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Original Article

Tetracarpidium Conophorum (African Walnut) Seeds Protects Against Diabetes-Induced Liver Damage in Rats Treated with Streptozotocin

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Abstract

Introduction: This study evaluated the protective potential of Tetracarpidium conophorum seeds against liver damage in rats treated with a single intraperitoneal dose of 75 mg/kg/body weight of streptozotocin. **Material and Methods:** The rats were divided into five (n=5) groups: A - normal control, B - diabetic control, C - diabetic rats treated orally with a Tetracarpidium conophorum seeds extract (500 mg/kg/body weight), D - diabetic rats treated orally with 7 mg/kg/body weight of metformin and E - diabetic rats treated subcutaneously with 0.3 IU/kg/body weight of Humulin R. Treatment was done once daily for 2 weeks. A blood sample was collected for biochemical estimations. The liver and pancreas were also harvested for biochemical/histological studies. **Results:** The blood glucose reduction percentage was 41%, 34%, and 36% in rats treated with Tetracarpidium conophorum seeds, metformin and insulin, respectively. Tetracarpidium conophorum seeds significantly reduced ($p<0.05$) thiobarbituric reactive substances, serum transaminases, gamma-glutamyl transferase levels, and the percentage of hepatic fragmented DNA while it significantly decreased ($p<0.05$) glutathione levels and increased superoxide dismutase activity. Histological observations showed varying degrees of liver and pancreas damage in the diabetic group that was untreated, while the administration of Tetracarpidium conophorum seeds significantly improved the general histoarchitecture of tissues relative to control group and other treatment groups. **Conclusions:** Tetracarpidium conophorum seeds possess good glycemic control of diabetes mellitus and protect the liver against oxidative damage induced by hyperglycemia.

Keywords: Tetracarpidium conophorum seed, diabetes, biomarkers, liver damage.

Introduction

Diabetes mellitus (DM) is an oxidative metabolic disorder whose prevalence has continuously increased worldwide over the past few decades. It is characterized by hyperglycemia that results from an absolute or relative insulin deficiency and is associated with long-term complications that affect the eyes, kidneys, heart, liver, blood vessels, and nerves [1].

The liver is involved in the regulation of blood glucose levels in physiological and pathological states such as DM [2, 3]. The spectrums of biochemical changes that occur in DM resemble those of liver diseases [4, 5]. The important indicators in assessing liver injury are levels of plasma alanine transaminase, aspartate transaminase, alkaline phosphatase, and gamma-glutamyl transferase [6-8]. Elevated levels of serum transaminases are a common sign of liver disease and are observed more frequently among people with diabetes than the general population [9].

Pharmaceutical industries have come to consider traditional medicine as a source of identification of bioactive agents that can be used to design synthetic drug products. Extracts from medicinal plants are now being sold in the purified form for the treatment and prevention of all types of diseases. Tetracarpidium

conophorum, known as African walnut, is called "asala" by Yoruba people in South-Western Nigeria and "ukpa" by the Igbo ethnic group in South-Eastern Nigeria. T. conophorum is an industrial plant widely cultivated for the production of nuts and is used as a delicacy. The nuts are eaten either raw or cooked [10, 11].

Diabetic patients are hindered from accessing effective medications due to financial constraints. Hence, there is a need for an affordable and accessible medicinal plant with little or no side effects to manage the disease [11]. Therefore, this study was conducted in order to investigate possible protective potentials of T. conophorum seeds against diabetic liver damage in a streptozotocin-induced diabetic rat model.

Material and Methods

Reagents and chemicals

Methanol, streptozotocin, citric acid, sodium citrate, normal saline, hydrochloric acid, Tris, EDTA, Triton x-100, Diphenylalanine, glacial acetic acid, sulfuric acid, acetaldehyde, trichloroacetic acid, sodium hydroxide, Humulin R and Metformin were obtained from either the Sigma chemical company, St. Lious, Mo, U.S.A., or British Drug House (BDH) chemical Ltd., Poole, England. The diagnostic kits were obtained from Randox Laboratories Ltd., Crumlin, Co. Antrim, U.K., or Agappe Diagnostics, Switzerland. All reagents and chemicals used were of analytical grade.

Methanolic extraction from Tetracarpidium conophorum

T. conophorum nuts were purchased from a local market in Osogbo, Osun State, Nigeria. The plant was identified and authenticated at IFE Herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria, where specimen copy was deposited. The herbarium identification number was 17713. The nuts were prepared and extracted with 100% methanol. Acute toxicity and determination of safe doses of the extract have been described in our previous study [11].

Phytochemical screening of extract and fractions

The preliminary phytochemical tests were carried out on the methanol extract using standard procedures, as described by Harborne and Turner [12].

Determination of total antioxidant capacity and reducing power assay

Total antioxidant capacity was assayed using the phosphomolybdenum method as described by Prieto et al. [13] while reducing power assay was carried out as described by Oyaizu [14].

Grouping and treatment of experimental animals

Experimental animals were used following the institution guidelines and the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. Thirty albino rats were kept in clean plastic cages and fed with rat food and water ad libitum. The animals were acclimatized to standard laboratory conditions. Diabetes was induced in twenty-five rats with a single injection of streptozotocin (75 mg/kg/body weight), as described by Ajilore and Adesokan [11]. The rats were randomly divided into five groups (n=5) as follows:

Group A: Normal control rats;

Group B: Diabetic control rats;

Group C: Diabetic rats treated with 500 mg/kg body weight of Tetracarpidium conophorum seed extract (TECOSE);

Group D: Diabetic rats treated orally with 7 mg/kg body weight of metformin;

Group E: Diabetic rats treated subcutaneously with 0.3 IU/kg body weight Humulin R;

All the rats were treated once daily for two weeks. The weights and blood sugar of each rat were recorded before induction of diabetes, after induction of diabetes, and at the end of treatment. Blood samples were collected by ocular puncture before sacrificing the animals, and liver tissues were harvested.

Preparation of the blood sample

A fresh blood (5 ml) sample was collected by ocular puncture from each rat into a clean plain labeled tube, allowed to clot, and was then centrifuged at 3000 rpm for 10 minutes in a bench centrifuge at room temperature. The clear serum was separated and kept at -20°C until assay.

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Determination of thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH) levels and superoxide dismutase (SOD) activity

The extent of lipid peroxidation in the liver, brain, and heart was determined by measuring the release of TBARS using the procedure described by Ottolenghi [15] and expressed as nanomolar of malondialdehyde (MDA)/g tissue. GSH level was determined by the method described by Sapakal et al. [16], while SOD activity was estimated according to the method described by Micord and Fridovich [17]. Tissue total protein level was determined according to the method described by Gornal et al. [18] using the Randox diagnostic kit.

DNA fragmentation assay

Immediately after sacrificing the animals, liver samples were harvested, and 0.5 g of the tissues were homogenized with 5 ml buffer solution containing 10 mM tris-HCL (PH.8), 1 mM EDTA, 0.2% Triton X-100. The homogenate was centrifuged at 10000 rpm for 20 minutes (at 4˚C). The pellet was re-suspended in 2.5 ml of the previous buffer solution. To the pellets (P) and supernatants (S), 1.5 ml of 10% tri-chloro-acetic acid (TCA) was added and incubated at 44˚C for 10 minutes. Then 0.75 ml of 5% TCA was added, and the assay mixtures were incubated again at 100˚C for 20 minutes. As described by Gibb et al. [19], DPA (diphenylamine) solution was subsequently added to each sample: 2 ml of DPA [200 mg DPA in 10 ml glacial acetic acid; 0.15 ml of sulfuric acid and 0.06 ml acetaldehyde]; then, the samples were incubated at room temperature for 24 hours. The proportion of fragmented DNA was calculated from absorbance reading at 600 nm using the formula:

$$\text{DNA fragmentation} = \frac{\text{OD (S)}}{\text{OD (S)} + \text{OD (P)}} \times 100$$

OD (S) is the proportion of fragmented DNA

OD (P) is the proportion of intact DNA

Estimation of liver function indices

Serum alanine transaminase and aspartate transaminase activities were determined according to the method described by Reitman and Frankel [20] using the Randox diagnostic kit, while gamma-glutamyl transferase activity was determined according to the method described by Szasz [21] using the Agappe diagnostic kit.

Histological studies

At the end of the second week of treatment, liver and pancreas tissues were harvested from the sacrificed rats and immediately fixed in 10% formalin and used for histomorphological studies.

Statistical analysis

Data obtained were analyzed using One Way Analysis of Variance (SPSS version 20.0). Levene statistic was used for tests of homogeneity of variance. Tukey's test was used for multiple comparisons and homogenous subsets. A p-value of less than 0.05 was considered statistically significant.

Results

Phytochemical composition of the methanol extract from the T. conophorum seeds

The T. conophorum seed extract gave a positive test reaction to alkaloids, cardiac glycosides, steroids, flavonoids and terpenoids, and a negative test reaction to saponins, tannins, and phlobotannins (Table 1).

Table 1: Phytochemical composition of the methanol extract from the Tetracarpidium conophorum seed.

		Standard Snack	Results
		Alkaloids	+
		Cardiac glycoside	+
		Steroids	+
		Tanins	–
		Phlobotanins	–
		Flavonoids	+
		Terpenoids	+
		Saponins	–
+ Present	- Absent	Alkaloids	+

Reducing power activity and total antioxidant capacity of Tetracarpidium conophorum seed extract.

The ability of T. conophorum seed extract to reduce ferric chloride and their total antioxidant contents were dose-dependent with 60 mg/ml as the highest, followed by 40 mg/ml and 20 mg/ml (Table 2)

Values are expressed as mean ± S.D (n=3). Means of sample concentrations with different Tukey superscripts along the row are statistically significant at $p<0.05$.

Table 2: Reducing power activity and total antioxidant capacity of Tetracarpidium conophorum seed extract.

	Concentrations of Extract		
Samples	**20mg/ml**	**40mg/ml**	**60mg/ml**
Reducing Power Activity	0.75 ± 0.00[a]	0.78 ± 0.01[a]	0.83 ± 0.06[b]
Total Antioxidant Capacity	0.63 ± 0.01[a]	0.75 ± 0.00[ab]	0.80 ± 0.02[b]

Table 3: Average body weight (g) in the control and treatment groups.

	Average Body Weight (g)			
Treatment Group	**Before Induction**	**After Induction**	**After Treatment**	**Percentage Weight Change**
Normal Control	122.80±31.23[a]	135.00±27.61[b]	146.00±26.55[c]	8 %
Diabetic Untreated	118.00±17.89[b]	96.80±10.64[a]	90.80±10.85[a]	- 6 %
Diabetic + 500 mg/kg of Methanol Extract	116.40±13.74[b]	107.20±10.64[a]	120.60±18.22[b]	13 %
Diabetic + 7 mg/ kg of Metformin	120.00±15.81[ab]	115.00±15.83[a]	124.00±17.52[b]	9 %
Diabetic + 0.3 unit/ kg of Humulin R	129.60±22.24[b]	105.60±12.12[a]	112.40±9.24[ab]	7 %

Note:Values are expressed as mean ± S.D (n=5). Means with different Tukey superscripts along the row are statistically significant at $p<0.05$.

Table 4: Average blood glucose (mmol/L) levels in the control and treatment groups.

	Average Blood Glucose (mmol/L)			
Treatment Group	**Before Induction**	**After Induction**	**After Treatment**	**Percentage Blood Glucose**
Normal Control	3.53±0.54a	3.54±0.54a	3.69±0.52a	Not Sig.
Diabetic Untreated	2.98±0.60a	15.47±2.36ab	20.05±2.76b	+ 30 %
Diabetic + 500 mg/kg of Methanol Extract	4.46±1.47a	27.75±8.51c	16.41±9.65b	- 41 %
Diabetic + 7 mg/ kg of Metformin	4.55±0.47a	24.11±0.47c	15.98±6.65b	- 34 %
Diabetic + 0.3 unit/ kg of Humulin R	4.19±1.12a	21.07±5.48c	13.52±1.34b	- 36 %

Note:Values are expressed as mean ± S.D (n=5). Means with different Tukey superscripts along the row are statistically significant at $p<0.05$.

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Average body weight of rats in the control and treatment groups

The percentage weight gain was significant ($p<0.05$) and the highest (13%) was seen in rats treated with 500mg/kg/body weight of methanolic extract of TECOSE when compared to the metformin (9%) and insulin (7%) treatment groups. There was significant ($p<0.05$) weight loss in untreated diabetic rats (Table 3).

Average blood glucose of rats in the control and treatment groups

At the end of the second week of treatment, there was a significant ($p<0.05$) decrease in the blood glucose levels following the treatment with the Tetracarpidium conophorum seed extract, metformin and insulin. The percentage reduction in blood glucose levels was highest (41 %) in rats treated with 500mg/kg/body weight of methanol extract of TECOSE, followed by insulin (36%) and metformin (34%), respectively (Table 4).

Thiobarbituric acid reactive substances, reduced glutathione levels and superoxide dismutase activity in the control and treatment groups

Figures 1-3 showed the degree of lipid peroxidation (using TBARS), GSH levels and SOD activity in the brain, liverand heart tissues in control and treatment groups. TBARS concentration was significantly ($p <0.05$ higher in all tissues of untreated diabetic rats relative to control and the treatment groups. There was no significant ($p>0.05$) difference in the levels of GSH in the liver and heart in all treatment groups. The level of GSH in the brain was significantly ($p<0.05$) reduced in untreated diabetic rats but was significantly ($p<0.05$) increased in rats treated with extracts and metformin. SOD activity was significantly ($p <0.05$) higher in all tissues of untreated diabetic rats relative to control and the treatment groups.

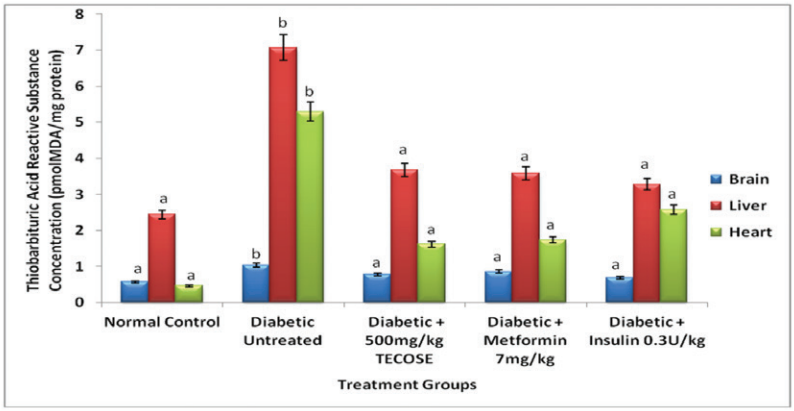

Figure 1: Thiobarbituric acid reactive substances concentration (pmolMDA/mg protein) in the brain, liver and heart. Values are expressed as mean ± S.D (n=5). Means of bars of the same legend with different Tukey superscripts are statistically significant at $p<0.05$.

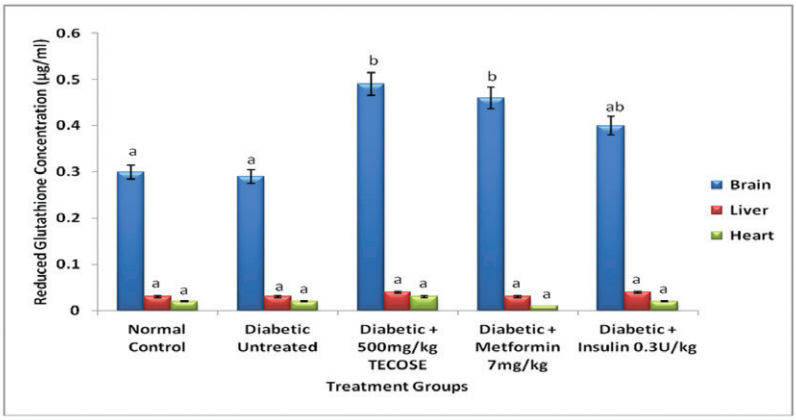

Figure 2: Reduced glutathione concentration (µg/ml) in the control and treatment groups. Values are expressed as mean ± S.D (n=5). Means of bars of the same legend with different Tukey superscripts are statistically significant at $p<0.05$.

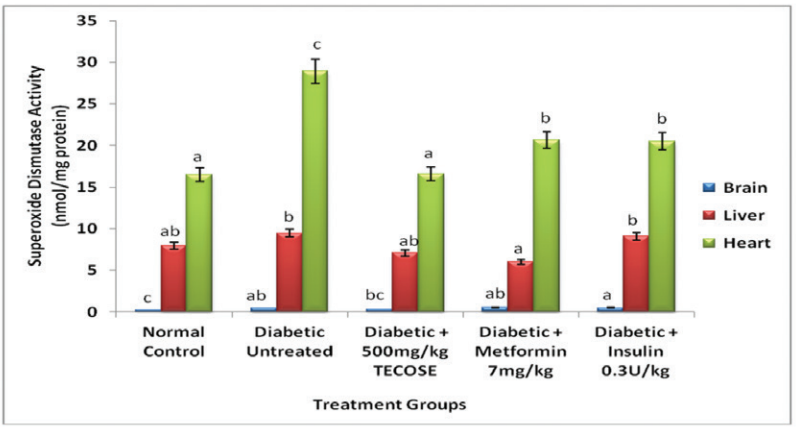

Figure 3: Superoxide dismutase activity (nmol/mg protein) in the brain, liver and heart. Values are expressed as mean ± S.D (n=5). Means of bars of the same legend with different Tukey superscripts are statistically significant at $p<0.05$.

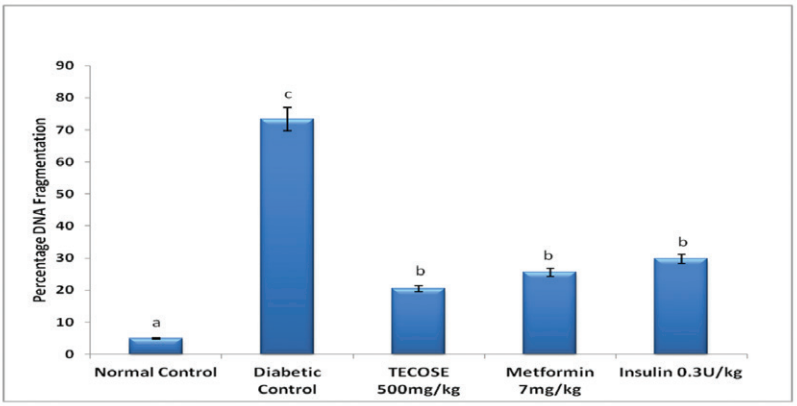

Figure 4: Percentage of DNA fragmentation in the control and treatment groups. Values are expressed as mean ± S.D (n=5). Tukey superscripts a, b, and c are significance homogenous subsets of means within groups bars with different Tukey superscripts and are statistically significant at $p<0.05$.

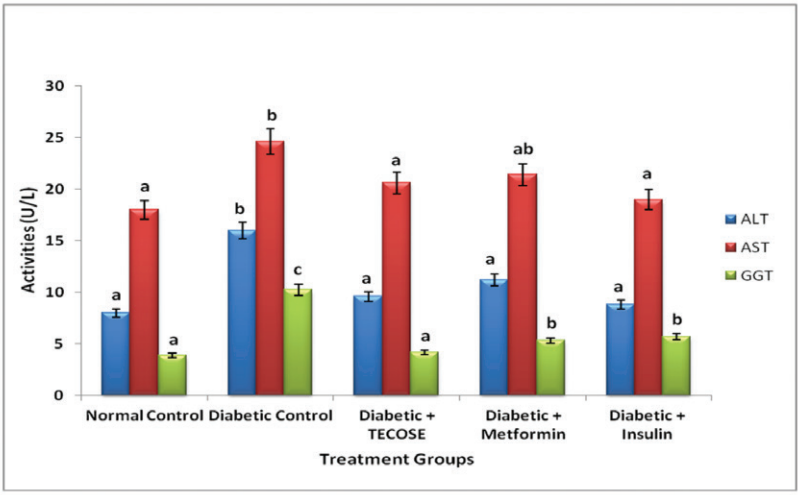

Figure 5: Liver function indices in the control and treatment groups. Values are expressed as mean ± S.D (n=5). Tukey superscripts a, b, ab and c are significance homogenous subsets of means within groups. Bars of the same legend with different Tukey superscripts are statistically significant at $p<0.05$.

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Percentage of DNA fragmentation in the control and treatment groups

The percentage of DNA fragmentation was significantly ($p<0.05$) increased in untreated diabetic rats relative to control and the treatment groups (Figure 4).

Liver function indices in the control and treatment groups

The serum activities of alanine transaminase (ALT), aspartate transaminase (AST), and gamma-glutamyl transaminase (GGT) were significantly ($p<0.05$) higher in the untreated diabetic group. At the same time, the levels were near normal in other treatment groups (Figure 5).

Histomorphological studies of the liver and pancreas in the control and treatment groups

The plate of hepatic cells from the untreated diabetic group relative to control group and other treatments was characterized by loss of liver parenchyma, disorganization, cell death, dilation of the central vein, severe hemorrhage and presence of inflammatory red cells within and around the central vein with sinusoids (red arrows) (Figure 6A). There were also variations in the sizes and shapes of the hepatic nuclei, poor staining intensity, as demonstrated by PAS stain (red arrows) (Figure 6B). The plate of hepatic cells from the TECOSE treatment group showed similar morphological organization to the control group

Figure 7 showed the panoramic views of the pancreas micromorphology of control and treatment groups. Pancreatic parenchyma (PP) showed mild infiltration of inflammatory cells. Red cells with normal serous acinar and zymogenic cells containing abundant eosinophilic cytoplasm with degenerative changes occurring in the islet of Langerhans (IL) marked with the loss of islet cells and distorted pancreatic acinar cells are seen in the diabetic control group (red arrows) (Figure 7A). The normal control group showed intact cytoarchitecture with intact IL, PP, and blood vessels (Bv). The rats treated with insulin and metformin

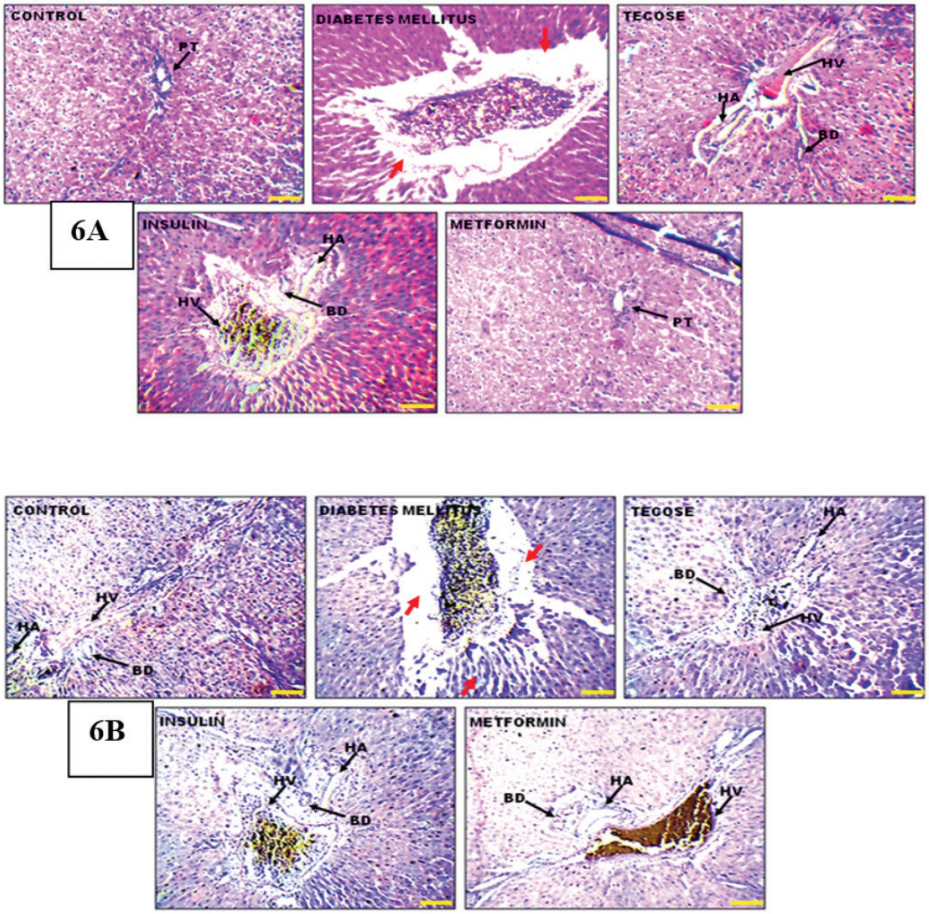

Figure 6: Photomicrographs of liver micromorphology panoramas across the study groups. The plates showed the hepatic duct (HD), portal triad (PT) composed of the hepatic vein (HV) and artery (HA) as well as the bile duct (BD), and well-distributed hepatocytes (H). General histoarchitecture distortion is obvious in the diabetes mellitus plate. Stains: Hematoxylin and Eosin stain (6A) and Periodic Acid Schiff (6B). Scale bar: 50um.

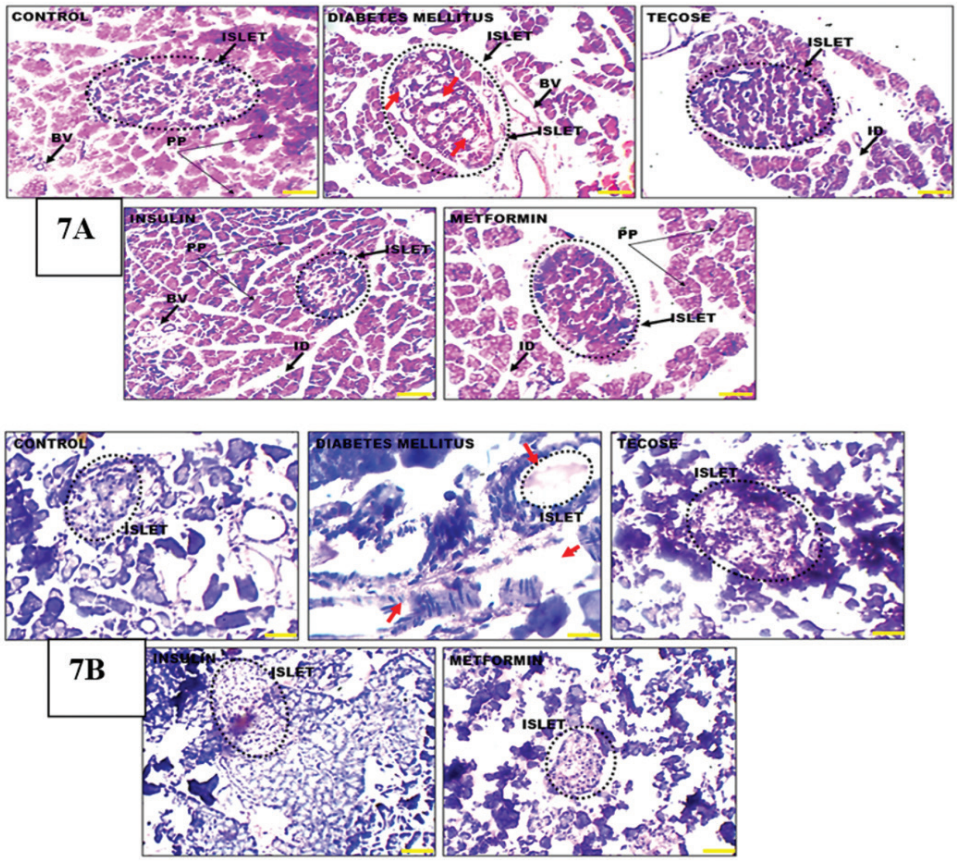

Figure 7: Photomicrographs of pancreatic micromorphology panoramas in the control and treatment groups. The plates showed the islets of Langerhans (IL) containing alpha, beta, delta and pancreatic polypeptide cells, an interlobular duct (ID), pancreatic parenchyma and blood vessels. Stains: Hematoxylin and Eosin stain (7A) and Periodic Acid Schiff (7B). Scale bar: 50um.

showed mild insignificant pancreatic degenerative changes, while the TECOSE treatment group showed a similar observable presentation to the control group (Figure 7B).

Discussion

This study was conducted in order to investigate the possible protective potentials of TECOSE against diabetic liver damage in a streptozotocin-induced diabetes rat model. The beneficial therapeutic effects of many plant extracts have been linked to their phytochemical compositions and bioactive compounds. In this study, we found the presence of alkaloids, flavonoids, terpenoids, cardiac glycosides and steroids in TECOSE. Alkaloids have both antibacterial and anti-fungal properties and have been used in the preparation of some drugs [22]. Flavonoids have many protective effects, including anti-inflammatory, antioxidant, anti-viral, and anti-fungal properties [22].

Flavonoids are well known for their health benefits. Still, they may also have adverse effects, such as antinutritional effects, namely reduced intake of glucose, slower absorption of glucose and, therefore, protection against diabetes mellitus [23]. Cardiac glycosides are phosphodiesterase inhibitors and direct adenylate cyclase stimulants. Alkaloids present in the seeds of T. conophorum are responsible for the bitter taste noticed when one drinks water after eating the nut [24]. The blood-glucose-lowering effect of the study plant could be a result of the presence of phytochemicals like flavonoids, alkaloids, cardiac glycosides and steroids. Many compounds isolated from plant sources with these organic compounds have been reported to show antidiabetic activity [25, 26]. Besides, TECOSE significantly reduced the power activity and total antioxidant capacity in this study.

A significant reduction in the body weight of rats following induction with streptozotocin (STZ) was also noted in previous studies [27-29]. However, the mechanism of weight loss in streptozotocin-induced diabetes is unclear. A study suggested that hypophagia associated with STZ administration and the inability

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of the affected animals to metabolize the carbohydrate fuel leads to a shift in reliance on fatty acid fuels, wasting of fat stores, and loss of weight [27]. TECOSE, metformin, and insulin administration significantly increased the weight of diabetic rats in the present study. Improved insulin sensitivity and lower blood glucose levels via the promotion of peripheral glucose uptake have been postulated as a possible mechanism responsible for gaining weight in diabetes [30].

Treatment of rats with STZ causes a significant increase in blood glucose levels by destroying pancreatic beta-cells [31]. TECOSE (500 mg/kg body weight) significantly ($P<0.05$) reduced blood sugar levels as effective as reference drugs, metformin, and insulin. The possible mode of action responsible for the antidiabetic effect and other therapeutic benefits attributed to Tetracarpidium conophorum and many other medicinal plants is missing in the literature [32], and this should be a subject of concern for future research.

The oxidative stress induced by hyperglycemia is the main pathway for the development of pathological changes in the affected organs [33, 34]. A significant increase in the levels of TBARS in the liver, heart and brain of untreated diabetic rats in this study supports previous studies that showed that diabetes mellitus generates free radicals that damage biomolecules like lipids, proteins, DNA and others [35, 36]. TBARS formed from lipid peroxidation is a critical biomarker of oxidative stress in hyperglycaemic conditions. TECOSE (500 mg/kg/body weight), insulin and metformin significantly reduced the levels of TBARS in all the three organs assessed in this study. In this study, reducing power and antioxidant properties exhibited by a crude methanol extract and fractions of TECOSE were responsible for inhibiting free radical toxicity generated by STZ administration propagation by significantly increasing SOD activity and decreasing GSH levels. SOD provides first-line defense against reactive oxygen species (ROS) that mediate cell injury. It is an enzyme responsible for the breakdown of the superoxide anion into oxygen and hydrogen peroxide [37, 38]. GSH is an intracellular reductant in the reduction of hydrogen peroxide to water by the glutathione peroxidase enzyme [39].

A significant increase in the percentage of fragmented DNA in this study following STZ administration is in accordance with previous studies [40-42]. DNA damage associated with diabetes mellitus and its complications happens mainly through oxidative stress [43]. The glycemic control potentials of TECOSE, metformin, and insulin mitigated ROS generation, which might have been responsible for the significant reduction in the percentage of DNA fragmentation.

Previous research has found elevated serum AST, ALT, and GGT levels following the induction of diabetes with STZ as well [44]. Elevated serum levels of ALT, AST, and GGT are markers of liver injury from induced oxidative stress [3]. Administration of TECOSE, metformin, and insulin significantly reduced the serum levels of these markers by mitigating the generation of ROS, as previously mentioned.

The histomorphological study of hepatic cells from the untreated diabetic group was characterized by loss of liver parenchyma, disorganization, cell death, dilation of the central vein, severe hemorrhage, and the presence of inflammatory red cells within and around the central vein with sinusoids. The histomorphological study of pancreatic parenchyma showed mild infiltration of inflammatory cells. Red cells with normal serous acinar and zymogenic cells containing abundant eosinophilic cytoplasm with degenerative changes occurring in the islet of Langerhans marked with the loss of islet cells as well as distorted pancreatic acinar cells are seen in the diabetic control group. Treatments with metformin and insulin showed mild insignificant histomorphological distortions. However, the administration of TECOSE significantly improved the general histoarchitecture of the liver and pancreas relative to the control group.

Conclusions

The results obtained in this study concluded that Tetracarpidium conophorum seeds have the potential to protect against liver damage associated with diabetes mellitus since it improves glycemic control and mitigates the generation of reactive oxygen species in hyperglycemic states.

Conflict of Interest

The authors declare that there is no conflict of interest.

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