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Research article

Alkaloidal extract from *Zanthoxylum zanthoxyloides* stimulates insulin secretion in normoglycemic and nicotinamide/streptozotocin-induced diabetic rats

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ABSTRACT

Introduction: Increase in the prevalence of type-2 diabetes in Sub-Sahara Africa has created the need for robust treatment and management programs. However, an effective diabetes management program requires a high annual budget that most countries in this region cannot afford. That said, various plants and plant products in this region have either been confirmed and/or ethnopharmacologically used for the management of type-2 diabetes.
Aim: To investigate the antidiabetic and insulin secretory effects of an alkaloidal extract derived from *Zanthoxylum zanthoxyloides* in normoglycemic and experimental diabetic rats.
Materials and methods: Alkaloidal extract was prepared from leaves of *Zanthoxylum zanthoxyloides* (ZZAE). Nicotinamide/streptozotocin-induced type-2 diabetes was modeled in male Sprague Dawley rats weighing between 130 to 150 g. The experimental diabetic rats were grouped into six treatment groups [Model, 20% Tween20, chlorpropamide, and ZZAE (50, 100, and 150 mg/kg)], and one control group. Fasting blood glucose (FBG), and body weight were measured weekly. Rats were sacrificed 2 days after treatment under chloroform anesthesia to collect blood samples and to isolate major organs for biochemical, and histological analyses respectively. Islets of Langerhans were isolated from normoglycemic rats and co-cultured with ZZAE and chlorpropamide (10 μg/mL) to assess the insulin secretory effect of ZZAE.
Results: ZZAE improved glucose kinetics curve in normoglycemic ($p < 0.001$) and experimental diabetic rats ($p < 0.05$) compared to the model. ZZAE (100 and 150 mg/kg) restored islets population, and improved kidney, and liver, histoarchitecture. ZZAE (150 mg/kg) improved post-treatment serum insulin levels compared to the model group ($p < 0.001$) and the Chlorpropamide group ($p < 0.05$). ZZAE also restored glycogen synthesis in skeletal muscles of experimental diabetic rats and stimulated insulin secretion in pancreatic islets of Langerhans isolated from normoglycemic rats.
Conclusion: These results showed that ZZAE has active alkaloids that can be explored for diabetes management.

1. Introduction

Diabetes is a metabolic disorder defined by hyperglycemia and its associated comorbidities like retinopathy, nephropathy, and neuropathy [1]. Diabetes is also associated with other complications such as non-alcoholic fatty liver disease [2], ischaemic heart disease [3], stroke [4], etc. that significantly affect the quality of life and life expectancy of diabetic patients [5]. It was estimated that approximately 451 million people were living with diabetes in the year 2017 with a projected increment in diabetes prevalence to about 693 million by the year 2045 [6]. It is worthy of note that in the past 2 decades, the number of diagnosed type-2 diabetes in developing regions such as Sub-Sahara Africa

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has more than doubled with several million others still undiagnosed [7]. In fact, over two-thirds of people living with type-2 diabetes (66.7%) in Sub-Sahara Africa are not aware of their diabetic status [8].

The global diabetes health-related expenditure for 2019 was estimated to be USD 760 billion and is projected to increase up to USD 825 billion by 2030 and USD 845 billion by 2045 [9]. Thus, effective diabetes management/treatment programs require huge budgets. In developing countries, such as those in Africa, it is practically impossible to designate a huge budget for any single disease for just a single year. This increases the risk of diabetes-related morbidities and mortalities in countries from developing regions. The foregoing facts warrant the need to research more indigenous and affordable treatment methods for diabetes in these regions.

In Africa, China, and Southeast Asia, herbal medicine practice continues to play a major role in primary healthcare. In some of these countries, the practice is either culturally or religiously linked with knowledge passed down from generation to generation [10, 11]. Herbal medicine practice involves the use of whole and/or part(s) of plant or the preparation of crude medicine from a collection of plants and plant parts with water, alcohol, or a combination of both [11]. These formulations are either ingested or topically applied to the affected part. Indeed, plants have served an important role in Pharmaceutical drug discovery with scaffold compounds from which drugs like Aspirin, Cocaine Digitalis, etc. were obtained [12].

Zanthoxylum zanthoxyloides [(L.) (family: *Rutaceae*)] contains a high diversity of essential oils, alkaloids, and several aliphatic and aromatic amides. It has been reported to possess antibacterial and cytotoxic properties [13] anti-fungal properties [14], and anti-inflammatory properties. In Nigeria, a decoction from the leaves of *Z. zanthoxyloides* is used for the treatment of diabetes. In Côte d'Ivoire, Mali, and Burkina Faso, the leaves and seeds of *Z. zanthoxyloides* are used as a spice for the preparation of diabetic diet [15] with antidiabetic confirmation report from preliminary studies [16]. The disadvantage of crude drugs is that they usually contain several bioactive secondary plant metabolites and may be prone to polypharmacy-related toxicities [12]. Here, we investigate the antidiabetic effect of alkaloidal extract from *Zanthoxylum* zanthoxyloides leaves. The anticipation is that findings from this study will advance frontiers for the isolation of alkaloids from *Zanthoxylum zanthoxyloides* that can serve as scaffolds for candidate drugs for diabetes treatment.

2. Materials and methods

2.1. Drugs and chemicals

Chlorpropamide (100 mg/kg) (LGM Pharma Florida, USA), Nicotinamide (Yashica Pharmaceuticals Private Limited, Thane, Maharashtra), streptozotocin (Pfanstiehl Inc, Ilinois, USA), Heparin (Duchefa Farma B.V. from the Netherlands), Plasma glucose kit and Rat insulin ELISA kit (Crystal Chem Inc, Illinois, USA), Tween20, ethanol, Sodium bicarbonate 7.5% solution and petroleum spirit (Thermo Fisher Scientific, Massachusetts, USA). All other chemicals and reagents used in this study were of analytical grade.

2.2. Collection, identification, and authentication of plant material

The aerial parts of *Zanthoxylum zanthoxyloides* were collected from Achimota Forest, a suburb of Achimota in the Greater Accra Region of Ghana, on 4th June 2018. Mr. Francis Otoo, the Curator at the herbarium unit, School of Biological Sciences, University of Cape Coast, Ghana, identified and authenticated the plant. A voucher specimen (SC/SBS/UCC/B41SH) was deposited in the herbarium for future reference.

2.3. Preparation of Zanthoxylum zanthoxyloides crude extract (ZZCE)

Preparation of ZZCE was carried out using standard calorimetric procedures as previously described [17] with few modifications. Briefly, the leaves of *Zanthoxylum zanthoxyloides* were washed under running water and air-dried for 4 weeks. The dried leaves were pulverized using a hammer mill (Polymix Micro Hammer Cutter Mill, Glen Mills Inc, USA). A 6.5 kg of the powdered leaves was defatted with petroleum ether at 60–80 °C for 9 h. A crude extraction was performed from the defatted leaves with 70% ethanol in a Soxhlet apparatus (L3 Soxhlet extractor, Ergotech Soxhlet Apparatus Co, UK) for 22 h. The extract was concentrated on a water bath (Premiere HH-4 Digital Water Bath, C & A Scientific Co Inc, USA). The crucible containing the concentrated extract was transferred into a 40 °C hot air oven (Oven 300 plus series, Gallenkamp, England) for 24 h leaving a dark-green gummy paste. The dark-green gummy paste was weighed and named *Zanthoxylum zanthoxyloides* crude extract (ZZCE) and stored in a desiccator at 25 °C until it was ready to be used.

2.4. Qualitative phytochemical screening of ZZCE

Qualitative phytochemistry was conducted on ZZCE using standard qualitative colorimetric methods as described by Trease and Evans [18]. Terpenoids, carbohydrates, alkaloids, saponins, tannins anthraquinones, flavonoids, phenols, and glycosides, were qualitatively screened and results were recorded (+) if present and (–) if absent.

2.5. Animal acquisition and husbandry

Healthy 10–12 weeks old male Sprague-Dawley rats with an average weight of 182.16 ± 31.64 g) were used for the preliminary glucose kinetics effect of the ZZCE and 7–8 weeks old weighing between 130 and 160 g for the rest of the experiments. Rats were kept in the School of Biological Sciences experimental animal house in stainless steel cages (34 cm × 47 cm × 18 cm) with wood shavings as bedding. Animals were fed standard rodent pelleted chow (GAFCO, Tema, Ghana), and had access to water *ad libitum*. Conditions were varied whenever necessary to suit the specific requirements of some experiments. To adjust to environmental conditions, rats were kept for a week in the experimental animal house; School of Biological Sciences, University of Cape Coast, Ghana before the commencement of all experiments. Rats were maintained under normal ambient laboratory conditions of temperature, humidity, and a 12-hour dark-light cycle.

2.6. Ethical clearance

All animal experiments, procedures, and techniques used in this study were approved by the University of Cape Coast Institutional Review Board (UCCIRB) with an approval number, UCCIRB/CHAS/2018/13. Animal experimental procedures were also in compliance with the guidelines on the use of animals in scientific experiments as described by OPRR Public Health Service Policy on Humane Care and Use of Laboratory Animals (Rockville, MD: NIH/Office) for protection from research risks, 1996.

2.7. Intravenous glucose tolerance test for ZZCE

A previously described method for an intravenous glucose tolerance test (IVGTT) [19] was adopted for this study with few modifications. Briefly, rats that have been fasted overnight were assigned into 5 groups of 4 [Negative control, Chlorpropamide (100 mg/kg), ZZCE (100, 200, and 400 mg/kg)]. The rats were put under anesthesia (Phenobarbital 30 mg/kg, i.p) and a 0.2 mL of blood sample was collected from each rat's tail vein using a chilled syringe containing 10 IU heparin (Duchefa Farma B.V., Netherlands) for time zero FBG. A solution of 1 mL/kg of ZZCE (100, 200, and 400 mg/kg) was given to rats in the respective groups 30 min before the IVGTT and 1 mL/kg Chlorpropamide (100 mg/kg) 10 min before the IVGTT experiment. A solution of 2 g/kg glucose was injected into experimental rats' femoral veins within 2 min time window and an equivalent volume of saline into the controls. Tail vein blood sample (0.2 mL) was collected from each rat of each group in the following time intervals: 5, 10,

15, 30, 60, 90, and 120 min with a syringe containing 10 IU heparin. Blood samples contained in 10 IU heparin (Duchefa Farma B.V., Netherlands) were centrifuged at 13,000 rpm for 5 min and an aliquot (10 μL) of the plasma added to 1 mL of glucose kit reagent (Randox Laboratories Ltd. 140 London Wall, London) and incubated at 37 °C in a water bath (Premiere HH-4 Digital Water Bath, C & A Scientific Co Inc, USA) for 5 min. The concentration of plasma glucose was then estimated in quadruplicates by measuring the optical density of the colored complex with an autoanalyzer (Biosystems 300; Barcelona, Spain) [20].

2.8. Preparation of Zanthoxylum zanthoxyloides alkaloidal extract (ZZAE)

The alkaloidal extraction procedure was carried out as previously described [21]. Briefly, ZZCE was acidified with 15% acetic acid in a separator funnel (Fristaden Lab, Chicago, USA) and thoroughly shaken every 10 min for 2 h. Chloroform was added to the acidified ZZCE and thoroughly shaken at 1 h intervals for 6 h. The aqueous layer from the previous step was basified with 10% ammonia; thoroughly shaken before it was left on an orbital shaker (GYROMAX 818, California, USA) overnight. The organic layer was extracted with chloroform the next day until there was no color formation after the addition of chloroform. The chloroform was then evaporated and the gummy green extract obtained was stored in a desiccator for 7 days. The identity of the extract was confirmed with Mayer's test and Wagner's test and named *Zanthoxylum zanthoxyloides* alkaloidal extract (ZZAE). HPLC analysis was performed on the alkaloidal extract according to the procedure previously described [22], with slight modifications. Shimadzu LC-20 AD HPLC system, equipped with a model LC- 20 AV pump, UV detector SPD-20AV, Rheodyne fitted with a 5 μl loop, lab solution, and auto-injector SIL-20AC. A hyper CTO-10AS C-18 100Å column (4.6 × 150 nm, 3μm size) was used to run the HPLC analysis.

2.9. Establishment of nicotinamide/STZ diabetes in rats

Experimental type-2 diabetes mellitus was induced in healthy overnight fasted male rats (120 g–150 g) with nicotinamide (100 mg/kg i.p.) 30 min before the administration of streptozotocin (55 mg/kg, i.p.) adopting a previously described method [23, 24] with few modifications. Briefly, five rats were selected 5 days post-diabetes induction and a lateral tail incision [25] made on their tails after disinfecting the tails with 70% ethanol. Their blood glucose concentrations were measured with an Accu-check performer glucometer (Accu-Chek® Performa, Roche Diabetes Care, Middle East) and glucose test strips. Five other rats were randomly selected seven days post-diabetes induction and their blood glucose concentration tested again to confirm the establishment of type-2 diabetes. Rats were then randomly reassigned into one of 7 groups of 7 [model, chlorpropamide (100 mg/kg), 20% tween20, and ZZAE (50, 100, and 150 mg/kg)] and control (Figure S1).

2.10. Diabetes management, blood glucose monitoring, and body weight measurement

Apart from the control and the model, all groups received daily oral administration of 1 mL/kg of their respective treatment [chlorpropamide (100 mg/kg), 20% tween20, and ZZAE (50, 100, and 150 mg/kg)] for 28 days. Bodyweight (g) was measured with SCL-1015 (Kent Scientific Corporation, Connecticut, USA) FBG (mmol) was measured with Accu-check performer glucometer (Accu-Chek® Performa, Roche Diabetes Care, Middle East) and glucose test strips every 7 days for each rat in each experimental group. The relative Bodyweight unit food consumption was determined weekly. At the end of the 28 days treatment period, two rats were isolated from each group and fasted overnight for insulin sensitivity analysis (Post-treatment IVGTT) The remaining 5 rats from each group were sacrificed under chloroform anesthesia and blood collected by cardiac puncture for analysis of serum lipids, liver biomarkers, kidney biomarkers, and insulin. The liver, pancreas, and kidney were isolated for histological analysis [26].

2.11. Post-treatment insulin sensitivity test (IVGTT)

Two rats that had been isolated from each experimental group and fasted overnight were put under anesthesia (Phenobarbital 30 mg/kg, i.p). Two milliliters of a solution of 2 g/kg glucose was injected into experimental rats' femoral veins within 2 min the rats were left for 30 min before 0.5 mL of a blood sample was taken from the tail vein of each rat for blood glucose analysis.

2.12. Preparation of blood for biochemical analysis

The pre-collected blood samples were centrifuged at 3000 rpm at 4 °C (Ynigtai centrifuge TDL5M, Changsha Yingtai Instrument Co., Ltd, Hunan, China) for 5 min to obtain sera.

2.13. Measurement of serum insulin concentration

Commercial quantitative enzyme-linked immunosorbent assay [(ELISA) (Thermo Scientific, Austin, Texas. USA) (sensitivity: 5 μIU/mL) was used to determine serum insulin concentration of nicotinamide/STZ-induced diabetic rats following manufacturer's guidelines. Briefly, 50 μL of 0 uIU/mL, 8 uIU/mL, 16 uIU/mL, 32 uIU/mL, 80 uIU/mL, and 140 uIU/mL of the standard solutions were pipetted into the wells. 50 μL of diluted serum, and then 50 μL of HRP conjugated anti-Rat Insulin antibody was sequentially added to the test sample wells. The plate was covered and incubated at 37 °C for 60 min before the liquids in the wells were discarded. The plate was clapped on filter paper and washed 3 times with Tris buffer. Each wash was vortexed mildly for 3 min. TMB substrate A (50 μL) was added o each well, then TMB substrate B (50 μL) to each well and gently shaken for 30 s. The plate was covered and incubated in

Table 1. Effect of ZZAE on bodyweight changes of NIC/STZ-induced diabetes in rats.

Treatment groups	Initial body weight (g)	Final Bodyweight (g)	Change in body weight (g)	% change in body weight
Control	136.5 ± 2.453	185.1 ± 5.586	48.6	35.6
Model	135.1 ± 2.242	130.1 ± 3.482	- 5[a]	-3.7
20% Tween20	136.4 ± 4.394	127.6 ± 5.188	- 8.8	-6.4
Chlorpropamide (100 mg/kg)	134.5 ± 7.346	160.9 ± 0.965	26.4[b]	19.6
ZZAE (mg/kg)				
50	133.6 ± 6.252	142.8 ± 8.637	9.2[b]	6.8
100	133.2 ± 4.448	159.3 ± 6.783	26.1[b]	19.5
150	135 ± 9.175	174.3 ± 2.402	39.3[b]	29.1

a = model vs. control statistically significant, b = model vs. all treatment groups statistically significant, c = Chlorpropamide (100 mg/kg) vs ZZAE groups statistically significant. Values are reported as mean ± SD, n = 4.
ZZAE – *Zanthoxylum zanthoxyloides* alkaloidal extract.
NIC – Nicotinamide, STZ – streptozotocin.

the dark at 37 °C for 15 min. Stop solution (50 μL) was added to each well and thoroughly mixed. The optical density was read at an absorbance of 450 nm in a microplate reader (Victor® Nivo 212 TM multimode microplate reader, PerkinElmer, USA) within 30 min.

2.14. Measurement of skeletal muscle glycogen synthesis

Measurement of skeletal muscle glycogen synthesis was performed using a previously described method [27] with slight modification. Briefly, about 200 mg of skeletal muscle sample was taken and cut into 2–3 mm pieces. The samples were put in 100 mL of 1 M HCl and incubated for 3 h at 100 °C. The sample was allowed to cool to 25 °C and diluted with 300 mL of Tris/KOH (0.3 M sodium acetate, pH 4.8) containing amyloglucosidase [28]. The glucosyl units in the samples were measured using a quantitative glycogen assay kit (ab169558, Abcam, Cambridge, UK).

2.15. Determination of serum biochemical parameters

Serum lipids, liver enzymes, and kidney biomarkers were measured using a fully automated chemistry analyzer (YSTE261).

2.16. Preparation of organs for histological assessment

Routine histological assessment was performed following standard histological techniques as previously described [29]. The mean cross-sectional area of islets of Langerhans was estimated with ImageJ java image processing program v. 1.8.0_172 (Laboratory for Optical and Computational Instrumentation, University of Wisconsin, USA).

2.17. In vitro islet secretion activity of ZZAE

Measurement of pancreatic islet insulin secretion was determined using previously described methods [30, 31, 32] with slight modifications. The pancreas was isolated from ten healthy male rats (300 g) under anesthesia. Each pancreas was cut into 2–4 mm pieces and digested in collagenase (Genaxxon Bioscience GmbH, Söflinger, Germany) [33] in an enclosed weighing bottle and stirred with a magnetic stirrer. The sample was incubated at 37 °C for 30 min before the mixtures were diluted with 25 mL of Hank's solution in a graduated canonical cylinder and allowed to settle for 20 min. The islet cells settle at the bottom and the supernatant was aspirated with a pipette. Approximately 30–35 islet cells were transferred from the fifth Hank's solution into a 100 μL of 7.5% bicarbonate buffer solution [32] in a micro incubator flask. Five of the micro incubator flasks were supplemented with 50 μg/mL ZZAE. All isolated islet cells were incubated in a Dubnoff metabolic shaker (72 cycles per minute at 37 °C). A 50 μL aliquot was aspirated from each sample at 60 min into the incubation and stored at -80 °C. The remaining 50 μL was allowed to continue the incubation for another 60 min. Insulin production was assayed with quantitative enzyme linked immunosorbent assay (ELISA)) (Thermo Scientific, Austin, TX. USA) for the insulin concentration from the 60^{th} and 120^{th} min.

2.18. Statistical analysis

Data were analyzed using GraphPad Prism v8.0 (GraphPad, La Jolla, CA, USA). The data were reported as means ± SD of each group and statistical significance determined using analysis of variance (ANOVA). The level of statistical significance was set at ($p < 0.05$) with Tukey's multiple comparison analysis for groups that showed significance.

3. Results

3.1. Qualitative analysis of ZZCE and ZZAE

Qualitative analysis of ZZCE revealed the presence of monoterpenes, carbohydrates, alkaloids, and cardiac glycosides in ZZCE (Table S1). The overall yield of ZZAE was 168.439 g which represents 2.52% of the total quantity of leaves extracted (Table S2). An HPLC analysis of ZZAE revealed 11 different alkaloids in ZZAE. Most of which were present in

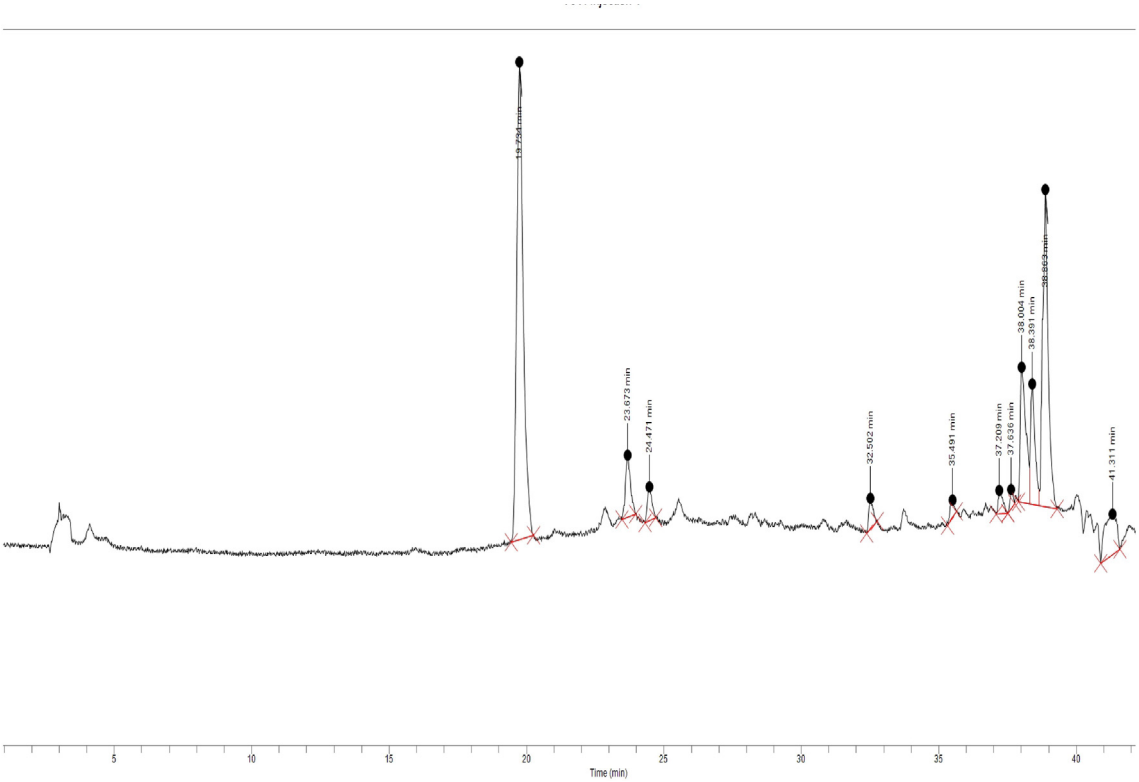

Figure 1. HPLC profile of ZZAE.

fairly high amounts and detected at reasonable time intervals to allow for isolation (Figure 1). The most abundant alkaloid was recorded at 19.734 min with a peak height of 22,935.3 and an area of 382,569.4 (Table S3). The second most abundant alkaloid in ZZAE was recorded at 38.863 min with a peak height of 15,214.9 and an area of 205,594.5.

3.2. ZZCE improved glucose response curve in normoglycemic rats after intravenous glucose challenge

Blood glucose levels for all treatment groups peaked within the first five minutes and began to show a monophasic decline time-response curve. The Chlorpropamide (100 mg/kg)-treated group reached a normoglycemic concentration at the 60th min with serum glucose concentration statistically significant from the control and ZZCE [(100 mg/kg and 200 mg/kg) ($p < 0.01$) but not the ZZCE (400 mg/kg) (Figure S2). The ZZCE (400 mg/kg) reached a normoglycemic concentration at the 90th min. and remain so to the 120th min.

3.3. ZZAE improved loss in bodyweight in NIC/STZ-induced diabetes in rats

The quantity of food taken per kg per week for the control group for the first week was 402.64 ± 23.28 g/kg/week and even fell under that for the rest of the week (Figure S3) and was statistically significant ($p < 0.0001$) compared to the model (512.51 ± 30.03 g/kg/week). Even though, all but, the control group had a very close range of food consumption (507–522 g/kg/week), the ZZAE (100 and 150 mg/kg) and the chlorpropamide (100 mg/kg) significantly ($p < 0.01$) reduced from week 3 (411–448 g/kg/week) to week 4 (402–382 g/kg/week) of treatment. Despite the high consumption of food by the model and the 20% Tween20 (Table S4), the model group recorded a 3.7% reduction in body weight and this was significantly lower compared to the control ($p < 0.001$). However, treatment with ZZAE (50, 100, and 150 mg/kg) led to a 6.8, 19.5, and 29.1% increase in body weight respectively after the 28-day treatment course (Table 1). The change in bodyweight between the ZZAE (100 and 150 mg/kg) was significantly higher ($p < 0.05$) compared to the model group.

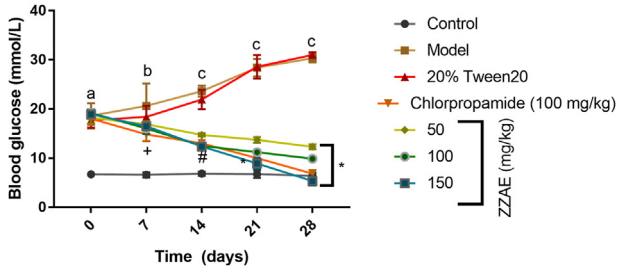

Figure 2. Glucose lowering effect of ZZAE and Chlorpropamide in NIC/STZ-induced diabetes rats. a = model vs control, ($p < 0.01$), b = model vs control, ($p < 0.001$), c = model vs control, ($p < 0.0001$), + = model vs. all treatment groups ($p < 0.05$), # = model vs. all treatment groups ($p < 0.01$), * = model vs. all treatment groups ($p < 0.001$), n = 4.

3.4. ZZAE ameliorated elevated blood glucose levels in NIC/STZ-induced diabetes in rats

The average fasting blood glucose (FBG) of the control group was significantly lower than the model from day 0 ($p < 0.01$) however, this had significantly increased by the end of the study ($P < 0.001$) (Figure 2) (Table S4). The average FBG of the ZZAE (150 mg/kg) group was significantly lower than that of the model group ($p < 0.05$) on day 14, and ($p < 0.001$) on days 21 and 28. For the positive control group administrated Chlorpropamide (100 mg/kg), the average FBG level of the rats was significantly lower than the highest dose of the ZZAE (150 mg/kg) ($p < 0.05$) group on day 14 but was not day 28.

3.5. ZZAE improved insulin sensitivity after treatment of NIC/STZ diabetes in rats

As expected, the control group showed the highest post-treatment insulin sensitivity (372 mg/dL) (Figure S4). The model recorded mean glucose of 637 mg/dL which was significantly higher compared to the control ($p < 0.001$). The insulin sensitivity of the ZZAE (150 mg/kg) significantly improved (408 mg/dL) ($p < 0.01$) compared to the model but not the Chlorpropamide (100 mg/kg).

3.6. ZZAE improved pancreas histology in NIC/STZ-induced diabetes in rats

Histology of H & E stained pancreas showed the control group with high islets of Langerhans cellularity compared to the model group (Figure 3). The model group had a high number of necrotic islets of Langerhans leading to a significantly reduced mean area of islet cellularity (Table S5). The ZZAE (50, 100, and 150 mg/kg)-treated groups had a dose-dependent restoration of islets cellularity and significantly low numbers of acini with pyknotic nuclei. The Chlorpropamide (100 mg/kg) and the ZZAE (150 mg/kg) had significantly low macrophage infiltration into the pancreatic interlobar ducts compared to the control as shown in Figure 3.

3.7. ZZAE improved glycogen synthesis of skeletal muscle in NIC/STZ-induced diabetes in rats

After the 28-day treatment plan, the average skeletal muscle glycogen level of the model group was significantly lower compared to the control

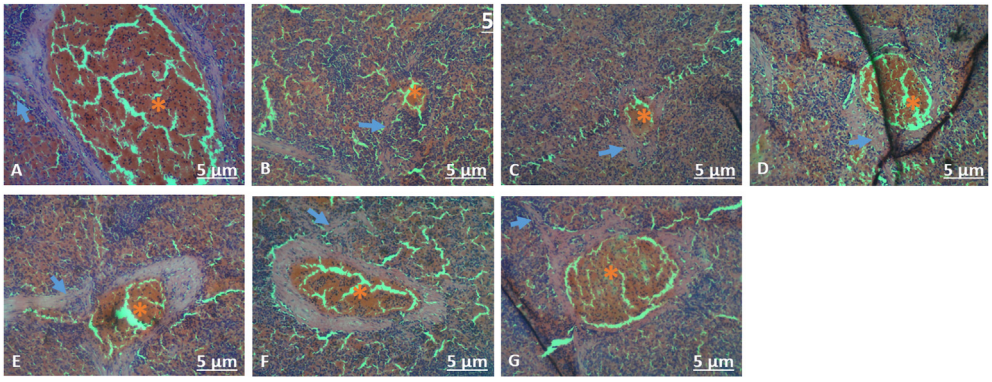

Figure 3. Effect of ZZAE and Chlorpropamide on pancreatic histoarchitecture of NIC/STZ-induced diabetic rats. A; control, B; model, C; 20% tween20, D; chlorpropamide, E; ZZAE 50 mg/kg, F; ZZAE 100 mg/kg, G; ZZAE 150 mg/kg. ZZAE: *Zanthoxylum zanthoxyloides* alkaloidal extract; NIC: nicotinamide; STZ: streptozotocin. Star = area of islets of Langahans, arrow = interlobar duct, cross = necrotic islets of Langahans.

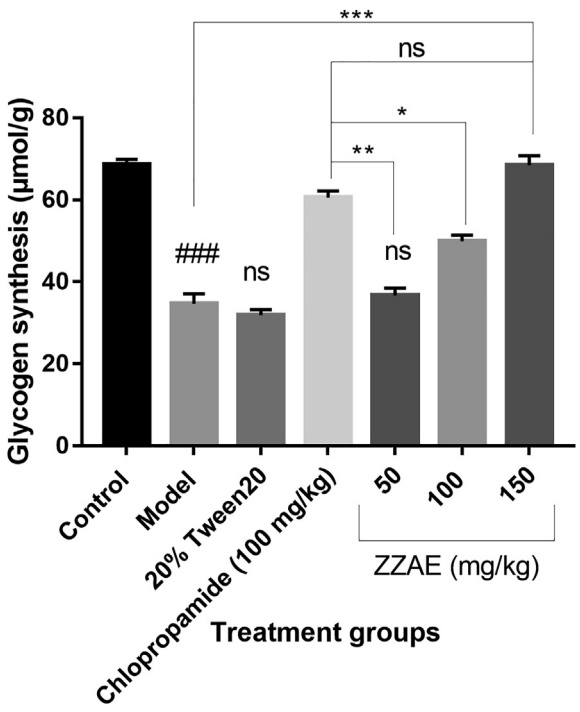

Figure 4. Effect of ZZAE and Chlorpropamide on skeletal muscle glycogen synthesis on NIC/STZ-induced diabetic rats. Each bar is the mean ± SD, n = 4, ### = $p < 0.01$, * = $p < 0.05$, *** = $p < 0.001$, ZZAE: *Zanthoxylum zanthoxyloides* alkaloidal extract.

group ($p < 0.001$) as shown in Figure 4. The Chlorpropamide (100 mg/kg) group had a significantly high average skeletal muscle glycogen level compared to the model group ($p < 0.001$). The ZZAE (150 mg/kg) had a significantly higher skeletal muscle glycogen than the model group ($p < 0.0001$) and the Chlorpropamide (100 mg/kg) groups ($p < 0.05$). ZZAE (150 mg/kg)-treated group significantly improved post-treatment insulin sensitivity at 80 min and 90 min compared to the model ($p < 0.001$) and the Chlorpropamide (100 mg/kg) ($p < 0.01$).

3.8. ZZAE increased serum insulin levels in NIC/STZ-induced diabetes in rats

The mean post-treatment serum insulin level of the control group (21.18 ± 0.838 µIU/mL), was significantly higher ($p < 0.001$) than the model group (7.225 ± 0.330 µIU/mL). The ZZAE (50, 100 and 150 mg/kg)-treated groups exhibited a dose-dependent improvement in serum insulin levels with the ZZAE (150 mg/kg) demonstrating the highest mean serum insulin levels (18.3 ± 0.294 µIU/mL) and significantly higher than the model ($p < 0.001$) but not the Chlorpropamide (100 mg/kg) (Table 2).

3.9. ZZAE inhibited diabetic dyslipidemia in NIC/STZ-induced diabetes in rats

The mean high density lipoprotein (HDL) (1.008 ± 0.075) was significantly higher ($p < 0.05$) compared to the model group (0.718 ± 0.086). Interestingly, the ZZAE (50 mg/kg) had mean HDL (1.29 ± 0.066 mg/dL) which was significantly higher than the control group, the Chlorpropamide (100 mg/kg) and the ZZAE (100 mg/kg) ($p < 0.05$). The mean serum low density lipoprotein (LDL) was significantly higher in the model (1.358 ± 0.013) compared to the control ($p < 0.001$). The Chlorpropamide (100 mg/kg) group recorded a mean serum LDL levels of 0.348 ± 0.015 which was statistically different from the model group ($p < 0.001$). However, the ZZAE (100 and 150 mg/kg) both showed a dose-dependent reduction in total cholesterol (0.833 ± 0.038 and 0.805 ± 0.051 mg/dL) respectively (Table 3).

3.10. ZZAE improved kidney histology and biomarkers in NIC/STZ-induced diabetes in rats

After the 28-day treatment, the average urea level of the model group (129.77 ± 6.38 mg/dL) was significantly higher than the control group (34.83 ± 3.64 mg/dL) ($p < 0.001$) as shown in Table 5. The ZZAE (50, 100, and 150 mg/kg) showed a dose-dependent regulation in serum levels of urea with the ZZAE (150 mg/kg) group recording the highest reduction in mean serum urea (54.24 ± 3.04 mg/dL), compared to the model group ($p < 0.001$) and almost parallel with the Chlorpropamide (100 mg/kg). The control group had an intact glomerular apparatus each bordered by the distal- and proximal-convoluted tubules. The ZZAE demonstrated a dose-dependent improved glomerular filtrate architecture. In particular, ZZAE (150 mg/kg) had a tuft-like vascular structure and intact glomerular capsular space compared to the model (Figure 5). The chlorpropamide (100 mg/kg) and the ZZAE (150 mg/kg) had more compact podocytes compared to the control.

3.11. ZZAE improved liver histology and functional status in NIC/STZ-induced diabetes in rats

The control group had regular liver histoarchitecture with sheets of hepatocytes radiating from the central vein and regular sinusoids. The model group and the 20% tween20 group had macrovascular steatosis with neutrophilic lobular inflammation compared to the chlorpropamide and the ZZAE treated groups (Figure 6). The ZZAE (50, 100, and 150 mg/kg)- treated groups showed a dose-dependent reduction in Mallory-Denk bodies compared to the control. There was also a large number of necrotic cells and inflammatory cells in the model and 20% Tween20 groups. These results were corroborated with liver

Table 2. Effect of ZZAE on serum insulin levels of NIC/STZ-induces diabetes in rats.

Treatment groups	Pre-treatment (µIU/mL)	Post-treatment (µIU/mL)	P value
Control	21.33 ± 0.619	21.18 ± 0.838	NS
Model	6.8 ± 0.294	7.225 ± 0.330[a]	NS
20% Tween20	6.775 ± 0.435	7.1 ± 0.245	NS
Chlorpropamide (100 mg/kg)	6.65 ± 0.208	16.825 ± 0.45	<0.01
ZZAE (mg/kg)			
50	6.55 ± 0.238	10.05 ± 0.3[bc]	<0.05
100	6.65 ± 0.341	13.4 ± 0.44[b]	<0.01
150	6.8 ± 0.356	18.3 ± 0.294[b]	<0.001

a = model vs. control statistically significant, b = model vs. all treatment groups statistically significant, c = Chlorpropamide (100 mg/kg) vs ZZAE groups statistically significant. Values are reported as mean ± SD, n = 4.
ZZAE – *zanthoxylum zanthoxyloides* alkaloidal extract, NIC – Nicotinamide, STZ - streptozotocin.

Table 3. Effect of ZZAE on serum lipid profile in NIC/STZ-induced diabetes in rats.

Treatment groups	TC (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	TG (mg/dL)
Control	1.21 ± 0.104	1.008 ± 0.075	0.435 ± 0.026	0.618 ± 0.022
Model	1.143 ± 0.051	0.718 ± 0.086[a]	1.358 ± 0.013[a]	1.2 ± 0.061[a]
20% Tween20	1.138 ± 0.070	0.618 ± 0.029	1.385 ± 0.013	1.153 ± 0.017
Chlorpropamide (100 mg/kg)	0.94 ± 0.147[b]	0.948 ± 0.025[b]	0.348 ± 0.015[b]	0.538 ± 0.028[b]
ZZAE (mg/kg)				
50	1.065 ± 0.062	1.298 ± 0.066[b]	0.628 ± 0.033[b]	0.378 ± 0.026[b]
100	0.833 ± 0.038[b]	0.928 ± 0.026[b]	0.483 ± 0.049[b]	0.312 ± 0.022[b]
150	0.805 ± 0.051[b]	1.403 ± 0.035[b]	0.235 ± 0.019[b]	0.548 ± 0.017[b]

a = control vs. model ($p \leq 0.05$), b = model vs all treatment groups ($p \leq 0.05$), ns = model vs treatment groups ($p > 0.05$).
TC: total cholesterol; HDL:high-density lipoprotein; LDL: low-density lipoprotein.
TG: triglycerides; NIC: nicotinamide; STZ: streptozotocin.

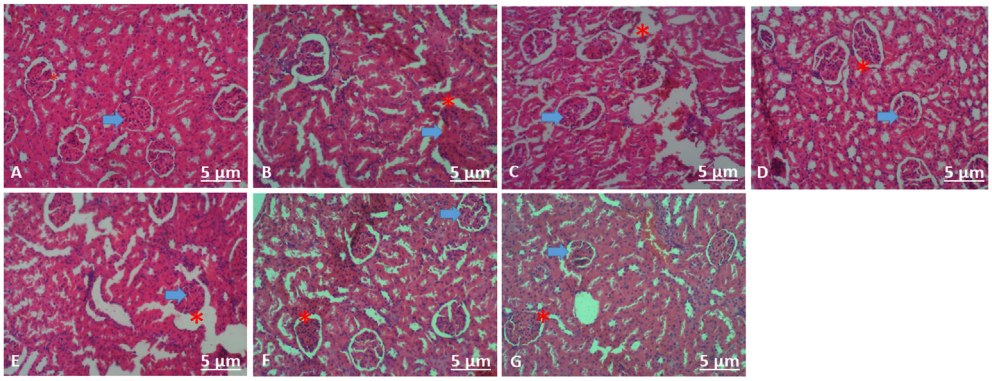

Figure 5. Effect of ZZAE and Chlorpropamide on kidney histoarchitecture of NIC/STZ-induced diabetic rats. A (control, ×100), B (model, ×100), C (20% tween20, ×100), D (chlorpropamide, ×100), E (ZZAE 50 mg/kg, ×100), F (ZZAE 100 mg/kg, ×100), G (ZZAE 150 mg/kg, ×100). ZZAE: *Zanthoxylum zanthoxyloides* alkaloidal extract; NIC: nicotinamide; STZ: streptozotocin, Star = glomerular capsular space, arrow = glomerula apparatus.

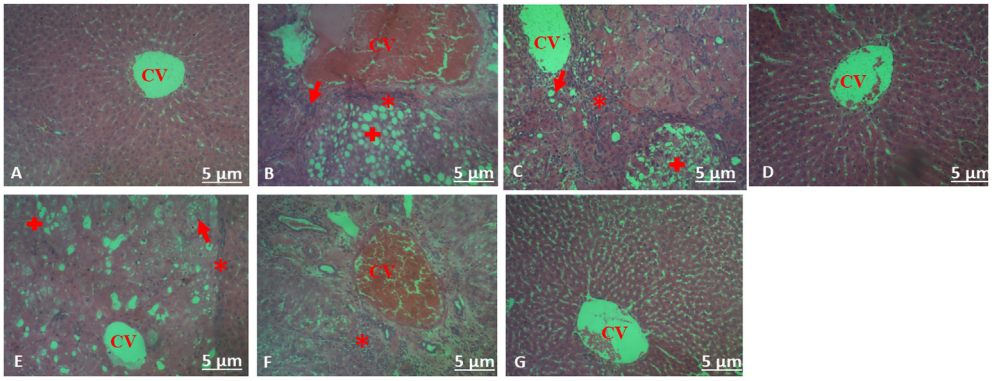

Figure 6. Effect of ZZAE and Chlorpropamide on liver histoarchitecture of NIC/STZ-induced diabetic rats. A (control, ×100), B (model, ×100), C (20% tween20, ×100), D (chlorpropamide, ×100), E (ZZAE 50 mg/kg, ×100), F (ZZAE 100 mg/kg, ×100), G (ZZAE 150 mg/kg, ×100). Arrows, (central vein), ZZAE: *Zanthoxylum zanthoxyloides* alkaloidal extract; NIC: nicotinamide; STZ: streptozotocin, Star = Inflammatory cells, arrow = mallorry denk bodies.

biochemical results. For instance, the mean serum GGT of the control (3.66 ± 0.17 IU/L) was significantly lower compared to the model (8.56 ± 0.32 IU/L) ($p < 0.01$). The average GGT level of the Chlorpropamide (100 mg/kg) group (4.90 ± 0.20 IU/L) was not statistically different from the control but was statistically different from the model ($p < 0.001$). The ZZAE demonstrated a dose-dependent regulation of the GGT. There was no significant difference between ZZAE (150 mg/kg) and Chlorpropamide (Table 5). The average AST and ALT for the control were statistically different from the model group ($p < 0.01$). Treatment with Chlorpropamide (100 mg/kg) and the ZZAE (150 mg/kg) significantly improved mean ALT and mean AST compared to the model ($p < 0.01$).

3.12. ZZAE stimulated insulin secretion in the islet of langerhans

The concentration of insulin produced after 120 min of incubation in a bicarbonate buffer 7.5% solution supplemented with ZZAE (50 µg/mL) (46.31 ± 3.4 µIU/islet) was significantly high ($p < 0.001$) compared to islets of Langerhans incubated without ZZAE (21.6 ± 3.1 µIU/islet) (Table 6).

4. Discussion

In this study, we report that alkaloidal extract from leaves of *Zanthoxylum zanthoxyloides* (ZZAE) ameliorates nicotinamide/streptozotocin-

Table 4. Effect of ZZAE on kidney biomarkers of NIC/STZ-induced diabetes in rats.

Biomarker (units)	Control	Model	20% Tween20	Chlorpropamide (100 mg/kg)	ZZAE (mg/kg)		
					50	100	150
Protein (g/dL)	5.73 ± 0.11	3.11 ± 0.09	4.1 ± 0.02	5.72 ± 0.07^{b}	4.28 ± 0.16	4.26 ± 0.09	4.12 ± 0.31^{b}
creatinine (mg/dL)	0.81 ± 0.07	4.05 ± 0.07^{a}	3.91 ± 0.04	2.05 ± 0.05^{b}	2.88 ± 0.09^{c}	1.71 ± 0.04	1.33 ± 0.04^{b}
Urea (mg/dL)	34.83 ± 3.64	129.77 ± 6.38^{a}	136.22 ± 9.14	58.16 ± 4.76^{b}	108.66 ± 4.29^{c}	84.51 ± 0.68^{b}	54.24 ± 3.04^{b}

a = model vs. control $p < 0.05$, b = model vs. all treatment groups, $p < 0.05$, c = Chlorpropamide (100 mg/kg) vs ZZAE groups, $p < 0.05$. values are reported as mean ± SD, n = 4.
ZZAE: *Zanthoxylum zanthoxyloides* alkaloidal extract; NIC: nicotinamide; STZ – streptozotocin.

Table 5. Effect of ZZAE on liver biomarkers in NIC/STZ-induced diabetes in rats.

Biomarker (units)	Control	Model	20% Tween20	Chlorpropamide (100 mg/kg)	ZZAE (mg/kg)		
					50	100	150
ALP (IU/L)	13.11 ± 0.30	31.46 ± 0.27^{a}	30.72 ± 0.84	16.44 ± 0.32^{b}	29.33 ± 0.42^{bc}	24.24 ± 0.55^{b}	17.03 ± 0.46^{b}
AST (IU/L)	61.37 ± 0.19	142.19 ± 0.66^{a}	137.25 ± 0.93	82.66 ± 0.94^{b}	138.17 ± .31^{c}	104.72 ± 0.91^{b}	76.49 ± .11^{b}
ALT (IU/L)	47.91 ± 0.55	137.39 ± 0.61^{a}	126.31 ± 1.04	56.53 ± 0.87^{b}	93.18 ± 0.33^{b}	105.72 ± 1.11^{b}	77.20 ± 0.32
GGT (IU/L)	3.66 ± 0.17	8.56 ± 0.32^{a}	9.01 ± 0.69	4.90 ± 0.20^{b}	7.06 ± 0.33^{c}	6.32 ± 0.26^{b}	5.60 ± 0.32^{b}
Protein (g/dL)	5.73 ± 0.11	3.11 ± 0.09	4.1 ± 0.02	5.72 ± 0.07^{b}	4.28 ± 0.16	4.26 ± 0.09	4.12 ± 0.31^{b}
Albumin (g/dL)	5.04 ± 0.43	2.27 ± 0.20^{a}	2.19 ± 0.14	3.43 ± 0.17	2.52 ± 0.13	2.87 ± 0.10	3.03 ± 0.16^{d}
Globulin (g/dL)	2.2 ± 0.02	0.88 ± 0.03^{a}	0.77 ± 0.03	1.99 ± 0.03^{b}	0.99 ± 0.03^{c}	1.16 ± 0.03^{c}	1.72 ± 0.04^{b}
Bilirubin (mg/dL)	0.81 ± 0.07	2.05 ± 0.07^{a}	2.1 ± 0.04	1.05 ± 0.05^{b}	1.88 ± 0.09^{c}	1.71 ± 0.04	1.33 ± 0.04^{b}

a = model vs. control statistically significant, b = model vs. all treatment groups statistically significant, c = Chlorpropamide (100 mg/kg) vs ZZAE groups statistically significant. Values are reported as mean ± SD, n = 4.
ZZAE – *Zanthoxylum zanthoxyloides* alkaloidal extract, ALT – alanine transaminase, AST – aspartate transaminase.
ALP – alkaline phosphatase, GGT – gamma-glutamyltransferase.

Table 6. Effect of ZZAE on islet secretion.

	No ZZAE		ZZAE (50 μg/mL)	
	60 min	120 min	60 min	120 min
Insulin release, μU/islet	13.2 ± 1.4	21.6 ± 3.1	34.21 ± 1.7$^{###}$	46.31 ± 3.4***

= no ZZAE 60 min. vs ZZAE (10 mg/kg) 60 min. ($p < 0.001$), *** no ZZAE 120 min vs ZZAE (10 mg/kg) 120 min. $p < 0.001$. values are reported as mean ± SD, n = 4.
ZZAE – *Zanthoxylum zanthoxyloides* alkaloidal extract.

induced diabetes in rats and improved the histoarchitecture of the pancreas, liver, and kidney following a 28-day treatment regimen. We also report that ZZAE stimulates insulin secretion in islets of Langerhans isolated from normoglycemic rats and proposed that stimulation of insulin secretion could be one of the major mechanisms by which the anti-diabetic effect of ZZAE is conferred.

It has been established that around 80% of the global population have ever used and/or use natural product medicine for or as part of their primary healthcare [34]. In the past two decades, research and development in herbal medicine have received tremendous attention, and public interest in natural therapies is greatly accepted [12]. With regards to diabetes treatment, plant-based alkaloids have been demonstrated to have extreme prospects [35, 36, 37]. In some parts of Africa, plants believed to have anti-diabetic properties are routinely added to food and taken for prophylactic purposes [38], and thus increase the need to scientifically assess the safety and efficacy of these herbal preparations.

Diabetes is characterized by hyperglycemia and its associated complications such as retinopathy, nephropathy, non-alcoholic fatty liver disease, dyslipidemia, etc. Intravenous glucose tolerance test (IVGTT) is routinely used to measure the lag time to sufficient concentration of insulin secretion upon glucose exposure. This method is extremely accurate because it eliminates the bias of intestinal absorption differences in different animals [39] which is associated with oral glucose tolerance test. Poor insulin response and elimination kinetics of glucose can be used as a prediction for insulin resistance and/or poor islet secretion [40]. In this study, the highest dose of *Zanthoxylum zanthoxyloides* crude extract (ZZCE 400 mg/kg) significantly improved insulin sensitivity in normoglycemic rats compared to the control at the 30th min. and Chlorpropamide at the 120 min in an IVGTT (Figure S2). These findings provide leads for further research in the formulation of diets and food supplements for prediabetic individuals.

Under regular physiological conditions, excess blood glucose is converted to glycogen by hepatocytes under the stimulatory effect of insulin. After the glucose has been converted into glycogen, a significant amount is stored in skeletal muscles and anything more than 5% of liver mass left in the liver is converted to fatty acids for storage as adipocytes [41]. Type-2 diabetes is associated with either insulin insensitivity and/or reduction in insulin production [42] which leads to a disruption in skeletal muscle glycogen synthesis and dislipidemia. In the case of NIC/STZ-induced diabetes, the STZ induces pancreatic β cell apoptosis. Thus, reduction in active islets of Langerhans results in the underproduction of insulin causing a reduction in hepatic conversion of glucose to glycogen. Insulin is also known to inhibit intracellular lipase that converts triglycerides to fatty acids [43]. Thus, diabetes results in reduced skeletal muscle glycogen synthesis, dyslipidemia, and weight loss. This also means that any agent or natural product capable of regulating the foregoing indices could be a potential intervention in diabetes.

In this study, ZZAE significantly improved pancreatic histoarchitecture and increased islets cellularity in NIC/STZ-induced diabetes in rats. This

caused a downstream effect in insulin secretion and its effect on weight management, lipid synthesis, and blood glucose concentration. For instance, while the model group suffered a significant chronic mean weight loss compared to the control, the ZZAE (150 mg/kg) significantly restored weight gain compared to the model group and the chlorpropamide (100 mg/kg) (Table 2). Exposure to NIC/STZ also caused a significant reduction in mean serum insulin levels in the model group compared to the control (Table 3). ZZAE (150 mg/kg) demonstrated to be effective in moderating mean serum insulin levels, mean serum triglycerides, and high-density lipoproteins (HDL) compared to the model (Table 4). It must be said that regulation of insulin secretion within physiological ranges promotes effective lipid synthesis, skeletal muscle glycogen synthesis and subsequently regulates blood glucose concentrations. The ability of ZZAE (especially 150 mg/kg) to effectively increase serum insulin secretion also led to a corresponding significantly better skeletal muscle glycogen synthesis compared to the model group (Figure 4).

Increased blood glucose stimulates physiological osmoregulatory mechanisms that trigger osmometric thirst and a consequence of polyuria and subsequently glomerulus collapse [44]. Studies have also found that secretion of proinflammatory cytokines stimulates hepatic insulin resistance that causes the release of fibroblast growth factor receptor-21 primarily causing nonalcoholic fatty liver disease (NAFLD) in about 70% of diabetic patients, [45, 46]. A similar outcome was seen in the model group which had endocapillary hypercellularity with a significant number of glomerular congestion than the control in our study (Figure 4) and elevated levels of serum urea and creatinine (Table 5). These histomorphological alterations that were seen in the model were significantly improved particularly by the ZZAE (150 mg/kg). It is also worthy of note that a corroboratory result was recorded in the serum urea and creatinine levels for the ZZAE treatment groups. Indeed, these findings are similar to those reported elsewhere [47]. ZZAE was able to significantly reduce the levels of the gamma-glutamyl transferase, alanine transaminase aspartate aminotransferase, and other liver enzymes seen in the model group, an indication of the restoration of liver health.

Deregulated glucose metabolism is the causative mechanism of diabetes microvascular disorders. Indeed, in the management of type-2 diabetes, the ability to restore normoglycemia is paramount. Thus, any drug compound and/or natural product that can regulate blood glucose can be relied upon to mitigate concomitant morbidities of diabetes. Among the various pathways that can be interfered with to ensure optimum glucose biosynthesis, stimulation of islet secretion is arguably the most effective. Our study showed that ZZAE had a good islet secretion stimulatory activity (Table 6). ZZAE has several alkaloids that may be working along different pathways to synergistically demonstrate the observed anti-diabetic effect. However, our study has provided leads for further studies to isolate and investigate the individual alkaloid(s) responsible for the observed antidiabetic effect in NIC/STZ-induced diabetes in rats.

5. Conclusion

Our study has shown that ZZAE blood glucose regulatory activity in NIC/STZ-induced diabetes in rats and its associated dislipidimia, and nephropathy. ZZAE has also demonstrated pancreatic islet stimulatory effect and improvement in skeletal muscle glycogen synthesis which could spark frontiers for the advancement in the development of drug candidates for type-2 diabetes.

Declarations

Author contribution statement

Isaac Kyei-Barffour: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Roselind Kyei Baah Kwarkoh; Ophelia Duke Arthur; Samuel Addo Akwetey; Christian Kweku Adokoh; Isaac Kingsley Amponsah: Performed the experiments.

Desmond Omane Acheampong: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Benjamin Aboagye: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Augustine Suurinobah Brah: Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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