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Ameliorative activity of standardized *Coccoloba uvifera* leaves extract against streptozotocin-induced diabetic rats via activation of IRS-1/PI3K/AKT/GLUT2 pathway in liver

Fatma Abdelhakim Mohamed[1], Rabab H. Sayed[2,5], Mohammed N. A. Khalil[3], Mohamed A. Salem[4,6], Amira S. El Senousy[3] and Ali M. El-Halawany[3*]

Abstract

Background *Coccoloba uvifera* L. (Family: Polygonaceae) known as sea grape is natively distributed in middle and south America. The aqueous leaf extract showed inhibitory activities against *α*-glucosidase and *α*-amylase in previous reports. Moreover, the hydroalcoholic leaves extract ameliorated hyperglycemia in the oral glucose tolerance test. Despite these promising results, the extracts used in these studies were not standardized, nor was their mechanism of action elucidated. The current study aims to standardize the ethanolic *C. uvifera* leaves extract (CU) using markers, and assess its ameliorative activity against diabetes and its hepatoprotective activity against diabetic complications.

Results Standardized leaves' ethanolic extract contained 0.09 ± 0.00057 and 0.23 ± 0.0011 mg/g gallic acid and rutin, respectively, as estimated by HPLC. Administration of CU (100, 200 and 400 mg/kg) for 6 weeks ameliorated DM manifestations in STZ-induced diabetic rats in a dose-dependent manner. The ethanolic extract reduced fasting blood glucose, increased serum insulin and reduced elevated liver enzymes. CU counteracted oxidative stress, promoted glucose metabolizing enzymes and reduced gluconeogenesis enzymes. The underlying mechanism involved increased expression of IR, IRS-1, IRS-2 and GLUT2 in liver tissue through activation of PI3K/AKT signaling. The histopathological study demonstrated reduced inflammation and hepatocyte degeneration.

Conclusion CU could be used as a promising antidiabetic drug with hepatoprotective activity in diabetes hepatic complications. The standardized CU ethanolic extract should be further assessed clinically alone or in combination with other antidiabetic remedies.

Keywords *Coccoloba uvifera*, Antidiabetic, PI3K/AKT, Hepatoprotective, Antioxidant, IR/IRS-1/IRS-2

*Correspondence:
Ali M. El-Halawany
ali.elhalawany@pharma.cu.edu.eg
Full list of author information is available at the end of the article

Background

Combating diabetes mellitus (DM) and its complications is one of the major challenges facing the healthcare system worldwide. DM has a high prevalence among all ages. It afflicted 463 million people in 2019, projected to increase to 578 million (10.2%) by 2030 [1]. DM complications due to persistent hyperglycemia harmfully affect vital physiological systems involving the Kidney, neurons, heart and liver [2]. One of these complications is hepatotoxicity [3].

DM is associated with multiple liver abnormalities such as abnormal glycogen deposition, non-alcoholic fatty liver disease, fibrosis, cirrhosis, abnormal elevated hepatic enzymes, acute liver disease and viral hepatitis [4]. Excessive accumulation of fats in the liver with persistent hyperglycemia may worsen insulin resistance leading to severe metabolic dysfunction. Consequently, this would increase the mortality and morbidity within diabetic patients through the damage of hepatocytes. The biochemical alterations induced by DM are comparable to those observed in serious liver diseases, e.g., the formation of hepatocellular carcinomas (HCCs) and even end-stage liver failure [5].

A combined pharmacological therapy approach is applied to have a better control on the serum glucose level and to limit diabetes complications, e.g., metformin and pioglitazone (thiazolidinediones), together with other drugs such as atorvastatin, betaine, losartan and orlistat [6, 7]. However, these synthetic drugs possess not only undesirable side effects but also high cost. Herbal plants are safe and cost-effective complementary therapy for diabetic patients [8, 9].

Coccoloba uvifera L. (Family: Polygonaceae) known as sea grape which is natively distributed in Middle and South America [10]. The sea grape was traditionally used by the Native Americans to make medicinal teas from its leaves, bark and roots. The sea grape's astringent juice and decoctions of wood, bark and roots were used to treat diarrhea, dysentery, hemorrhages and venereal diseases; they were also applied externally for rashes and other skin afflictions. A tea made from the leaves was used to treat hoarseness and asthma, as well as to bathe wounds. The resinous gum of the bark was also used against throat ailments, and the root decoction was used to treat dysentery [11].

Leaves of sea grape have many biological activities such as antidiabetic, antioxidant, anti-inflammatory, antimicrobial and cytotoxic activities [12–14]. The aqueous extract of leaves showed inhibitory activities against α-glucosidase and α-amylase enzymes [15]. Moreover, the leaf's hydroalcoholic extract ameliorated hyperglycemia in the oral glucose tolerance test [14]. In addition, a patent application demonstrated effective treatment of a diabetic condition by the daily ingestion of a tea brewed from *Coccoloba uvifera* leaves ethanolic extract [16]. Several compounds, such as flavonoids, anthraquinones, anthocyanins and terpenoids, were isolated from the plant [17, 18]. Moreover, different phenolic acids were quantified from *C. uvifera* leaves ethanolic extract which have been reported to control DM and its complications [12, 19]. Despite these promising results, the ethanolic extracts were not standardized, nor was their mechanism of action elucidated. The current study aims to (i) standardize the ethanolic *C. uvifera* leaves extract (CU) using markers, (ii) assess the ameliorative activity of the standardized extract against diabetes and (iii) assess its hepatoprotective activity against diabetic complications.

Materials and methods

Chemical and reagents

For extraction and HPLC analysis, respectively, solvents of analytical and HPLC grades were used. They were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant material and extraction

Leaves (2 kg) were air-dried and grinded to coarse powder. Powdered leaves were exhaustively extracted with 95% ethanol by maceration (5 X 10L). Under reduced pressure, the solvents were distilled off using Buchi Rotavapor (Switzerland) to yield 75 g of greenish-black extract of CU, and it was stored in closed airtight container at 4 °C. Aliquots were used for the chemical analysis and the pharmacological study.

Chemical standardization of *Coccoloba* uvifera L. extract

Determination of total phenolic and flavonoid content

Total phenolics was assessed using Folin–Ciocalteu reagent and it was expressed as mg/g gallic acid equivalents (GAE) [20]. Gallic acid standard curve was set up at concentration range (250–15.6 µg/mL). The assay was constituted by mixing 10% Folin–Ciocalteu reagent (200 µL) with CU extract, gallic acid or methanol (100 µL) to form sample, standard and blank assay mixtures. Next, saturated Na_2CO_3 solution (7%, 800 µL) was added and mixed. The mixture was incubated for an hour at room temperature in a dark place. Aliquots of the assays (200 µL) were transferred to a transparent 96-well plate to measure the absorbance at 630 nm using plate reader (ELx 808, BIO-TEK Instruments, US).

Besides, the total flavonoid content was investigated using aluminum chloride colorimetric assay [21]. The flavonoid–aluminum complexes were measured at absorbance 403 nm. Quercetin (100–10 µg/mL) was chosen as a reference standard. 250 µL $AlCl_3$ aqueous solution 10% was combined and vortexed with 500 µl extracts,

methanol or quercetin and 250 µL of water. Following a 10-min incubation period at room temperature, 250 µL of the aliquots was transferred to a transparent 96-well plate, water was replaced with the same volume as $AlCl_3$ in the blank. The amount of flavonoid compounds in the dry extract was represented as milligrams equivalents of quercetin per gram (mg/g QE).

High-performance liquid chromatography (HPLC) analysis

Aliquots of CU (121 mg) were dissolved in methanol:water (50:50) at final concentration 50 mg/ml, filtered using Millex-HV 0.45-mm membrane filters (Millipore, Bedford, MA, USA). HPLC analysis was carried out on waters 2690 Alliance HPLC system equipped with a Waters 996 photodiode array detector PDA (Milford, MA, USA). The flow rate was maintained at 1.0 mL/min; 10 µL sample was injected; and the chromatogram was traced at 280 nm. Chromatographic separations were performed on a Kromasil C-18 guard column (5 µm, 4.6 × 250 mm) (Eka Chemicals, Sweden). For the standardization process, rutin and gallic acid were selected as markers for the standardization of CU. A standard calibration curve was performed in the detector linear range from 10 to 50 µg/mL for both standards (supplementary data, Figures S1, S2). The mobile phase comprises of mixture A (0.1% phosphoric acid in water) and B (methanol) run in gradient mode. The following gradient elution was used: 95/5% A/B for 3 min, then increased to 50% B at 50 min, then elevated to 30/70% A/B at 55 min, followed by 10/90% A/B in 75 min. Under the previous conditions, gallic acid and rutin were eluted at 13.60 and 51.16 min, respectively (Fig. 1).

The pharmacological study

Animal

Adult male Wistar albino rats weighing 150–200 g were used. Rats were kept under controlled environmental conditions, humidity (60 ± 10%), a light/dark cycle of 12/12 h and room temperature. All animals had free access to food and water throughout the study.

Experimental design

For diabetes induction, rats were fasted overnight then injected with STZ (40 mg/kg, i.p.) (Sigma-Aldrich Chemicals (St. Louis, MO, USA), which was freshly prepared in cold 0.1 M citrate buffer (pH 4.5) [22]. STZ-injected rats were allowed to drink 5% glucose solution for 24 h to overcome initial hypoglycemic mortality induced by STZ. After 48 h of injection, diabetic animals were identified by measuring blood glucose level using an analyzer (Roche Diagnostic Accu-Check test strips, Germany). For the experiment, rats with blood glucose levels more than 250 mg/dl were selected.

Rats were randomly divided into five groups ($n = 6$ per group) as follows: diabetic untreated group (STZ), diabetic CU at three dose levels (100, 200 or 400 mg/kg, p.o.) treated groups [14] and a fifth group that received a single citrate buffer i.p. and daily 0.1% tween 80 p.o and served as normal control. CU was dissolved in 0.1% tween 80 and administered for 6 weeks starting from the third day after the injection of STZ. Animals were weighed on the first and last day of treatment. The collection of blood was from the retro-orbital sinus under light anesthesia (sodium pentobarbital; 10 mg/kg, i.p), and serum was separated and stored at − 80 °C to be used for the estimation of insulin and liver enzymes. Animals were euthanized by cervical dislocation under anesthesia and the liver was removed and washed with saline. A part of liver was placed in 10% (v/v) formalin for 24 h for histopathological analysis. The other parts were stored at − 80 °C for biochemical analysis. Frozen tissues were homogenized in a buffer for biochemical testing.

Biochemical analysis

Measurement of fasting blood glucose and serum insulin levels

Blood glucose levels were determined from tail vein of overnight fasted rats using glucometer (Roche Diagnostic Accu-Check test strips, Germany). According to the manufacturer's instructions, serum insulin levels were assessed using a commercially available rat ELISA kit (Ray Biotech, Peachtree Corners, GA, USA).

Measurement of serum aminotransferases and liver oxidative stress biomarkers

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by kits supplied by Biodiagnostic (Cairo, Egypt). Besides, liver malondialdehyde (MDA) as an index of lipid peroxidation and reduced glutathione (GSH) contents were measured colorimetrically according to the methods of [23] and [24], respectively. Results are expressed as nmol/g for MDA and µg/g for GSH.

Estimation of liver carbohydrate metabolic enzyme activities and glycogen content

Carbohydrate metabolic enzymes such as hexokinase, glucose-6-phosphatase, fructose-1,6-bisphosphatase and glycogen content were measured by the method of [25–27] and [28], respectively.

Quantitative real-time polymerase chain reaction

Using an SV Total RNA Isolation System (Promega, Madison, WI, USA), total RNA was isolated from the liver tissues, and the purity of the resulting RNA was determined spectrophotometrically at 260/280 nm.

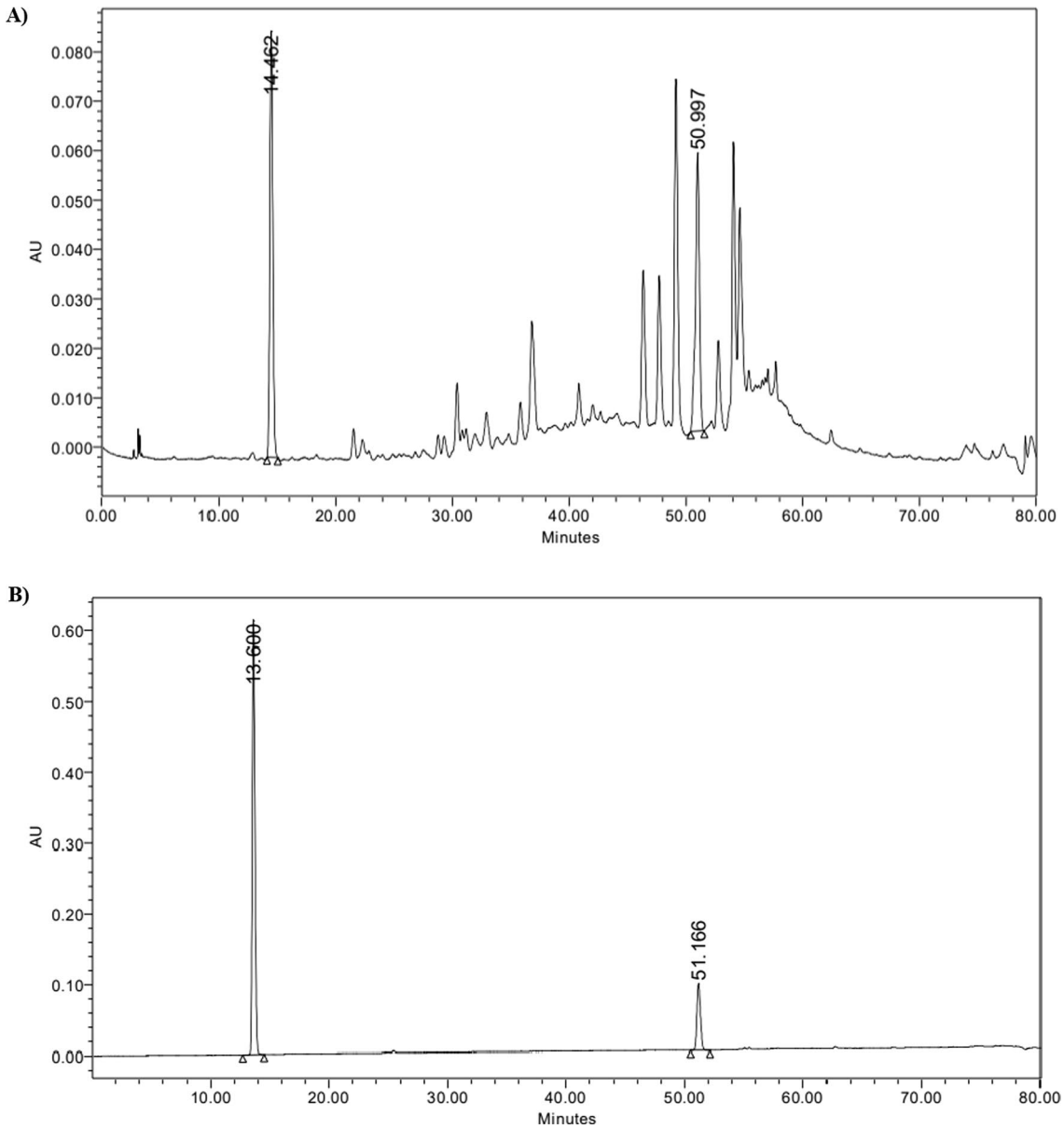

Fig. 1 HPLC standardization of CU leaves extract. **A** Chromatogram of total ethanolic leaves extract of *C. uvifera* (CU) traced at 280 nm. **B** Authentic gallic acid and rutin chromatogram eluted at 13.6 and 51.16 min at 280 nm, respectively

After that, the same quantities of RNA were reverse-transcribed into cDNAs using Reverse Transcription System (Promega). Quantitative RT-PCR of IR, IRS-1, IRS-2, GLUT2 and β-actin mRNAs was performed using SYBR green (iTaq Universal SYBR Green Supermix, Bio-Rad, Hercules, CA, USA). Table 1 describes the primer sequences employed. The thermal cycler protocol's initial enzyme activation stage was performed for 10 min at 95 ∘C. This was followed by 40 cycles of 15 s denaturation at 95 ∘C and 60 s annealing/extension at 72 ∘C. Using the $2^{-\Delta\Delta CT}$ formula, the

Table 1 Primer sequences used in RT-PCR

Gene	Primer sequence (5′-3′)
IR	F: TGGACATCCGGAACAACCTG R: TCTGCAGATGGCCCTCAATG
IRS-1	F: TGTGCCAAGCAACAAGAAAG R: ACGGTTTCAGAGCAGAGGAA
IRS-2	F: CTACCCACTGAGCCCAAGAG R: CCAGGGATGAAGCAGGACTA
GLUT2	F: GTCAGAAGACAAGATCACCGGA R: AGGTGCATTGATCACACCGA
β-Actin	F: ATCCTGGCCTCACTGTCCA R: AACGCAGCTCAGTAACAGTC

relative expression of target genes was determined. Each value was expressed as a fold change and normalized to β-actin levels.

Western blot analysis

After the total protein was extracted from the liver tissue, equal amounts of protein (10 µg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein was transferred to a nitrocellulose membrane (Amersham Bioscience, Piscataway, NJ, USA). Later, the membranes were washed with phosphate-buffered saline (PBS) and blocked via immersion in 5% (w/v) skim milk powder overnight. The membranes were then incubated with primary antibodies against p-PI3K (Tyr458, Tyr199) (1:1000, Cat. No. PA5-17,387), p-AKT (Ser473) (1:1000, Cat. No. 44-621G) and β-actin (1:500, Cat. No. PA1-183) obtained from Thermo Fisher Scientific Inc. (Rockford, IL, USA). Subsequently, the membranes were washed, and secondary antibodies labeled with peroxidase were added and they were incubated for one hour at 37 ∘C. The ChemiDoc™ imaging equipment with Image Lab™ software version 5.1 (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used to analyze the band intensity. The results were expressed using arbitrary units following normalization to the expression of the β-actin protein.

Histopathological analysis

The fixed specimens of the liver were processed overnight for dehydration, clearing and impregnation using an automatic tissue processor. Using an embedding station, the specimens were embedded in paraffin blocks, and a microtome was used to cut serial sections with a thickness of 5 µm. Hematoxylin and eosin staining were used for routinely staining of the tissue sections. Using the light microscopy, the mounted specimens were observed and scored as previously described [29]; a semi-quantitative comparison was conducted for the structural changes, and the abnormalities in the tissue sections were graded from 0 (normal structure) to 3 (severe pathological changes).

Statistical analysis

Data are expressed as mean ± standard deviations (S.D.). All results obtained were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical analyses were performed using GraphPad Prism software (version 9; GraphPad Software, Inc., San Diego, CA, USA). A probability level of < 0.05 was accepted as statistically significant in all statistical tests.

Results

Characterization of *Coccoloba* uvifera L. ethanolic leaves extract.

It was imperative to standardize the CU extract before the pharmacological study, The total phenolic content CU was determined to be 205.26 ± 0.362 mg of GAE/g of dry extract. At the same time, the total flavonoid content assay revealed the presence of 32.756 ± 0.507 mg QE/g of dry extract. HPLC analysis revealed the presence of rutin as a major flavonol glycoside and gallic acid as a major phenolic acid in *C. uvifera* leaves with concentration of 0.23 mg ± 0.0011 and 0.09 ± 0.00057 mg per gram of extract, respectively (Fig. 1).

Effect of CU extract on STZ-induced changes in body weight, fasting blood glucose and serum insulin

STZ diabetic rats exhibited a significant body weight loss at the end of the 6-week experiment (Fig. 2). However, treatment with CU extract (100, 200 or 400 mg/kg) increased the body weight of animals as compared to STZ diabetic rats. Further, STZ produced a marked elevation in serum fasting blood glucose level to reach 4.9-fold

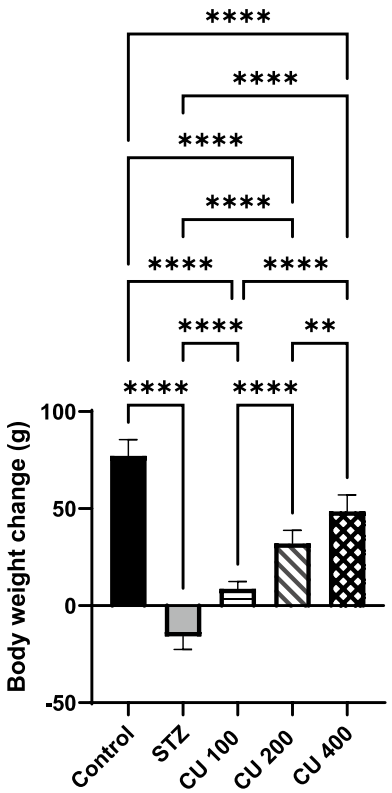

Fig. 2 Effect of CU extract on STZ-induced changes in body weight. Each bar with a vertical line represents the mean of experiments ± S.D. ($n = 6$). Statistical analyses were performed using the one-way ANOVA followed by Tukey's multiple comparison test, with the criterion for statistical significance as follows: $^{**}p < 0.01$ and $^{****}p < 0.0001$

along with a decline in serum insulin level to reach 48.6% of the control group values (Fig. 3A, B). Treatment with STZ increased blood glucose level to 485.20 mg/dL compared to control group showing 98.33 mg/dL. Treatment with CU extract (100, 200 or 400 mg/kg) lowered fasting blood glucose to attain 382.20, 368.70 and 195.30 mg/dL, respectively. Administration 200 or 400 mg/kg CU only increased serum insulin to reach 1.5-fold and 1.8-fold, respectively, compared to the STZ group Table S1. It was obvious that CU was effective in a dose-dependent manner.

Effect of CU extract on STZ-induced changes in liver enzymes and oxidative stress biomarkers

STZ diabetic rats showed markedly elevated serum activity of AST and ALT to reach 3.5-fold and 2.8-fold the control values, respectively (Fig. 4A, B). Administration of CU extract (100, 200 or 400 mg/kg) to diabetic rats resulted in a significant decrease in the serum activity of AST to reach 61.1%, 51.7% and 39.2%, and ALT to reach 88.8%, 71.3% and 44.1%, respectively, compared to the STZ group values (Table S1). Likewise, induction of diabetes using STZ increased lipid peroxidation as evidenced by elevated hepatic MDA level to reach 2.9-fold and depleted hepatic GSH content to reach 34.5% as compared to diabetic rats (Fig. 4C, D). CU extract (100, 200 or 400 mg/kg) treatment was effective in reducing MDA level to reach 76.8%, 53.7% and 44.7%, and replenishing GSH content to reach 1.6-fold, 2-fold and 2.4-fold, respectively, as compared with STZ-treated rats. As previously elucidated, the effect of CU was dose-dependent.

Effect of CU extract on STZ-induced changes in hepatic carbohydrate metabolic enzyme activity and glycogen content

The liver tissue of STZ diabetic rats revealed a significant inhibition in the activity of hexokinase and glycogen content to reach 61.4% and 45.1%, respectively, along with a substantial increase in the activity of glucose-6-phosphatase and fructose-1,6-bisphosphatase to reach 2.6-fold and 3.1-fold, respectively, in comparison with the control group (Fig. 5). On the other hand, treatment with CU extract (200 or 400 mg/kg) inversed the decrease in hexokinase to reach 1.3-fold and 1.5-fold, as well as

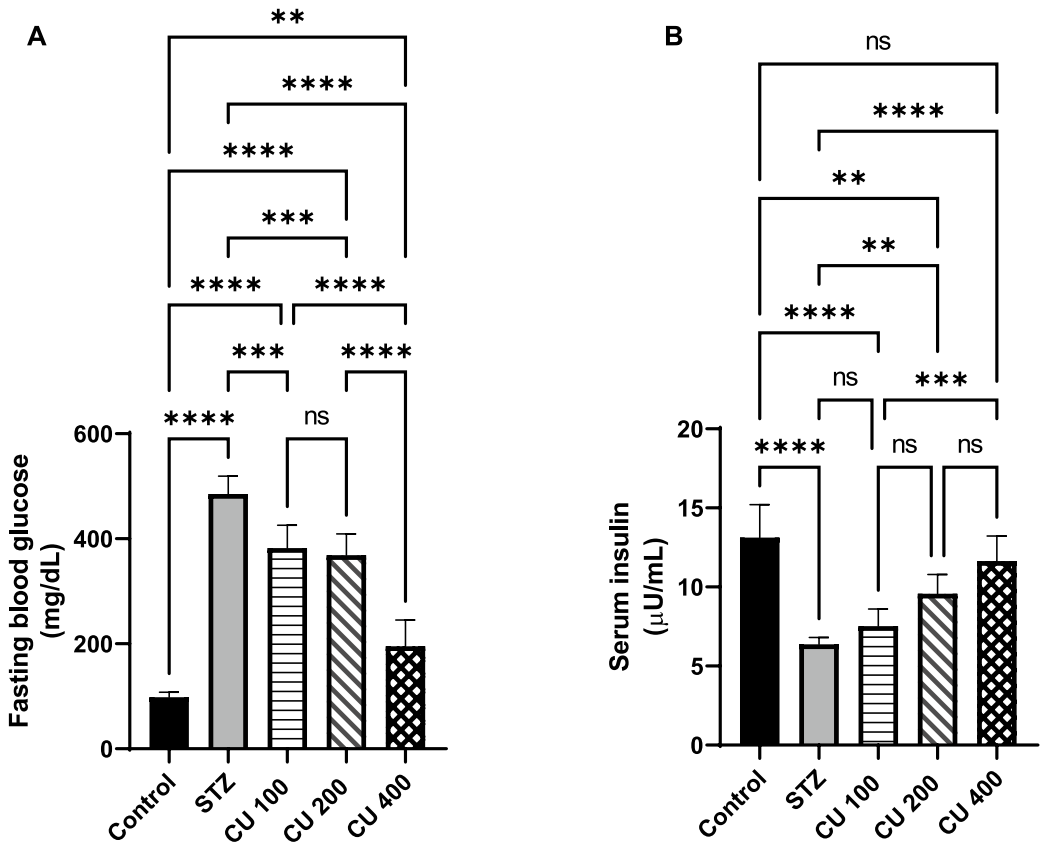

Fig. 3 Effect of CU extract on STZ-induced changes in fasting blood glucose (**A**) and serum insulin (**B**). Each bar with a vertical line represents the mean of experiments ± S.D. ($n = 6$). Statistical analyses were performed using the one-way ANOVA followed by Tukey's multiple comparison tests, with the criterion for statistical significance as follows: $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$ and ns = no significance

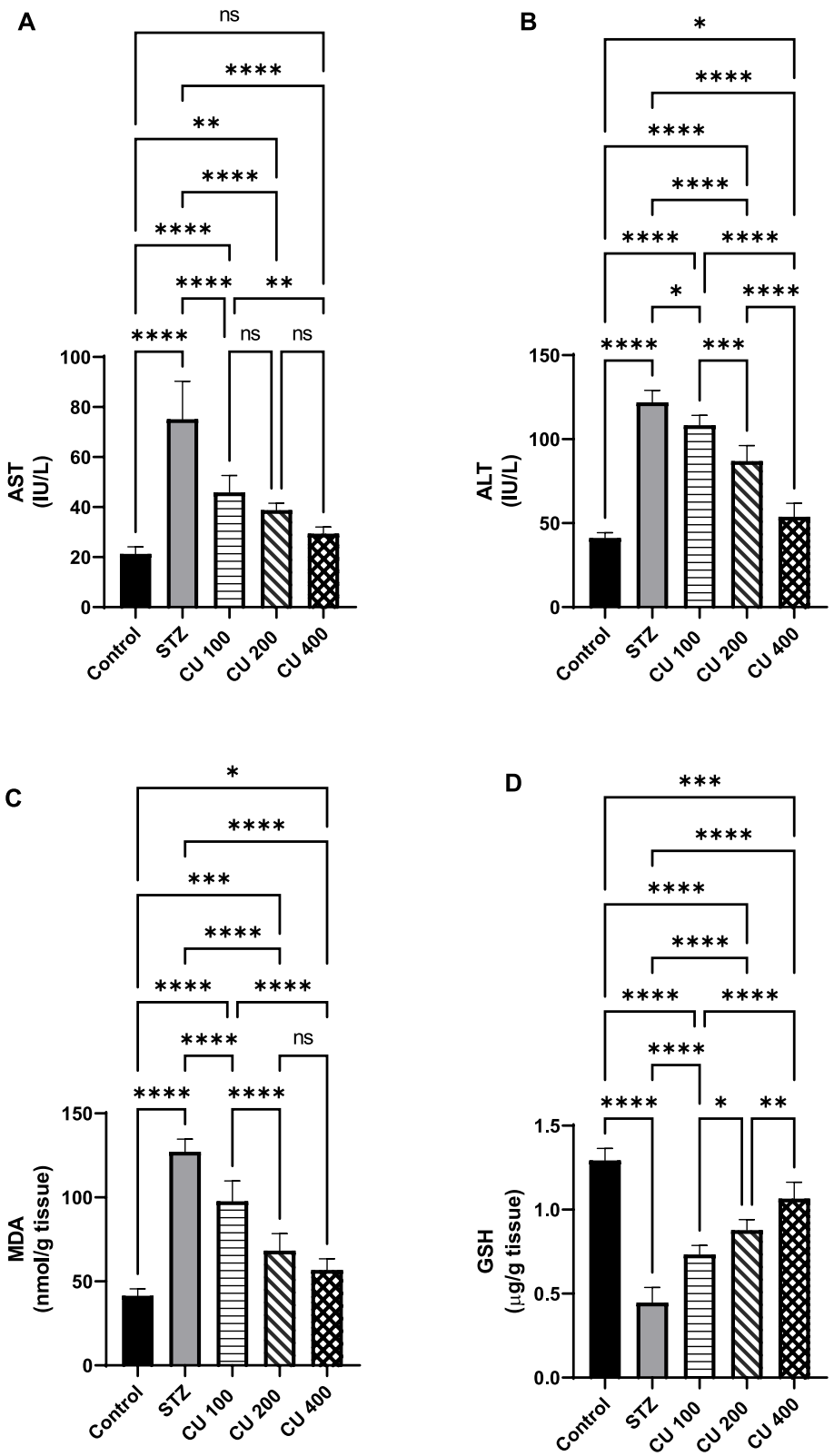

Fig. 4 Effect of CU extract on STZ-induced changes in serum AST (**A**), serum ALT (**B**) activities, hepatic MDA (**C**) and hepatic GSH (**D**) contents. Each bar with a vertical line represents the mean of experiments ± S.D. ($n = 6$). Statistical analyses were performed using the one-way ANOVA followed by Tukey's multiple comparison test, with the criterion for statistical significance as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$ and ns = no significance

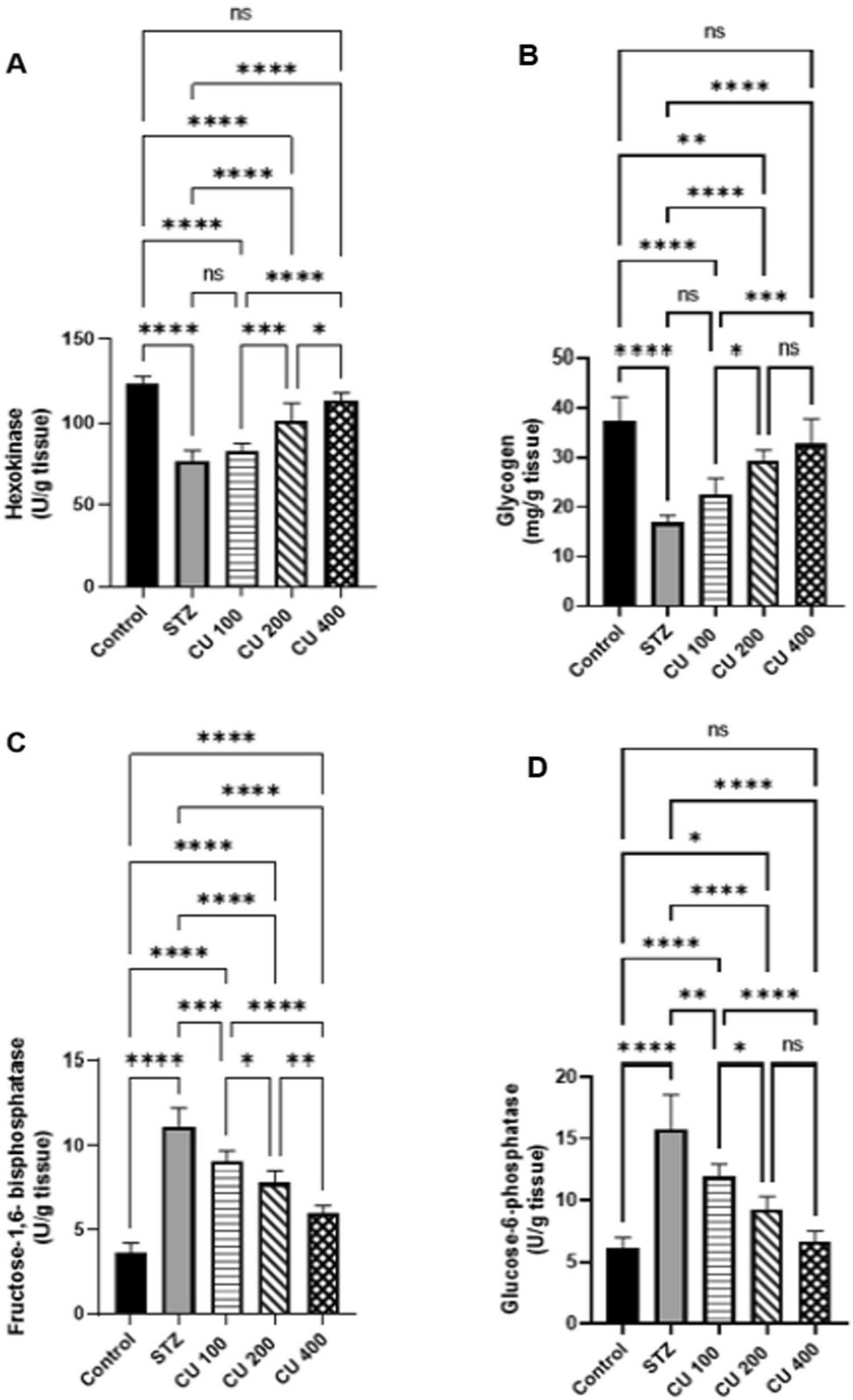

Fig. 5 Effect of CU extract on STZ-induced changes in the hepatic activity of hexokinase (**A**), glycogen content (**B**), fructose-1,6-bisphosphatase (**C**) and glucose-6-phosphatase (**D**). Each bar with a vertical line represents the mean of experiments ± S.D. ($n = 6$). Statistical analyses were performed using the one-way ANOVA followed by Tukey's multiple comparison test, with the criterion for statistical significance as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$ and ns = no significance

glycogen to reach 1.7-fold and 1.9-fold, respectively, as compared to STZ group (Fig. 5A, B). Similarly, administration of CU extract (100, 200 or 400 mg/kg) to diabetic rats decreased the glucose-6-phosphatase activity to reach 75.8%, 58.7% and 42.4%, respectively, and fructose-1,6-bisphosphatase to reach 80.9%, 69.8% and 53.8%, respectively, as compared to STZ group (Fig. 5C, D).

Effect of CU extract on STZ-induced changes in mRNA expression of IR, IRS-1, IRS-2 and GLUT2 in liver tissue

Induction of diabetes by STZ significantly downregulated the mRNA expression of hepatic IR, IRS-1, IRS-2 and GLUT2 to reach 28.1%, 16.1%, 38.4% and 30.7%, respectively, in comparison with control rats (Fig. 6). Treatment of diabetic rats with CU extract (100, 200 or 400 mg/kg) significantly improved the expression of IR to reach 2.2-fold, 2.3-fold and 2.8-fold, respectively, IRS-1 to reach 3.3-fold, 3.4-fold and 4.3-fold, respectively, IRS-2 to reach 1.9-fold, 1.9-fold and 2.3-fold, and GLUT2 to reach 2.2-fold, 2.2-fold and 2.7-fold, respectively, compared to STZ group values.

Effect of CU extract on STZ-induced changes in PI3K/AKT signaling in liver tissue

STZ diabetic rats demonstrated a decreased expression of p-PI3K and p-AKT to reach 30.3% and 16.8%, respectively, compared to normal rats (Fig. 7). In contrast, CU extract (100, 200 or 400 mg/kg) administration effectively ameliorated STZ-induced downregulation of p-PI3K to reach 2.6-fold, 2.5-fold and 2.5-fold, respectively, and p-AKT to reach 3.6-fold, 3.6-fold and 4.7-fold, respectively, compared to STZ group.

Effect of CU extract on STZ-induced histopathological changes in liver tissue

Control group (Fig. 8A, B) showed normal hepatic parenchyma without any detectable histopathological alteration. On the other hand, examination of hepatic tissue of STZ group (Fig. 8C, D) showed serious injuries in the hepatic parenchyma. Multifocal random and portal mononuclear inflammation infiltration were detected in the several examined sections accompanied by increased portal fibroplasia and necrobiotic changes in the surrounding hepatocytes. Marked oval cells hyperplasia were commonly observed among several affected sections. Mild improvement was detected in CU (100 mg/kg) group (Fig. 8E, F) that revealed moderate to high number of inflammatory cells infiltration accompanied by mild oval cells hyperplasia and limited degeneration of the hepatocytes. Comparable results were detected in CU (200 mg/kg) group (Fig. 8G, H), except for fewer sections that exhibited fewer inflammatory cells infiltration when compared to CU (100 mg/kg) group. Meanwhile, marked improvement was reported in CU (400 mg/kg) group (Fig. 8I, J) revealing apparently normal hepatic parenchyma in several examined sections. A sporadic case in CU (400 mg/kg) group showed limited area of sinusoidal dilation. The statistical analysis of lesion score of STZ-induced hepatic injuries was conducted in different experimental groups. STZ group exhibited a significant increase in lesion score compared with other groups. Meanwhile, all groups of CU treatment showed a significant decrease in lesion score when compared to STZ group (Fig. 8K).

Discussion

Globally, diabetes has become a serious health concern, with over 90% of cases of DM being type 2 diabetes [30]. About one-third of patients with cirrhosis also have diabetes, and people with type 2 diabetes have a higher risk of developing chronic liver disease, including steatohepatitis and non-alcoholic fatty liver disease [31]. This study assessed the CU's in vivo antidiabetic and hepatoprotective effect in STZ-induced diabetic mice. STZ is a cell-specific toxin that causes DNA damage and free radical production in pancreatic islets, which results in permanent damage [32]. Prior to the pharmacological study, the extract was chemically characterized. The HPLC chromatogram of the CU showed that gallic acid and rutin are among the major constituents of the extract. They were selected as markers because of their wide availability, cost-effectiveness and efficacy in clinical trials, which are important factors for QC protocols. The biochemical, molecular and histopathological levels evidenced the CU efficacy. Notably, the extract showed a dose-related activity, with 400 mg/kg being the most active dose. The study disclosed the possible underlying molecular mechanisms and assessed the histopathological changes.

The ameliorative CU activity against diabetes and diabetes liver complications.

CU had effectively restored serum insulin, resulting in a significant decrease in serum glucose. These effects were reflected in increased body mass. These effects suggest CU-protected Langerhans cells against STZ toxicity, maintaining insulin secretion. CU would be beneficial for type 2 DM patients regarding these aspects. Elevated liver enzymes are correlated with higher odds of diabetes [33]. This could be related to oxidative stress induced by DM [34]. It was reflected in the current study by low GSH and high MDA levels (Fig. 4). CU phenolics had counteracted this oxidative stress leading to restoration of GSH levels and reduction in the oxidative stress marker MDA.

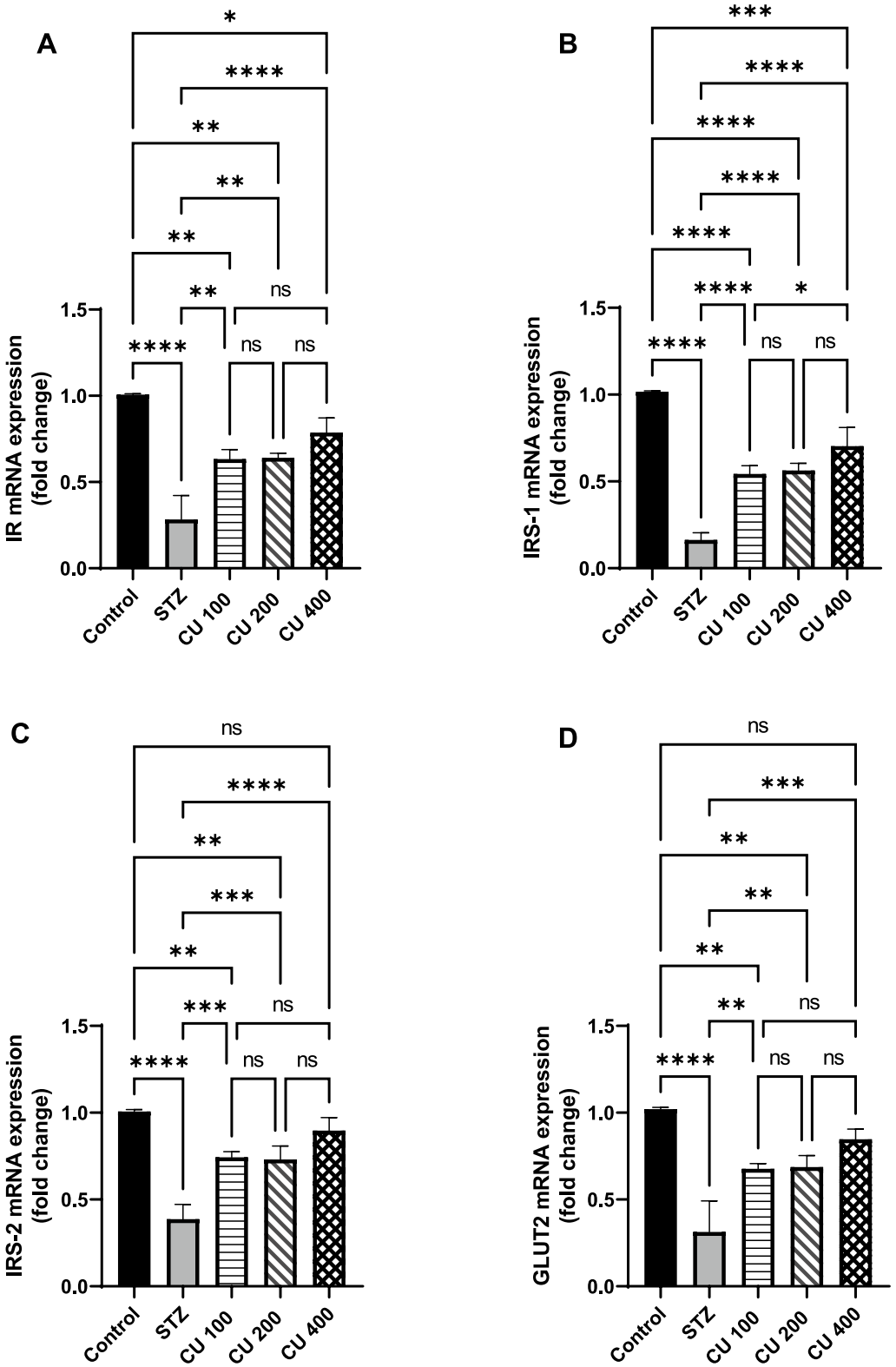

Fig. 6 Effect of CU extract on STZ-induced changes in mRNA expression of IR (**A**), IRS-1 (**B**), IRS-2 (**C**) and GLUT2 (**D**) in liver tissue. Each bar with a vertical line represents the mean of experiments ± S.D. ($n = 3$). Statistical analyses were performed using the one-way ANOVA followed by Tukey's multiple comparison test, with the criterion for statistical significance as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$ and ns = no significance

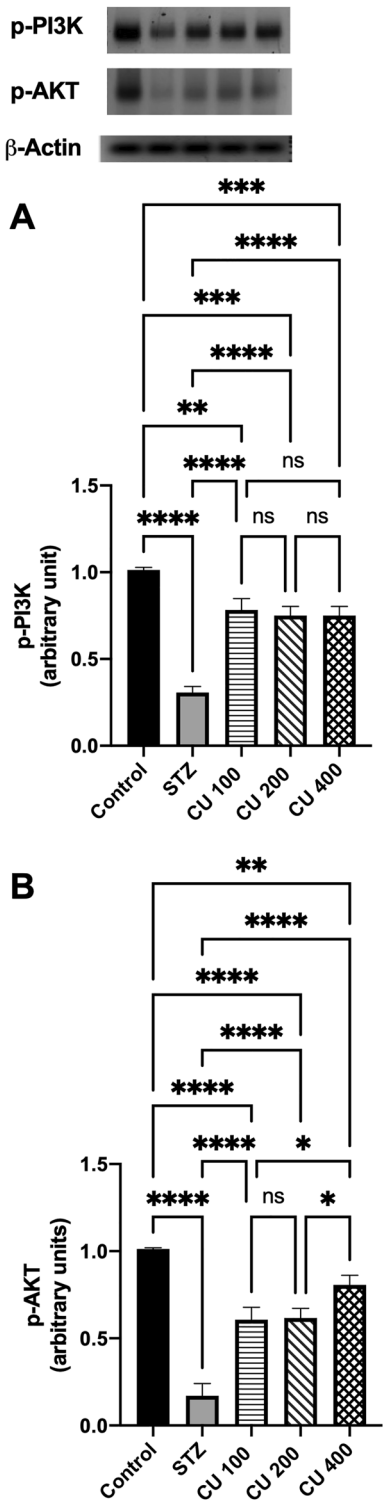

Fig. 7 Effect of CU extract on STZ-induced changes in p-PI3K (**A**) and p-AKT (**B**) expression in liver tissue. Each bar with a vertical line represents the mean of experiments ± S.D. ($n = 3$). Statistical analyses were performed using the one-way ANOVA followed by Tukey's multiple comparison tests, with the criterion for statistical significance as follows: *$p < 0.05$, **$p < 0.01$, ****$p < 0.001$ and ns = no significance

CU normalized the hepatic carbohydrates metabolic enzymes and replenished the glycogen content

The liver is involved in glycogen formation and controls postprandial hyperglycemia. It is well recognized that diabetes mellitus affects the liver's normal ability to synthesize glycogen [35]. Furthermore, partial or complete insufficient amounts of insulin in DM disrupt the metabolism of carbohydrates and lower the activity of the enzymes phosphofructokinase, hexokinase and glucokinase, which depletes muscle and liver glycogen. These enzymes provide an approach for evaluating the peripheral utilization of glucose [36]. CU effectively normalized the hepatic carbohydrate metabolic enzyme hexokinase and restored the glycogen content almost to the normal level. These activities in turn revealed the CU insulin-mimetic activity and its ability to facilitate the glucose uptake by the cells. On the other hand, glucose-6-phosphatase and fructose-1,6-bisphosphatase are crucial enzymes in gluconeogenesis [37]. The activity of these enzymes was reduced significantly by CU. Consequently, CU will result in the inhibition of gluconeogenesis and, hence, lower endogenous glucose synthesis.

CU elevated the expression of insulin receptors and glucose transferase

Insulin receptors (IRs) and insulin receptor substrates (IRSs), two important proteins in the insulin signaling system, are downregulated in the livers of diabetic rodents and humans [38]. The maintenance of glucose metabolism largely depends on IRSs, the major mediators of insulin signaling [39]. Tyrosine phosphorylation of the IRS protein is necessary for the metabolic effects of insulin [40]. This phosphorylation triggers a signaling cascade by activating PI3K and the serine/threonine kinase Akt/PKB. Furthermore, hepatic nutritional homeostasis is particularly dependent on IRS-2, as it mediates the anabolic effects of insulin via the PI3K-AKT cascade [41]. In the current study, CU upregulated IR, IRS-1 and IRS-2 mRNA expression.

The glucose transporter gene GLUT2 regulates the hepatocytes' ability to absorb and release glucose across the plasma membrane. It preserves the balance of glucose inside and outside liver cells. However, the expression of GLUT2 is drastically decreased in DM [42]. CU increased GLUT2 levels in the hepatocyte membrane by increasing mRNA expression.

CU upregulated PI3K and p-AKT signaling in liver tissue

Insulin initiates the PI3K/AKT signaling pathway by binding to insulin receptors on the cell membrane. The main mechanism of insulin signal transduction, which controls glucose uptake, glycogen formation and

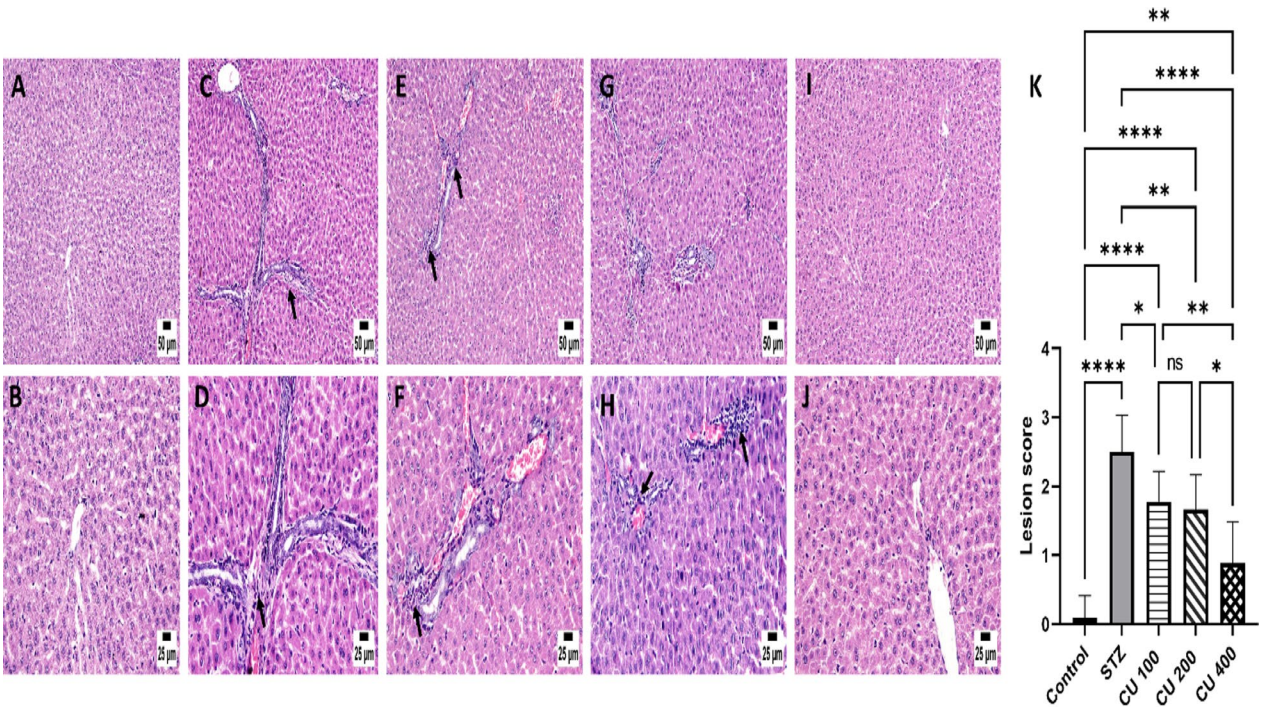

Fig. 8 Effect of CU extract on STZ-induced histopathological changes. H&E staining of the liver tissues from different experimental groups. **A, B** The control group showed normal hepatic parenchyma with healthy hepatocytes. **C, D** STZ group showing portal fibroplasia with mononuclear inflammatory cell infiltration (arrow). **E, F** CU (100 mg/kg) group showing limited portal inflammatory cell infiltration (arrow) with congested blood vessels. **G, H** CU (200 mg/kg) group showing mild portal hepatitis (arrow) with limited oval cell hyperplasia. **I, J** CU (400 mg/kg) group normal hepatic parenchyma. **K** Liver lesion score in different groups represented as mean ± S.D. ($n = 3$). Statistical analyses were performed using the one-way ANOVA followed by Tukey's multiple comparison tests, with the criterion for statistical significance as follows: *$p < 0.05$, **$p < 0.01$, ****$p < 0.001$ and ns = no significance

breakdown, is the PI3K/AKT signaling pathway [43]. Insulin binds to the α subunit of IR on liver cells, activating IRS in the process. Subsequently, IRS binds to PI3K's regulatory subunit, p85, and activates the catalytic subunit, p110. Phosphatidylinositol (3,4)-bisphosphate and phosphatidylinositol (3,4,5)P3, which are produced by activated PI3K, promote the activation of AKT [44].

Activated AKT results in the following: Extracellular glucose is more easily transported into cells when active AKT encourages the translocation of glucose transporter 4 to cell membranes. In addition, it facilitates the synthesis of glycogen and inhibits hepatic gluconeogenesis. Thus, the control of the liver's glucose metabolism is mostly dependent on the insulin-PI3K/AKT signaling pathway. CU effectively ameliorated STZ-induced downregulation of p-PI3K and AKT.

CU retrieved histopathological changes in liver tissues

Previous findings showed that STZ causes histological changes in the liver of diabetic mice. Liver cells of the STZ diabetic animals after 4 weeks from injection displayed severe congestion, necrotic foci, hydropic changes and aggregation of lymphocytes between the hepatocytes, among other more advanced abnormalities. Furthermore, after six weeks of STZ injection, mononuclear inflammatory cell infiltration, severe hydropic degeneration alterations and kupffer cell hyperplasia can be identified [45]. CU retrieved histopathological changes caused by STZ through inhibition of inflammatory cell infiltration and hyperplasia as well as CU limited degeneration of the hepatocytes, showing apparently normal hepatic parenchyma at 400 mg/Kg dose.

Different phenolic acids were quantified from *C. uvifera* aqueous, ethanolic and acetone leaves extracts such as syringic, ferulic, gallic, *o*/*p*-coumaric, ellagic, caffeic acids and others [12]. In the following sections, the antidiabetic activity of these constituents will be discussed. It is reported that high consumption of polyphenols may reduce the risk of diabetes incidence, control the postprandial glycemia and prevent the onset of glucose intolerance. These effects are mediated by facilitating the insulin response and attenuating the release of glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1 [46].

Gallic acid (GA) was quantified in CU with a concentration of 0.09 mg/g extract, and also, it was isolated

from *C. uvifera* leaf extract [47]. A study showed that GA increased glucose uptake by promoting the translocating of GLUT4 to the plasma membrane of rat adipocytes in a dose-dependent manner. Indeed, authors had concluded that the antidiabetic activity of mulberry leaves could be attributed to its gallic acid content [48]. Interestingly, GA alleviated DM detrimental effects in high-fat diet-fed streptozotocin-induced insulin resistance in diabetic rats. GA-treated rats had lower body weight gain, lower fasting blood glucose and lower insulin resistance [49]. Furthermore, the levels of glycogen content and the activity of glucose-6-phosphatase, fructose-1,6-bisphosphatase and hexokinase were dramatically restored to almost normal levels. These beneficial effects were mediated by increased expression of PPARγ (peroxisome proliferator-activated receptorγ) in adipocytes; consequently, GLUT4 was translocated and activated in PI3K- p-AKT dependent pathway [49]. Molecular docking showed that GA had promising interaction against GLUT4, GLUT1, PI3K and p-AKT. Moreover, histological examination revealed consistent distributions of pancreatic islets, adipose and hepatic cells with seemingly normal structures [49, 50]. Moreover, a study evaluated the antidiabetic activity of different hydroxybenzoic acid derivatives. GA suppressed the overexpression of microRNA-1271 generated by free fatty acids and upregulated its targets, such as p-IRS, p-PI3K, p-AKT and p-FOXO1, accompanied with the modulation of glucose metabolism genes [51].

GA was also found to be effective in clinical trials. In single-cell gel electrophoresis assays, oxidized purines are significantly reduced by 31% and pyrimidines by 2% after administration of GA, 15 mg for 7 days for patients of type 2 diabetes [52]. Moreover, following the intervention, the plasma concentrations of C-reactive protein and oxidized LDL were decreased by 39% and 24%, respectively. Consequently, a small quantity of GA (within the daily consumption range in Central Europe) lowers markers reflecting inflammation and elevated risks of CVD and cancer, as well as preventing oxidative DNA damage [52].

Several phenolic acids ameliorated DM manifestations. HbA1c and fasting plasma insulin levels were dramatically reduced by syringic acid, with improvement in liver glucose homeostasis [53, 54] Furthermore, ferulic acid (FA) has been demonstrated to increase the levels of insulin, glycogen and glucokinase, as well as lower the enzymes glucose-6-phosphatase and phosphoenolpyruvate carboxykinase in mice fed on high-fat diet [55]. Furthermore, FA was reported to regulate the gene expression of GLUT2 in the liver of diabetic rats [56]. Moreover, it raised the protein expression levels of IRS-1, PI3K and AKT in the muscles and brains of obese mice treated with FA, which could relieve obesity-related insulin-resistant case [57].

Ellagic acid (EA) enhanced the expression of IRS-1, AKT and ERK in HepG2 cells exposed to high glucose levels; simultaneously, EA downregulated the MDA level, counteracted oxidative stress and increased the glucose consumption by cells [58]. In DM-type 2 rats, EA raised GSH levels while suppressing serum MDA, TNF-α and IL-6. It also decreased liver enzymes ALT, AST and blood glucose [59].

Furthermore, caffeic acid caused upregulation of the expression of IRS-1, AKT, PI3K and GLUT4 [60]. In another study, caffeic acid lowered plasma glucose and glucose-6-phosphatase while increasing body weight and plasma insulin in diabetic rats [61]. Protocatechuic acid found in CU extract [47] has been shown to be able to counteract insulin resistance in obese volunteers by raising the levels of p-Tyr-IRS-1 and p-AKT in the visceral adipose tissue. Furthermore, protocatechuic acid reduced the inflammation and PTP1B activity [62]. In addition to lowering blood glucose levels and gluconeogenic enzymes, *p*-coumaric acid was reported to modify lipid and glucose metabolism via activating GLUT2.

Flavonoids are ubiquitous phenolic compounds in plants and their beverage products. Numerous in vitro and animal studies support that dietary flavonoids positively impact glucose homeostasis. In addition, through a variety of intracellular signaling mechanisms, flavonoids have been demonstrated to control carbohydrate digestion, insulin secretion, insulin signaling and glucose uptake in insulin-sensitive tissues [63]. Several flavonoids were isolated from *C. uvifera* leaves extract such as quercetin, myricetin and kaempferol derivatives [47, 64]. Their impact on DM and its complications would be displayed in the following paragraphs.

Rutin was quantified in this study in CU with a concentration of 0.23mg/g extract. A recent review explored the diverse mechanisms of action of rutin against DM. First, rutin reduces glucose level by reducing its absorption from the intestine, increasing the tissue glucose uptake, reducing gluconeogenesis, increasing insulin secretion and protecting islets of Langerhans against deterioration [65]. Moreover, rutin protects against DM complications by reducing sorbitol accumulation, reactive oxygen species, advanced glycation end-products and inflammatory cytokines [66]. Rutin was reported to increase the liver's antioxidant status by raising catalase, glutathione peroxidase and superoxide dismutase levels [67]. It also reduced serum levels of liver enzymes and corrected the histological damage to hepatocytes [68]. Rutin-induced insulin receptor kinase activity and GLUT4 translocation in

differentiated myotubes enhance glucose uptake [69]. Furthermore, querectin, isoquercetin and rutin had α-glucosidase inhibiting activity [70].

In STZ diabetic rats treated with rutin (100 mg/kg), there was a decrease in plasma glucose and a rise in insulin levels, as well as a restoration of glycogen content and the activity of carbohydrate metabolic enzymes. The pancreas' histological examination demonstrated rutin's protective function. Meanwhile, the islets became larger, and the fatty infiltration of the islets decreased. [71]. In another study, flavonoids extract from mulberry leaves containing rutin as the main ingredient (1mg/mL) remarkably increased the protein expression levels of p-IRS-1, p-PI3K, p-AKT, total GLUT4 and membrane GLUT4 in 3T3-L1 adipocytes insulin resistance model [72]. Moreover, it has been shown that rutin (23 µg/mL) reversed the high glucose-induced insulin resistance caused in hepatic FL83B cells via enhancing AKT phosphorylation, which thereby enhanced GLUT2 translocation and glucose uptake [73].

Rutin was effective in clinical trials. Consuming 1g of rutin in patients with type 2 diabetes mellitus resulted in a significant decrease in heart rates, mean arterial pressure, pulse pressure and blood pressure. Moreover, there is a substantial rise in the antioxidant enzymes catalase, glutathione peroxidase and superoxide dismutase as well as quality of life (QOL) parameters (emotional limitations, mental health, energy and freshness, social performance and general health) [74].

Rutin was effective when combined with other oral synthetic antidiabetics. When given separately, rutin effectively decreased hyperglycemia in acute assays in a way comparable to oral antidiabetic drugs (OADs). In the subchronic assay, rutin also helped to lower the HbA1c% and hyperlipidemia. In all treatments, rutin and OADs effectively reduced hyperglycemia, as seen by the decline in the percentage of HbA1c and lipid profile. consequently, rutin showed significant activity when combined with antidiabetic medications, which is a first step toward creating novel DM treatments [75].

Rutin and quercetin increased glucose uptake in insulin-resistant FL83B liver hepatocytes. This effect was mediated by the upregulation of p-AKT and GLUT2, reduction in oxidative stress and prevention of the degradation of PPARγ [76]. Quercetin aglycone and its derivatives (quercetin-3-O-glucoside and quercetin-3-O-galactoside) isolated from berry extract, enhanced insulin-independent glucose uptake and stimulated AMPK in muscle cells [77]. Over a period of 28 days, quercetin at dosages of 25 and 50 mg/kg significantly decreased blood glucose and glycosylated hemoglobin (Hb), while increasing the levels of Hb in plasma and hepatic glycogen. After receiving quercetin, the hexokinase and glucose-6-phosphatase activity in diabetic rats were also markedly restored [78].

When isoquercetin (quercetin-3-*O*-glucoside) was supplemented to STZ-induced diabetic rats, the insulin levels increased significantly, and the glucose levels returned to normal. Additionally, liver enzymes were reduced. Glycogen synthase GK and GLUT2 expressions were markedly upregulated, while glucose-6-phosphatase expressions were significantly downregulated. The levels of gluconeogenesis enzymes were significantly lower than those in negative control. Insulin, IR, IRS-1, IRS-2 and AKT mRNA expressions were also elevated. Indeed, isoquercetin efficacy was similar to that of glibenclamide [79].

Administration of kaempferol (K) and its glycosides rich fraction decreased the area under the curve in glucose tolerance test in genetically type 2 KK-A^y mice. Moreover, the liver had lower triglycerides level and fatty acid synthase activity [80]. By controlling mitochondrial calcium absorption, K stimulates Akt activation and enhances insulin production. Moreover, K directly restores AKT activation. Subsequently, reversing the effects of AKT inactivation causes the upregulation of gluconeogenesis, the downregulation of glycogen synthesis and the uptake of glucose. Additionally, the antioxidant K controls both apoptosis and autophagy [81].

Myricetin caused upregulation of p-IR, p-IRS-1 and p-AKT in the liver of high-fat diet-fed and STZ-induced type 2 diabetic rats. These effects were mediated by suppressing PTP1B's activity and expression; PTP1B is the tyrosine phosphatase that adversely regulates insulin signal transduction. These effects were evident when myricetin was administered alone and not in combination with horsegram protein [82]. Moreover, hexokinase, glycogen synthase, glycogen phosphorylase, glycosylated hemoglobin, glucose-6-phosphatase and plasma glucose were all inhibited from a significant increase [83]. It has been determined that myricitrin, glycosylated myricetin, activates the IRS-1/PI3K/AKT/GLUT4 pathway in the soleus muscle of type 2 DM-affected rats as well as in L6 muscle cells exposed to high glucose [84].

Conclusion

The current study provided an unequivocal experimental validation of the antidiabetic activity of standardized CU. Moreover, it deciphered the molecular mechanism of antidiabetic and hepatoprotective actions of CU for the first time. CU had ameliorated DM detrimental impact. The underlying mechanism involved the upregulation and induction of IRS isomers, GLUT2 and their upstream PI3K/AKT signaling pathway. GA and rutin were the major and key bioactive constituents of CU. Both could serve as QC markers in future analyses. As a

result, *C. uvifera* leaves extract could be used as a promising antidiabetic drug with hepatoprotective activity in diabetes hepatic complications. The current study laid the foundation for performing further assessment and clinical testing of the antidiabetic activity CU alone or in combination with other remedies.

Abbreviations

DM	Diabetes mellitus
CU	Ethanolic *Coccoloba uvifera* L. leaves extract
HPLC	High-performance liquid chromatography
STZ	Streptozotocin
AST	Serum aspartate aminotransferase
ALT	Alanine aminotransferase
MDA	Liver malondialdehyde
GSH	Reduced glutathione
RT-PCR	Reverse transcription polymerase chain reaction
IR	Insulin receptor
IRS-1	Insulin receptor substrate-1
IRS-2	Insulin receptor substrate-2
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
PI3K	Phosphatidylinositol 3-kinase
p-AKT	Phosphorylated protein kinase B
PPARγ	Peroxisome proliferator-activated receptor γ
HepG2	Hepatoblastoma cell line
ERK	Extracellular signal-regulated kinase
TNF-α	Tumor necrosis factor alpha
Il-6	Interleukin-6
PTP1B	Protein-tyrosine phosphatase 1B
FOXO1	Forkhead box protein O1

Supplementary Information

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Additional file 1.

Additional file 2.

Acknowledgements
Not applicable

Statement regarding plants
Coccoloba uvifera L., was authenticated by Mrs. Therese Labib, Botanical Specialist and consultant at Orman and Qubba Botanical Gardens. A voucher sample was kept at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University number (26.7.23).

Author contributions
Fatma Mohamed was involved in writing the original draft, methodology, formal analysis, data curation and conceptualization. Rabab Sayed contributed to biological activity, statistical analysis and writing the manuscript. Mohamed Khalil was responsible for original draft, reviewing, data analysis, chemical investigation and study design. Mohamed Salem took part in data analysis, writing-reviewing and editing, and study design. Amira El Senousy participated in data analysis, writing-reviewing and study design. Ali El-Halawany helped with supervising work and study design.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
The study protocol was approved by the Ethics Committee for Animal Experimentation at Faculty of Pharmacy, Cairo University MP (2964) 28/03/2022 and followed the guidelines of the US National Institutes of Health guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 2011). All protocols were followed to minimize the animals' suffering.

Consent for publication
Not Applicable.

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

Author details
[1]Department of Pharmacognosy, Faculty of Pharmacy, Heliopolis University , Cairo 11785, Egypt. [2]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Cairo University, Cairo, Egypt. [3]Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Kasr El-Aini, Cairo 11562, Egypt. [4]Department of Pharmacognosy and Natural Products, Faculty of Pharmacy, Menoufia University, Gamal Abd El Nasr St, Shibin El Kom, Menoufia 32511, Egypt. [5]Clinical Pharmacy Department, School of Pharmacy, Newgiza University, Giza, Egypt. [6]Present Address: The BioActives Lab, Biological and Environment Science and Engineering (BESE), King Abdullah University of Science and Technology(KAUST), Thuwal, Saudi Arabia.

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