxic effects of STZ on islets of Langerhans. The higher antihyperglycemic activity of AE-AI might also involve the increased utilization of glucose.

Moreover, histopathological data showed that diabetic animals had degenerative changes in islets of Langerhans and acinar cells. AE-AI treatment prevented STZ-induced pancreatic damage and therefore enhanced glucose regulation. STZ is

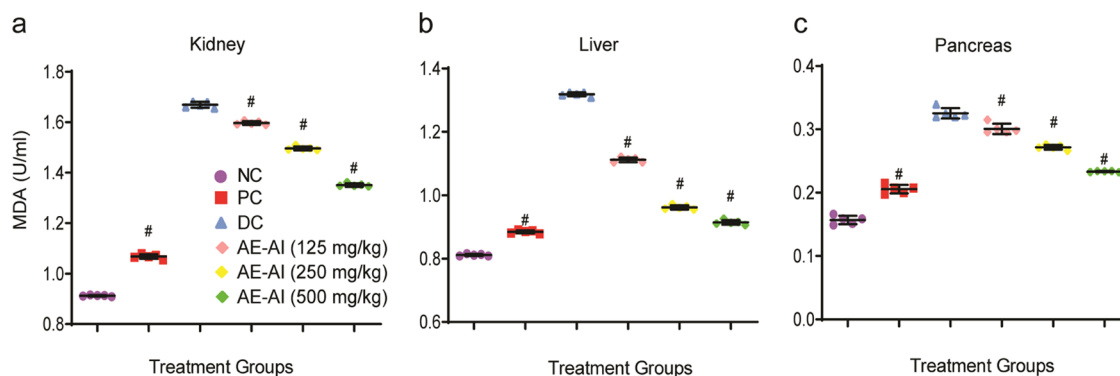


Figure 5. AE-AI reduced MDA levels in STZ-induced diabetic rats. AE-AI exhibited a dose-dependent decrease in MDA levels as compared to DC. (a) Kidney; (b) liver; (c) pancreas. NC = negative control, PC = positive control, DC = diabetic control. One-way ANOVA followed by Tukey's multiple comparison test, $n = 5$, $\# \leq 0.001$.

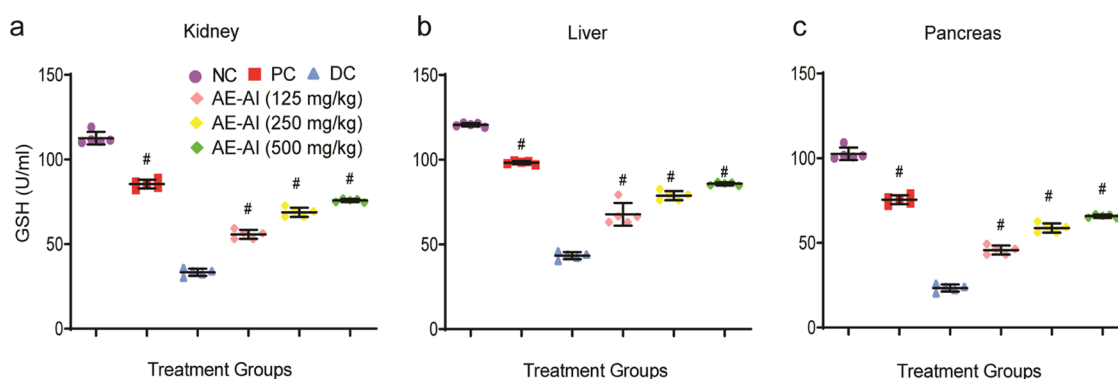


Figure 6. AI-AE-induced GSH levels in STZ-induced diabetic rats. AE-AI exhibited a dose-dependent increase in GSH levels as compared to DC. (a) Kidney; (b) liver; (c) pancreas. NC = negative control, PC = positive control, DC = diabetic control. One-way ANOVA followed by Tukey's multiple comparison test, $n = 5$, $\# \leq 0.001$.

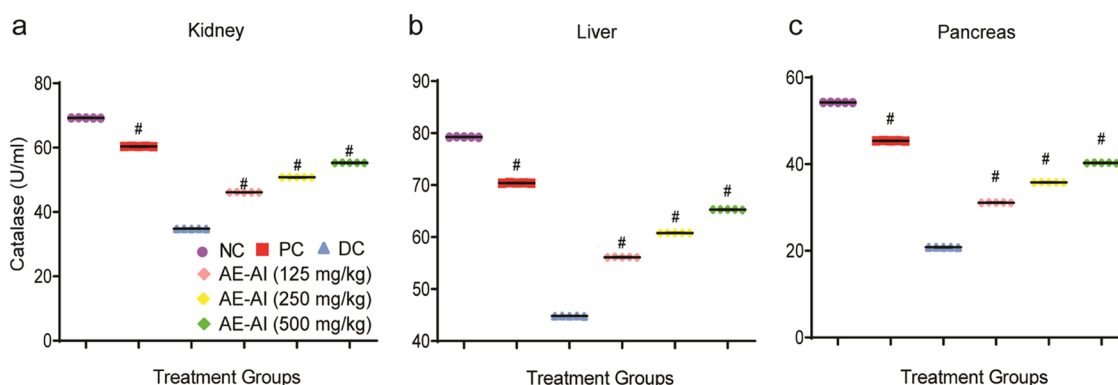


Figure 7. AI-AE-induced catalase levels in STZ-induced diabetic rats. AE-AI exhibited a dose-dependent increase in catalase levels as compared to DC. (a) Kidney; (b) liver; (c) pancreas. NC = negative control, PC = positive control, DC = diabetic control. One-way ANOVA followed by Tukey's multiple comparison test, $\# \leq 0.001$.

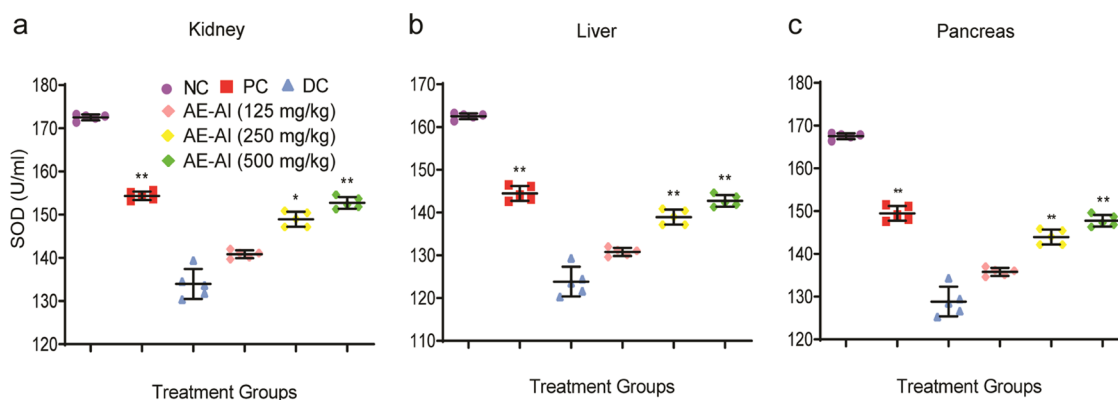


Figure 8. AI-AE-induced SOD levels in STZ-induced diabetic rats. AE-AI exhibited a dose-dependent increase in SOD levels as compared to DC. (a) Kidney; (b) liver; (c) pancreas. NC = negative control, PC = positive control, DC = diabetic control. One-way ANOVA followed by Tukey's multiple comparison test, $** \leq 0.01$, $* \leq 0.05$.

also known to damage hepatic cells and their mitochondrias by generating free radicals.⁵⁴ Previous studies have shown that STZ disturbs the hepatic architecture by inducing vacuolization of hepatocytes, congestion of blood vessels, and cellular infiltration.⁵⁵ We witnessed similar pathological changes by STZ administration and AE-AI effectively demonstrated a protective effect against TII DM-induced hepatic and renal damage. Biochemical parameters also revealed that AE-AI treatment reduced the levels of ALT, AST, and ALP, which are the indicators of hepatic damage. The improvement in the

hepatic enzyme levels can be attributed to the decrease in free fatty acid and peroxides in the serum and reduced oxidation.^{56,57} TII DM is linked with diabetic nephropathy, which normally leads to renal morbidity and mortality. In this ailment, glomerular sclerosis and fibrosis occur, which results in proteinuria. Reduced insulin concentration leads to minimal energy and increases the catabolism of proteins, ultimately resulting in dysfunctioning of the glomerulus.^{53,58} A reduction in urea and creatinine values was observed in the animals after treatment with AE-AI. This improvement in the renal

Table 2. AE-AI Restored Biochemical Parameters Associated with TII DM-Mediated Hepatic Damage and Dyslipidemias^a

parameters	PC	NC	DC	AE-AI(125 mg/kg)	AE-AI(250 mg/kg)	AE-AI(500 mg/kg)
ALP	89.2 ± 0.86	118 ± 1.53	185 ± 1.39	169.2 ± 1.24**	147.8 ± 2.08 [#]	126 ± 2.05 [#]
ALT	45.8 ± 1.11	63.2 ± 1.14	137.2 ± 3.80	116.8 ± 2.26*	100.0 ± 1.61**	79.1 ± 0.54 [#]
AST	142.0 ± 0.8	168 ± 1.74	214 ± 2.92	209.0 ± 1.18	201.8 ± 1.77*	186 ± 1.15
total lipids	347 ± 1.17	374 ± 1.46	448 ± 1.02	422.0 ± 0.86*	400 ± 2.13 [#]	388 ± 1.98*
TC	71.4 ± 1.50	86.2 ± 1.50	205 ± 2.42	133.1 ± 1.10*	123 ± 1.02 [#]	98.8 ± 0.73**
triglycerides	76.2 ± 1.28	86.2 ± 0.58	132 ± 1.52	121.2 ± 0.37**	115 ± 0.812 [#]	101 ± 1.38 [#]
HDL	28.8 ± 0.37	22.8 ± 0.73	16.1 ± 0.44	17.6 ± 0.67 [#]	18.8 ± 1.30 [#]	21.3 ± 0.54 [#]
LDL	34.0 ± 1.18	54.0 ± 1.30	171 ± 2.68	128 ± 2.35 [#]	93.4 ± 2.16 [#]	67.1 ± 0.89 [#]
VLDL	17.6 ± 0.51	29.6 ± 0.61	32.8 ± 0.58	36.6 ± 0.51 [#]	34.4 ± 0.67 [#]	32.2 ± 0.31 [#]

^aNC = negative control, PC = positive control, DC = diabetic control.

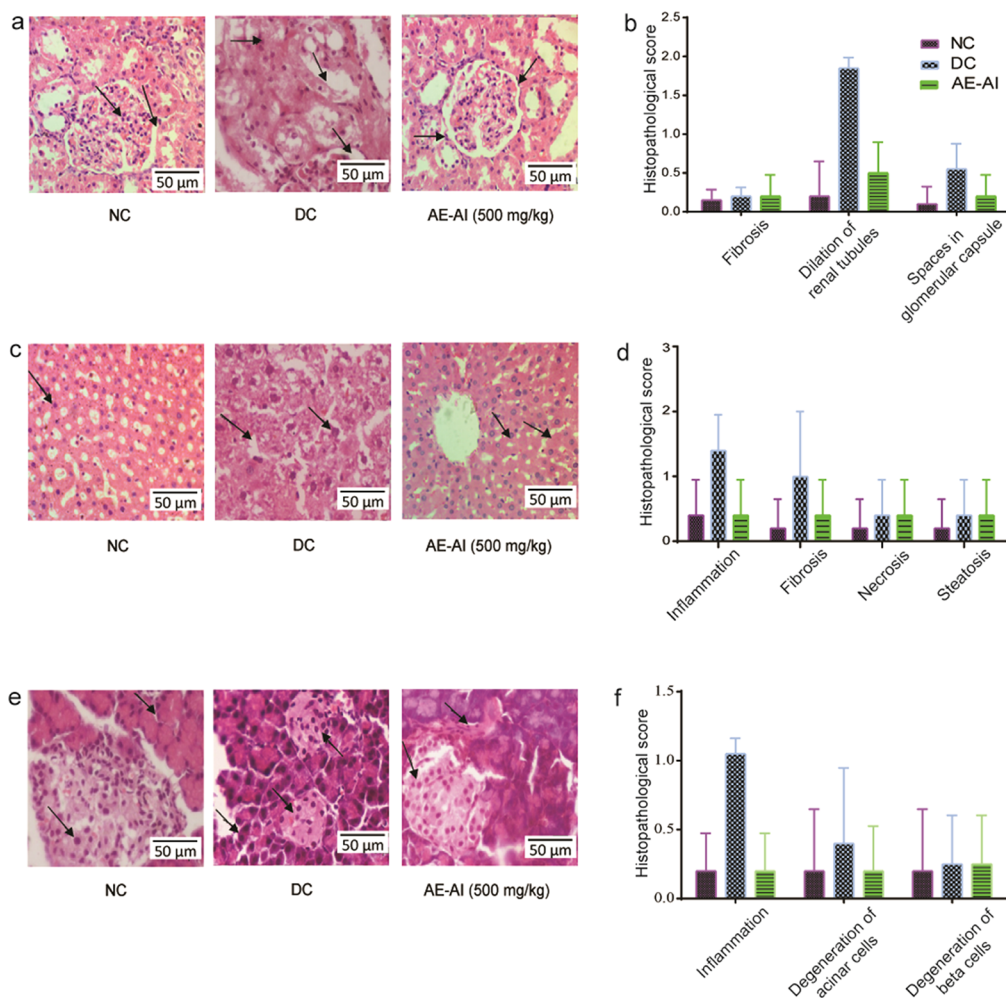


Figure 9. Histopathological analyses revealed the protective effect of AE-AI against STZ-induced tissue damage. (a, b) Kidney: NC group exhibited a normal renal capsule, nephrons, and tubules, while DC revealed a mild coagulative necrosis in proximal and distal convoluted tubules in the cortical area and tubulo-interstitial nephritis. Treatment with AE-AI showed normal architecture with intact basement membranes, Bowman's space, and Bowman's capsule. (c, d) Liver: NC exhibited normal hepatocytes, sinusoidal spaces, and hepatic plates, while DC showed moderate to severe hepatocytic degeneration in the peripheral zone. Treatment with AE-AI showed almost normal hepatocytes with few necrotic regions. (e, f) Pancreas: NC showed normal islets of Langerhans, whereas the DC group revealed severe inflammation, and degenerative changes in the islets of Langerhans along with mild necrosis of acinar cells. Treatment with AE-AI prevented severe inflammation and degeneration of acinar cells. NC = negative control, DC = diabetic control.

parameters was also supported by the histopathological findings, which showed that treatment with AE-AI resulted in the prevention of tubular degeneration and glomerular thickening.

In addition, risk of coronary heart disease increases with TII DM due to the altered lipid metabolism.^{59,60} Increase in

triglyceride, LDL, and TC levels along with reduction in HDL has been reported in DM.⁶¹ Restoring blood glucose and lipid levels is therefore essential in controlling TII DM and reducing the chances of contracting DM-associated diseases. In this study, AE-AI significantly normalized the lipid levels, indicating its beneficial effects against TII DM-mediated dyslipidemia.

These results resemble the previous findings that reported that higher doses of a hydro-alcoholic extract of *A. capillus-veneris* L. showed increase in HDL levels and significant reduction in TC, triglycerides, LDL, VLDL, and atherogenic coefficient levels.⁶²

AE-AI also showed higher levels of TPC and TFC, which were relatively more than that of *A. capillus-veneris* L.⁶³ This variation in antioxidant levels might be due to specie difference or the solvent used for extraction. These *in vitro* findings were further validated by the antioxidant activity of AE-AI in the STZ-induced TII diabetic model. AE-AI treatment of STZ-induced TII diabetic rats increased SOD, CAT, and GSH levels and decreased MDA in the liver, kidney, and pancreas. Free radical-mediated diseases like TII DM can be improved by consuming antioxidants. Antioxidant enzymes like SOD, CAT, and GSH are considered the primary line of antioxidant defense. Free radicals either increase insulin resistance or impair insulin secretion. Numerous studies have reported the possible contribution of free radicals and reduced levels of antioxidants in the induction of TII DM.^{40,64,65} Phenolic substances have been reported to rapidly inactivate lipid-free radicals and display antioxidant property.⁶⁶ Moreover, these compounds produce lactones that interact with nucleophilic groups of α -amylase, resulting in its inhibition.⁴⁰ Flavonoids also have antioxidant properties and normally increase insulin release, thereby producing antihyperglycemic effects.⁴⁰ Based on the above observations, it is conceivable that the presence of phenolic compounds and flavonoids in AE-AI might be responsible for improvement of the STZ-mediated oxidative stress and TII DM. The current study also revealed an encouraging correlation with an earlier research where it was claimed that flavonoids from *A. capillus-veneris* L. increased insulin serum levels and produced antidiabetic effects in rats.

CONCLUSIONS

It is conceivable from this study that AE-AI possesses certain biologically active compounds that might be involved in reducing oxidative stress and hyperglycemia in STZ-induced TII diabetic rats. Based on this study, further studies could be performed to isolate the active principles of AE-AI in order to identify the antidiabetic compounds and elucidate their possible mechanism of actions. Moreover, future studies are required to validate these findings in human models.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.2c04673>.

The supplementary data about the impact of AE-AI treatment on body weights of animals and GC-MS analysis of AE-AI can be found in supporting information file (PDF)

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Author Contributions

H.M.B. performed the research. H.M.Z. and M.N.H.M. analyzed the histopathological and antioxidant data. All authors participated in editing the manuscript. H.M.B. wrote the first version of the manuscript. A.S. designed and supervised the project. A.S. and M.N.H.M. edited the final version of the manuscript.

Notes

The authors declare no competing financial interest.

All of the experiments were performed according to the OECD guidelines and were approved by the Institutional Research Ethics Committee of the Faculty of Pharmacy, The University of Lahore (Approval number: IREC-2020-09).

Any other relevant material or data can be provided on request.

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