Biointerface Research in Applied Chemistry
Open-Access Journal (ISSN: 2069-5837)

Article
Volume 13, Issue 5, 2023, 445
https://doi.org/10.33263/BRIAC135.445

Earthworm Extract Enhanced Organ Functions in Diabetic Rats by Ameliorating Physiological and Structural Changes

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Scopus Author ID 56564677600
Received: 27.07.2022; Accepted: 5.10.2022; Published: 21.12.2022

Abstract: Diabetes mellitus is an endocrine disorder that affects various body functions. Earthworms are oligochaete soil macroinvertebrates with intrinsic antimicrobial, anti-inflammatory, antioxidant, and chelating abilities. This study aimed to examine earthworm extract's potential to treat diabetes in rats. There were three primary groups of six male Wistar albino rats: the control, the diabetic, and the earthworm extract groups. Streptozotocin (60 mg/kg, i.p.) was used to cause type 1 diabetes mellitus. The control and diabetic groups received 1 ml of distilled water, while the earthworm group received the earthworm extract (45 mg/Kg body weight) daily for four weeks. The earthworm extract group showed a significant reduction in glucose, arginase, alkaline phosphatase, alanine aminotransaminase, aspartate aminotransferase, total cholesterol, triglycerides, low-density lipoprotein, creatinine, urea, uric acid, malondialdehyde, and nitric oxide levels. On the contrary, the earthworm extract caused a significant increase in insulin, glucose-6-phosphate dehydrogenase, total protein, albumin, high-density lipoprotein, follicle-stimulating hormone, luteinizing hormone, testosterone, reduced glutathione, glutathione S-transferases, and catalase levels. The histopathological investigation illustrated the regeneration of damaged pancreatic beta cells and a clear improvement in the hepatic, kidney, heart, and testis structures. This study indicated the efficacy of earthworm extract in improving the biochemical and histopathological changes in diabetic rats' organs. The therapeutic effect of earthworm extract against diabetic complications results from its hypoglycemic activity, antioxidant impact, and regeneration of damaged tissues.

Keywords: earthworm; fertility; oxidative stress; streptozotocin; type 1 diabetes.

1. Introduction

As an endocrine disorder, diabetes mellitus (DM) impacts a lot of biological functions [1]. The disease is a major global health issue that affects around 180 million people globally [2]. Diabetes affects about 463 million, and there will be 578 million diabetic individuals globally by 2030 and 700 million by 2045 [3]. DM causes hyperlipidemia, weight loss, vascular

damage, nephropathy, and retinopathy [4]. High blood glucose levels (hyperglycemia) are the hallmark of Type 1 diabetes mellitus (T1DM). T1DM accounts for about 10% of cases and is caused by absolute insulin deficiency due to progressive and massive loss of insulin-secreting β-cells [5]. T1DM is a chronic condition characterized by a shortage of insulin and a rise in blood glucose levels (hyperglycemia) caused by the autoimmune death of pancreatic β-cells [6]. T1DM is one of the most frequent metabolic and endocrine disorders in children. In the vast majority of patients (70-90%), the loss of β-cells is the consequence of T1DM-related autoimmunity (concomitant with the development of T1DM-associated autoantibodies) [6]. T1DM is treated with pharmacological therapy: continuous insulin injections and non-insulin medicines. In addition, pancreatic and islet transplantation could be an alternative surgical therapy [7]. However, clinical guidelines have identified the adverse effects of continuous insulin infusion, including low blood glucose (hypoglycemia), weight gain, and low blood potassium (hypokalemia) [8]. Long-term consequences of diabetes include microvascular problems like nephropathy, retinopathy, and neuropathy and macrovascular complications like coronary heart disease, stroke, and peripheral vascular disease [9]. Destructive complications may also happen due to T1DM, like diabetic nephropathy (DN) that often results in end-stage renal disease [10] (ESRD), hepatomegaly, and elevated transaminases in a patient with glycogenic hepatopathy (GH) as a complication of uncontrolled diabetes [11]. In addition, endocrine disruption has also been reported to affect the hypothalamus–pituitary testicular (HPT) axis via induction of metabolic alterations in hormonal profile during diabetes conditions [12], and this could herald impairment of male reproductive function [13].

Streptozotocin (STZ) is an antibiotic discovered in the bacteria Streptomyces Achromogenes and a commonly used chemical in rodents to induce an experimental diabetes model [14]. STZ is specifically harmful to β-cells of the pancreas, which are responsible for insulin synthesis [15]. Hyperglycemia, glucosuria, polyphagia, polydipsia, polyuria, body weight loss, hypoinsulinemia, and hyperlipidemia define STZ-induced diabetes [16], which has been generally acknowledged as a model of type 1 diabetes mellitus for research of hyperglycemia and insulinopenia [17].

Natural extracts from plants and animals are natural remedies, considered complementary and alternative medicine that alleviates numerous adverse effects of contemporary medication [18-20]. One of the natural medicines accessible is the earthworm [21]. Earthworms are oligochaete soil macroinvertebrates that play an essential role in soil fertility and production [22]. Earthworms have numerous medicinal qualities; therefore, they have been frequently used in traditional medicine as a fibrinolytic, anticoagulative, and anticancer agent to treat fever, stomach discomfort, neck pain, brain disease, and digestive problems [23]. Earthworms have been utilized in treating gastric ulcers, liver disorders, and kidney diseases [24-26]

Additionally, earthworm extract has essential antimicrobial [27], anti-inflammatory [23], and powerful antioxidant effects, acting as a free radical scavenger by interacting with hydroxyl and singlet oxygen radicals [28]. One of its numerous advantages is its ability to activate natural antioxidant enzymes [25]. Considering that the primary goal of this research was to evaluate the antidiabetic efficacy of earthworm extract by representing its impact in streptozotocin-induced type I diabetic rats.

2. Materials and Methods

2.1. Chemicals and reagents.

Streptozotocin (STZ ≥ 99%) and sodium citrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). The rat insulin ELISA kit was purchased from SinoGeneClon Biotech Co., Ltd with Catalog No.: SG-20161 and Biochemical Kits were purchased from Biodiagnostic Company (Dokki, Giza, Egypt).

2.2. Earthworm extract preparation.

Earthworms were collected from commercial vermiculture in the Giza Governorate and stored in plastic tubs with decomposed organic material until further use. To get rid of the surface mucus on the earthworms, running water was used to wash them. After taking a soak in distilled water for 6 to 8 hours to clean their bodies, earthworms were cleaned again with distilled water. After cleaning, the worms were mashed by cutting them into little pieces, flattened, and placed in a glass tube. Then, 80% ethanol solvent was added and allowed to evaporate, leaving the crude extract. Soaking earthworms using 80% ethanol was carried out for two days.

2.3. Heavy metals and amino acid content of earthworm extract.

Metal contents were determined using a 304 u/c Atomic Absorption Spectrometer. In comparison, High-Performance Liquid Chromatography (HPLC) was used for the determination of the amino acid content of earthworm extract.

2.4. Experimental animals.

Male albino Wistar rats (*Rattus norvegicus*) weighing 140±10 gm were employed in the investigation. The National Research Center (NRC, Dokki, Giza) supplied the rats. In the well-ventilated animal house of the Zoology Department, Faculty of Science, Cairo University, they were housed in groups of six in polyacrylic cages. Rats were allowed unlimited access to food and water. Rats were kept in a comfortable setting with a 12-hour light/12-hour dark cycle at room temperature (22-25oC). Before the experiment began, they were exposed to lab conditions for seven days.

2.5. Ethical consideration.

The Institutional Animal Care and Use Committee (IACUC), Faculty of Science, Cairo University (Egypt) authorized the experimental protocols and practices applied in this study (CU/I/F/59/22). The international standards for the care and management of laboratory animals were followed during all experimental procedures.

2.6. Acute toxicity study (LD_{50}).

LD50 of AcCF was determined according to the method described by [29]. The rats were fasted overnight and then separated into four groups (2 rats/group). Different doses of the AcCF (10, 100, 300, and 600 mg/kgm) are administered to the rats. The animals were observed for o'clock post-administration and then 10 minutes every 2 hours for 24 hours. The animals were monitored for changes in behaviors such as paw licking, fatigue, semi-solid stool,

salivation, writhing, loss of appetite, and mortality. LD50 is calculated from the following formula (equation 1):

$$LD_{50} = \frac{M0+M1}{2} \quad (1)$$

where, M0: the highest dose of AcCF that gave no mortality.

M1: the lowest dose of AcCF that gave mortality.

2.7. Induction of Type 1 diabetes mellitus.

Prior to the experiment, all rats have fasted for 12 hours while still being given free access to water. T1DM was brought on by injecting 60 mg/kg of STZ intraperitoneally when it was dissolved in 0.1 mol/l sodium citrate buffer at pH 4.6. A blood glucose meter was used to monitor blood sugar levels 72 hours after STZ injection [30].

2.8. Experimental design.

Rats were divided into 3 groups (6 rats/subgroup):

Control group: rats were injected with a single dose of citrate buffer (0.1 mol/l, i.p) and then received 1 ml (dist. water, orally) daily for 4 weeks.

Diabetic group: rats were injected with a single dose of STZ (60 mg/kg, i.p) and then received 1 ml (dist. water, orally) daily for 4 weeks.

Earthworm extract group: rats were injected with a single dose of STZ (60 mg/kg, i.p) and then received earthworm extract (45 mg/kg, orally) daily for 4 weeks.

2.9. Animal handling and specimen collection.

At the end of the experiment, the chest was opened after the rats had been given 3% sodium pentobarbital to anesthetize them completely. The heart was punctured with a needle through the diaphragm. Once the heart had been pierced, light negative pressure was applied, and the needle was moved as necessary until blood began to flow into the syringe. The blood collected from the rats was separated by centrifugation at 3000 rpm for 15 minutes to obtain sera stored at -80 °C for further use.

Section of the cauda epididymis was isolated in a dish containing 1mL of Dulbecco's Modified Eagle Medium (DMEM). It remained in an incubator at 37°C to allow the spermatozoa to 'swim out' into the medium for approximately 10 min. One drop of caudal epididymal spermatozoa was diluted 1: 100 in DMEM supplemented with 1% BSA, then the diluted solution was put into the counting chamber, and the sperm number was counted using a hemocytometer under the light microscope.

The liver and pancreas were taken out and quickly wiped with filter paper to get rid of any blood residue. For biochemical testing, a portion of the liver was kept at -80 °C. In order to fixate them for histological analysis, the pancreas, kidney, testis, and liver were suspended in 10% formal saline.

2.10. Tissue homogenate preparation.

The tissue was homogenized (10% w/v) in ice-cold 0.1 M Tris–HCl buffer (pH 7.4). The homogenate was centrifuged at 3000 rpm for 15 minutes at 4 °C, and the resultant supernatant was used for the biochemical analyses [31,32].

2.11. Histopathological examination.

In 10% neutral-buffered formalin, the liver, kidneys, testes, and pancreas were fixed. The fixed specimens were cleaned, dehydrated, and paraffin wax-embedded. Hematoxylin and eosin were used to stain the tissues after sectioning them at a thickness of 4-5 μm [33].

2.12. Biochemical analyses.

The serum glucose, serum insulin, serum arginase, liver glucose-6-phosphate dehydrogenase (G6PD), serum alkaline phosphatase (ALP), serum aspartate aminotransferase (AST), and alanine aminotransferase (ALT), total serum protein, serum albumin serum total cholesterol (TC), serum triglycerides (TG), serum high-density lipoprotein-cholesterol (HDL-C), serum low-density lipoprotein-cholesterol (LDL-C), serum creatinine, serum urea, and uric acid were determined according to the manufacturer's instructions using Spectrum Diagnostics and Bio-diagnostic kits (Giza, Egypt). The malondialdehyde (MDA), glutathione reduced (GSH), glutathione-S-transferase (GST), catalase (CAT), and nitric oxide (NO) were determined in the liver and kidney homogenate supernatant according to the manufacturer's instructions using Biodiagnostic kits (Giza, Egypt). Luteinizing hormone (LH), follicular stimulated hormone (FSH), and testosterone were estimated by using ELISA sandwich immunoassay (BIO-RAD automated system).

2.13. Statistical analysis.

The mean ± SE was used to express values. One-way analysis of variance (ANOVA) was used to compare the group means for the comparisons within groups, and $p<0.05$ was used to denote statistical significance. The statistical evaluation was performed using SPSS for Windows (version 15.0).

3. Results and Discussion

3.1. Heavy metals and amino acid content of earthworm extract.

The heavy metal content of the extract is represented in Figure 1a. Heavy metals have been found in various antidiabetic medications, indicating their therapeutic effects [34]. According to some studies, heavy metals, especially zinc, could suppress gluconeogenesis and lipolysis and improve glucose transport, glycogen, and lipid synthesis [35,36]. The amino acid profile of earthworm extract revealed the presence of essential and no essential amino acids (Figure 1b). Amino acids play a key role in diabetes mellitus therapy, as they can boost insulin production, promote pancreatic β-cells growth, and decrease insulin resistance [37].

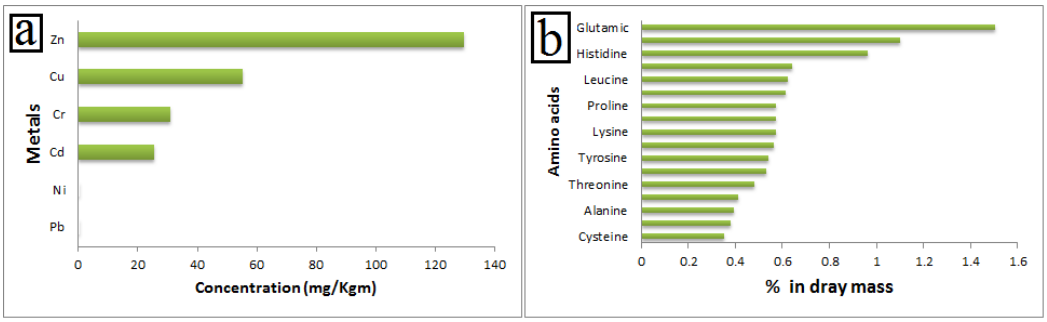

Figure 1. (**a**) Metal contents of earthworm extract and (**b**) amino acid content.

3.2. Diabetic markers.

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia and protein metabolism resulting from a disturbance in insulin release or activity [38]. As presented in Table 1, the STZ-diabetic group demonstrated a significant increase ($p<0.05$) in glucose and arginase levels. At the same time, G6PD and insulin concentration decreased significantly ($p<0.05$) compared to the control groups. On the contrary, earthworm extract treatment significantly reduced ($p<0.05$) the glucose and arginase measurements and significantly increased ($p<0.05$) G6PD and insulin levels compared to the diabetic group.

Table 1. The curative effect of earthworm on diabetic markers, liver function, lipid profile, kidney function, and fertility parameters of diabetic rats.

Parameters	Experimental groups		
	Control	**DM**	**DM+ earthworm**
Diabetic markers			
Glucose (mg/dl)	104.88±2.64^{a}	495.05±8.77^{c}	197.98 ±2.73^{b}
Insulin (µIU/ml)	7.69±0.40^{c}	1.82±0.12^{a}	4.18±0.17^{b}
Arginase(U/L)	129.69±4.01^{a}	357.82±10.65^{c}	179.92±19.87^{b}
G6PD (U/min/gm.tissue)	0.49±0.02^{c}	0.19±0.01^{a}	0.29±0.02^{b}
Liver Functions			
ALP (U/L)	100.49±7.98^{a}	1119.52±114.82^{c}	425.31±13.78^{b}
ALT(U/mL)	129.58±2.32^{a}	249.99 ±5.75^{c}	169.48±5.52^{b}
AST(U/mL)	36.14±3.78^{a}	110.86±1.54^{c}	60.09±4.54^{b}
Total protein (g/dl)	4.54±0.19^{c}	2.60±0.20^{a}	4.09± 0.15^{b}
Albumin (g/dl)	3.27±0.20^{b}	1.71±0.10^{a}	3.19± 0.15^{b}
MDA (nmol/g. tissue)	0.51±0.03^{a}	1.35±0.04^{c}	0.84±0.08^{b}
NO (µmol/L)	202.74±5.44^{a}	544.44±5.09^{c}	398.55±15.54^{b}
GSH (mg/g. tissue)	3.35±0.05^{c}	1.50±0.08^{a}	2.59±0.13^{b}
GST (U/g. tissue)	1.73±0.04^{c}	0.95±0.04^{a}	1.34±0.07^{b}
CAT (µmol/g tissue)	113.54±2.11^{c}	55.54±1.59^{a}	95.69±2.22^{b}
Lipid Profile			
Cholesterol (mg/dL)	42.36±2.84^{a}	78.70±4.10^{c}	54.80±2.62^{b}
TG (mg/dl)	54.14±2.35^{a}	189.55±4.79^{c}	84.83±4.49^{b}
LDL-C (mg/dl)	52.50±3.51^{a}	97.55±5.08^{c}	66.83±2.23^{b}
HDL-C (mg/dl)	35.30±3.24^{b}	15.90±1.65^{a}	29.55±1.04^{b}
Kidney Functions			
Creatinine (mg/dl)	0.50±0.02^{a}	1.39±0.03^{c}	1.08± 0.04^{b}
Urea (g/dl)	44.37±1.35^{a}	76.12±1.46^{c}	55.82± 3.76^{b}
uric acid (mg/dl)	1.14±0.06^{a}	2.05±0.05^{c}	1.56±0.07^{b}
MDA (nmol/g. tissue)	0.93±0.07^{a}	2.28±0.05^{c}	1.55±0.08^{b}
NO (µmol/L)	513.15±12.20^{a}	1165.59±48.06^{c}	878.95±36.08^{b}
GSH (mg/g. tissue)	2.94±0.20^{b}	0.53±0.03^{a}	0.86±0.04^{a}
GST (U/g. tissue)	0.35±0.01^{c}	0.17±0.01^{a}	0.24±0.02^{b}
CAT (µmol/g tissue)	310.40±9.32^{c}	146.78±5.70^{a}	192.09±4.48^{b}
Fertility parameters			
FSH (mIU/mL)	0.74±0.02^{b}	0.16±0.01^{a}	0.63±0.01^{b}
LH (mIU/mL)	0.58±0.03^{b}	0.15±0.01^{a}	0.51±0.02^{b}
Testosterone (ng/mL)	3.34±0.09^{c}	0.64±0.04^{a}	2.26±0.08^{b}
Sperm count (million/ml)	124.67±2.32^{b}	90.15±3.54^{a}	121.50±4.05^{b}

Values are means ± se (n = 6 per group). Each value not sharing a common letter superscript is significantly different ($p<0.05$). (a) represents the lowest value, (b) the middle value, and (c) the highest value. G6PD: Glucose-6-phosphate dehydrogenase, ALP: Alkaline Phosphatase, ALT: Alanine transaminase, AST: Aspartate transaminase, MDA: Malondialdehyde, NO: Nitric oxide, GSH: Glutathione reduced, GST: Glutathione S-transferase, CAT: Catalase, TG: Total triglycerides, LDL-C: Low-density lipoprotein-cholesterol, HDL-C: High-density lipoprotein-cholesterol, FSH: Follicle-stimulating hormone, LH: Luteinizing hormone.

β-cells of Langerhans' pancreatic islets produce and secrete insulin, an endocrine peptide hormone. Insulin regulates blood glucose levels by aiding cellular glucose absorption, metabolism of carbohydrates, lipids, and proteins, and encouraging cell division and proliferation. The present study proved the statement of others [39] that STZ is an effective diabetogenic drug characterized by the selective death of pancreatic β-cells, leading to hypoinsulinemia and hyperglycemia, imitating human T1DM [40]. In contrast, the earthworm extract is believed to have an insulin-like growth factor, boosting the insulin activity of the treated group. Moreover, earthworm extract stimulated the growth of the epithelial cells and enhanced the damaged tissue repair mechanisms [41]. Arginase is a manganese-metalloenzyme responsible for various physiological activities, such as converting L-arginine to L-ornithine and urea [42]. The rise in arginase activity in diabetic rats could have resulted from the increased amino acid metabolism and the elevated ratio of blood glucagon to the insulin that contributes to the increase in urea cycle activity [43]. However, the reduced arginase activity in treated rats could be attributed to the regulatory effect of the amino acids in the earthworm extract [37].

G6PD is an enzyme that catalyzes the first reaction in the pentose phosphate pathway, supplying energy to all cells in the form of NADPH [44]. In the current investigation, the reduction in G6PD activity in diabetic male rats might be attributed to insulin insufficiency [45]. The enhancement in the G6PD activity of the treated rats showed an increase in blood glucose absorption and prevention of diabetes.

The glucose, insulin, arginase, and G6PD activities in earthworm extract-treated rats significantly improved. Similarly, Ogasawara et al. indicated that earthworm extract inhibits α-amylase and α-glucosidase, leading to suppression in diabetes [46]. Earthworm extract enhances cellular regeneration and hinders tissue degradation and cellular apoptosis [47,48]. This hypoglycemic activity of earthworm extract in the treated group might be due to an insulin-like action of earthworm extract, the prevention of β-cells death, or the recovery of partially damaged β-cells as evidenced by the histological findings recorded in the present study.

3.3. Histopathology of the pancreas.

Microscopic examination of the control group (Figure 2 a, b) revealed no histopathological alterations in both exocrine and endocrine compartments. The pancreas of the diabetes group (Figure 2 c, d) showed marked histopathological changes represented by the marked reduction in both the number and size of islets of Langerhans in the examined sections. The cells within the islets decreased in number with the existence of numerous necrotic cells. Concerning the earthworm group (Figure 2 e, f), a few of the examined sections showed mild loss of endocrine cells within the islets; some others were normal without any detectable alterations. These findings suggested that the earthworm extract's therapeutic effects toward diabetes mellites were likely due to encouraging regeneration of the exocrine compartment of Langerhans islets. Once stimulation of cellular regeneration occurred, the newly developed exocrine compartment worked on lowering the blood glucose levels [49].

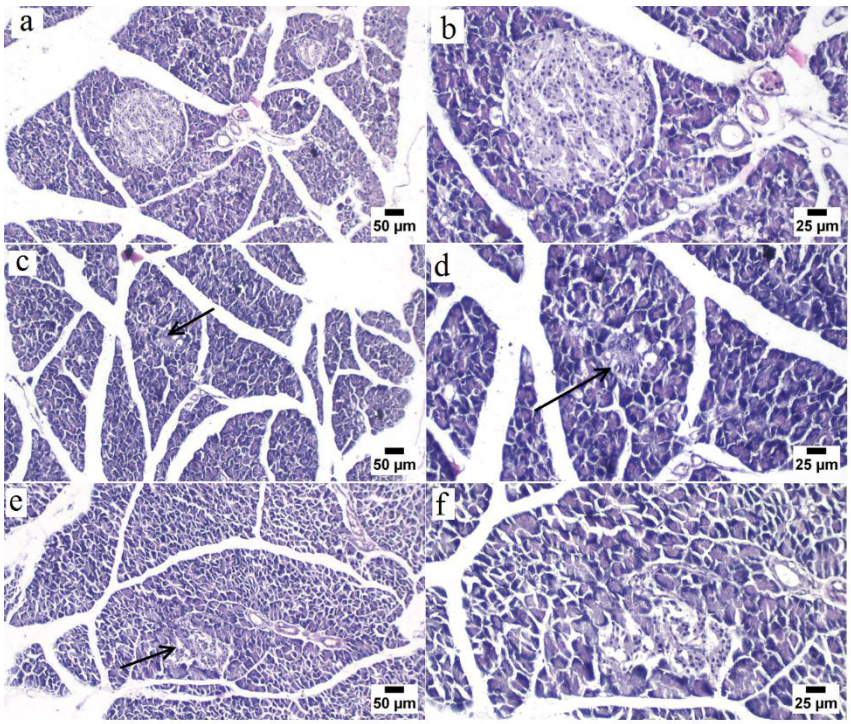

Figure 2. Photomicrograph of pancreas, Control group (**a, b**) showing normal exocrine and endocrine parts. Diabetes group (**c, d**) shows small islets of Langerhans (arrow) and necrosis in the cells of Langerhans islets (dotted arrow). Earthworm group (**e, f**) showing average size islets of Langerhans (arrow).

3.4. Liver function bioindicators.

The liver is a vital metabolic organ in the body that stores and generates glucose to maintain blood glucose levels. It is also in charge of preserving lipid and protein balance.

Table 1 revealed that ALP, ALT, and AST enzyme activities were significantly increased ($p<0.05$) in T1DM rats compared to the control group. However, total proteins and albumin decreased significantly ($p<0.05$) in diabetic rats. Otherwise, the administration of earthworm extract induced a significant decrease ($p<0.05$) in ALP, ALT, and AST activities compared to T1DM rats. Earthworm extract-treated rats showed a significant increase ($p<0.05$) in total protein and albumin concentrations.

These data showed that diabetes might be the cause of hepatic dysfunction. These results were supported by Allagui et al., who reported that the liver was necrotized in diabetes individuals [50]. As a result, the rise in ALP, ALT, and AST activity in serum might be due to the leakage of these enzymes from the cytosol of the liver into the circulation, showing that STZ has a hepatotoxic effect [51]. STZ-induced diabetes mellitus is associated with a disruption in glucose metabolism, resulting in the formation of protein glycation products, enhanced non-enzymatic auto-oxidative glycosylation, and the release of free radicals, which all contribute to oxidative stress [52]. Both β-cells death and liver damage have been linked to free radical generation [53]. However, the treatment with earthworm extract resulted in a significant reduction in the activities of serum ALP, ALT, and AST in diabetic rats, showing that hepatic cell function and structure were preserved. The mechanism might be owing to the earthworm extract inhibiting the progression of diabetes and acting as a potent antioxidant, which has the potential to neutralize reactive oxygen species (ROS) [25].

Albumin is the most abundant protein in the blood [54] and the most significant protein produced by the liver [55]. After STZ injection, serum total protein and albumin levels decreased significantly. Inhibition of oxidative phosphorylation has been related to decreased

protein synthesis, increased catabolic processes, and reduced protein absorption [56]. Regarding the effect of earthworm extract on STZ-treated rats, the results indicated a significant increase in the total protein and albumin levels. The rise in total protein and albumin levels in the earthworm extract-treated group suggests the possibility of liver function preservation [57].

3.5. Lipid profile biomarkers.

The liver is an insulin-dependent organ that helps maintain glucose and lipid balance. Therefore, insulin resistance or insufficiency is associated with hyperlipidemia [58,59]

The levels of TC, TG, and LDL-C in the diabetic rats were increased significantly ($p<0.05$), while HDL-C concentration decreased significantly ($p<0.05$) when compared to the control group (Table 1). Nevertheless, the earthworm extract-treated group showed a significant decrease ($p<0.05$) in TC, TG, and LDL-C levels and a significant increase ($p<0.05$) in HDL-C levels in comparison to the diabetic group.

These findings were consistent with the conclusions of Ozkol et al. [4] that insulin inhibits the hormone-sensitive lipase; therefore, the unusually high content of blood lipids in diabetes mellitus is mainly attributable to an increase in the mobilization of free fatty acids from peripheral fat depots. The unrestrained activities of lipolytic hormones on fat depots could cause the significant hyperlipidemia that characterizes the diabetic condition [60]. The present study showed reduced TC, TG, and LDL-C levels and increased HDL-C levels in the earthworm extract-treated group. The obtained data might be related to a glycolipoprotein mixture (G-90), which was isolated from earthworms and contains insulin-like growth factor (IGF) and epidermal growth factor (EGF). IGF is involved in glucose metabolism and lipid metabolism control. Moreover, IGF lowers total plasma cholesterol in treated rats. EGF was also attributed to mice's hepatic and plasma lipid level modulation. Thus, the lipid profile and liver function are regulated [61].

3.6. Histopathology of the liver.

Histopathological examination of the liver from the control group (Figure 3 a, b) revealed a normal structure of hepatic parenchyma in which the hepatocytes were arranged in parallel hepatic cords, and both centrilobular and periportal areas were free from any detectable alterations. The diabetes group (Figure 3 c, d) showed marked histopathological alterations. The portal areas were infiltrated by mononuclear inflammatory cells. Some of the examined sections showed hepatocellular necrosis with the existence of focal aggregations of mononuclear inflammatory cells within the hepatic parenchyma. Sporadic cell necrosis and hepatocellular degeneration were also frequently observed. According to another study, STZ caused liver function abnormalities and liver tissue destruction [62]. Earthworm treated group (Figure 3 e, f) exhibited an normal hepatic parenchyma that appeared free from any detectable alterations. The current data agreed with Kawakami et al., who claimed that the composite powder containing earthworm (CEP) protected against diabetic complications by improving lipid metabolism and suppressing liver deterioration progression [63]. Deng et al. believed that the earthworm extract had potent antioxidant properties attributed to minimized liver damage [61]. Additionally, the presence of several amino acids in the earthworm extract, such as glutamine, improved the healing ability of diabetic tissue injuries through increased collagen

production and decreased diabetic wound catabolism, leading to reduced wound deterioration [64].

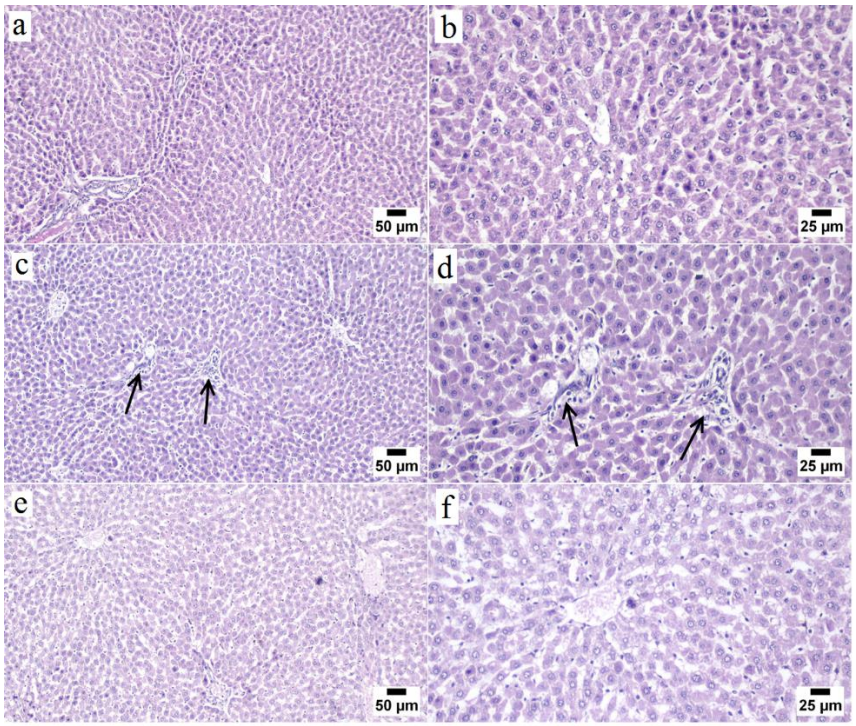

Figure 3. Photomicrograph of liver, control group (**a, b**) showing normal hepatic parenchyma. Diabetes group (**c, d**) showing mononuclear inflammatory cells infiltration at the portal areas (arrows) (H, E). Earthworm group (**e, f**) shows apparently normal hepatic parenchyma and centrilobular area (H, E).

3.7. Renal function markers.

Diabetes can damage hepatorenal function and lead to diabetic nephropathy (DN). DN affects 15-25% of type 1 diabetes patients and 30-40% of type 2 diabetic patients [65].

Significant increases ($p<0.05$) in serum creatinine, urea, and uric acid concentrations were observed in T1DM rats as compared to the control group (Table 1). Earthworm extract treatment significantly reduced ($p<0.05$) the serum creatinine, urea, and uric acid concentrations compared to the corresponding diabetic group.

Diabetic rats had significantly higher blood urea, creatinine, and uric acid, indicating kidney impairment [66,67]. Increased biomarkers confirm kidney damage caused by diabetes after injection of STZ. Treating diabetic rats with earthworm extract reduced urea, creatinine, and uric acid levels compared to the untreated diabetic rats. These results revealed that earthworm extract has renoprotective properties owning to the extract's mitogenic elements and growth factors [68], which can promote cell reproduction and regeneration. As a result, earthworm extract might play a role in tissue regeneration [26].

3.8. Histopathology of the kidney.

Microscopic examination of kidney sections from the control group (Figure 4 a, b) revealed normal renal cortex and medulla histological structure. Based on the literature, STZ induced renal failure and tissue damage [69], which is consistent with the results of the current study. The diabetes group (Figure 4 c, d) suffered from various histopathological alterations. The renal cortex showed perivascular edema with mononuclear inflammatory cell infiltration. The renal tubular epithelium suffered from degeneration and necrosis. Kidney sections from the earthworm group (Figure 4 e, f) were normal except for a sporadic case that exhibited

marked congestion in the renal cortex with mild mononuclear inflammatory cells in the renal medulla. Several studies indicated that substances within the earthworm extract were able to prevent organ fibrosis and reduce the deterioration of renal structure [70,71].

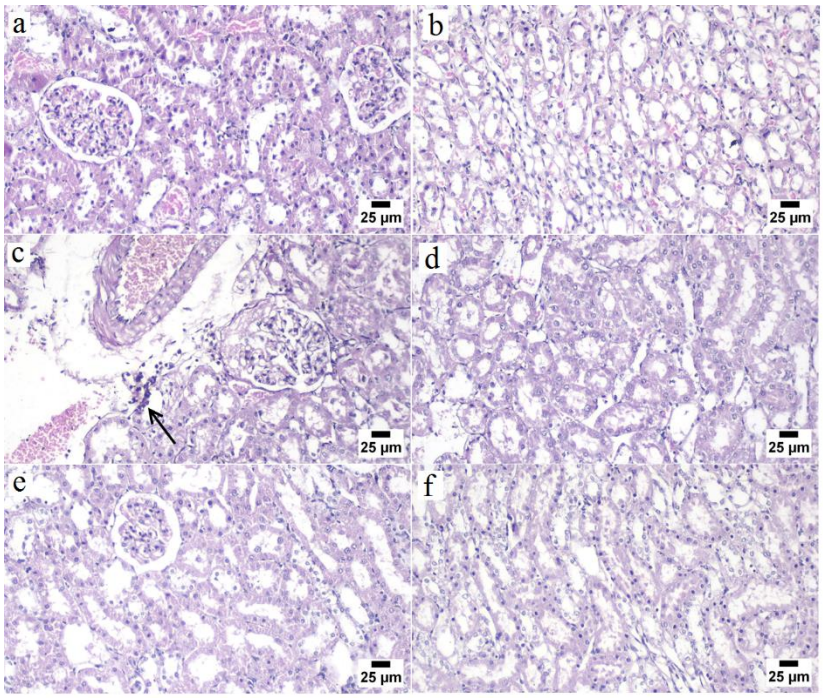

Figure 4. Photomicrograph of kidney, control group showing (**a**) normal renal cortex and (**b**) renal medulla;. Diabetes group) showing (**c**) perivascular edema with mononuclear inflammatory cell infiltration (arrow) and (**d**) degenerating renal tubules. Earthworm group (**e, f**) shows normal renal cortex (H, E).

3.9. Fertility markers.

Infertility is caused by endocrine abnormalities and also is one of the neurological complications of diabetes [72].

FSH, LH, testosterone, and sperm count decreased significantly ($p<0.05$) in T1DM rats as compared to the control group (Table 1). While earthworm extract treatment significantly increased ($p<0.05$) the FSH, LH, testosterone, and sperm count compared to the corresponding diabetic group.

Due to endocrine disruption and testicular oxidative stress, diabetes mellitus causes testicular damage, increases sperm abnormalities, and affects reproductive function [73,74]. On the other hand, earthworms extracted exhibited a protective effect on reproductive organ function and structure.

3.10. Histopathology of the testis.

Microscopic examination of testes sections from the control group (Figure 5 a, b, c) revealed normal histology of seminiferous tubules with active spermatogonial cells. The diabetes group (Figure 5 d, e, f) exhibited various histopathological alterations, including interstitial edema causing dispersion of the seminiferous tubules. The seminiferous tubules were small with irregular outlines and thickened basement membrane. In some instances, the spermatogonial cells were markedly reduced, leaving an empty tubule. The earthworm group (Figure 5 g, h, i) exhibited normal seminiferous tubules without any detectable alterations. According to another study, STZ induction in male rats induced oxidative stress, leading to injuries in the testicular tissues [75]. Therefore, some studies indicated that the treated diabetic

rats showed less severe testicular damage, probably because of decreasing oxidative stress [76,77].

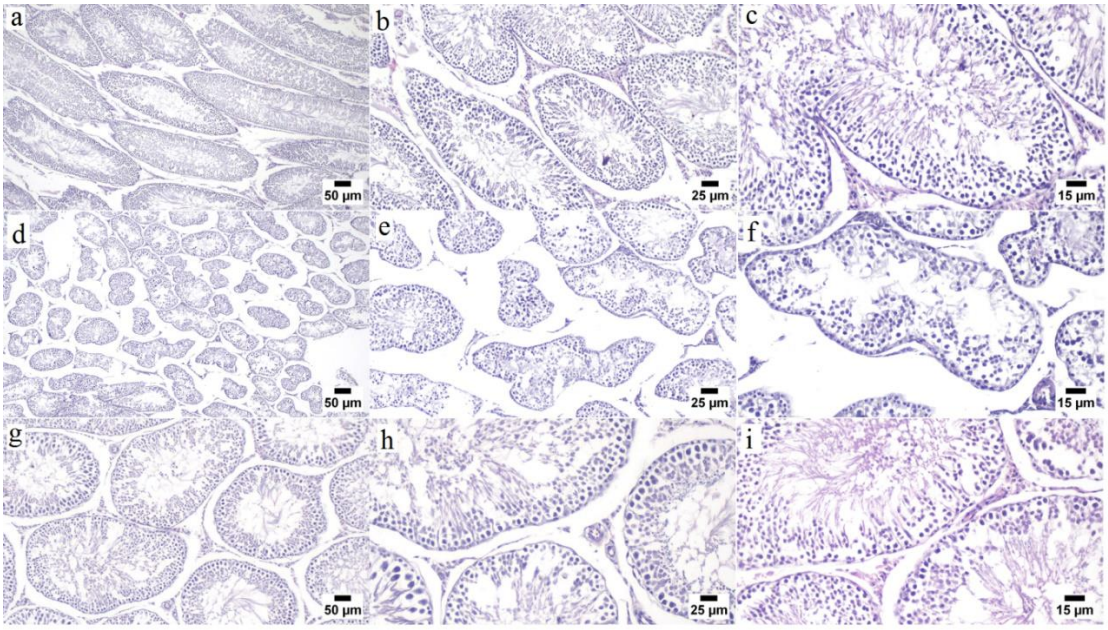

Figure 5. Photomicrograph of testes, control group showing (**a, b**) normal seminiferous tubules and (**c**) normal spermatogonial cells. Diabetes group showing degenerating spermatogonial cells within irregular small tubules (**d**) degenerating seminiferous tubules with irregular outlines (**e**) degenerating spermatogonial cells (**f**). Earthworm group showing (**g**) apparently normal seminiferous tubules, (**h**) seminiferous tubules, (**i**) normal seminiferous tubules.

3.11. Histopathology of heart.

Microscopic examination of heart sections from the control group (Figure 6 a, b) revealed normal histology of the myocardium.

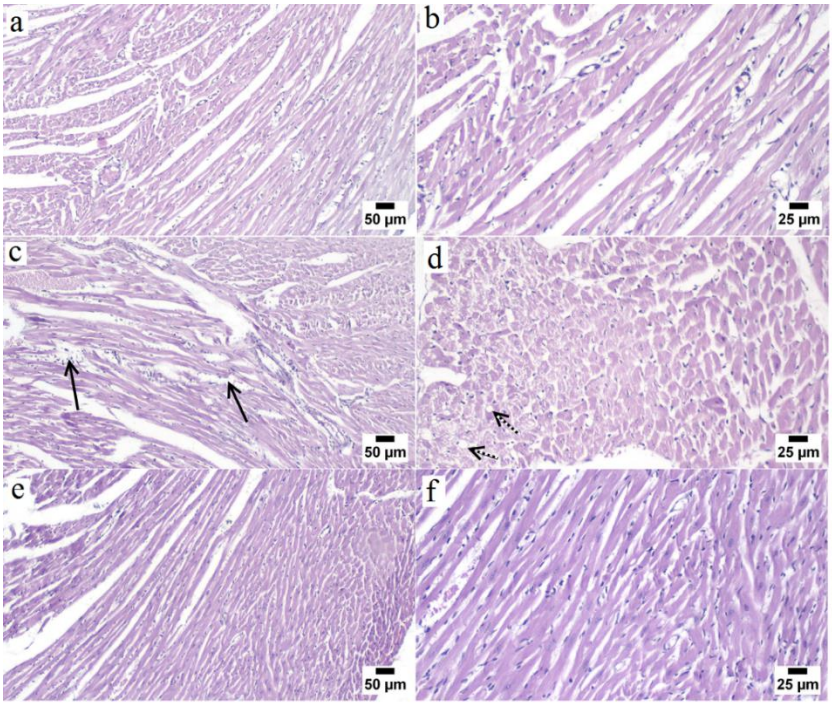

Figure 6. Photomicrograph of heart, Control group (**a, b**) showing normal myocardium. Diabetes group (**c, d**) showing muscular edema and mononuclear inflammatory cells infiltration (arrows) and vacuolation in the myocardium (dotted arrows). Earthworm group (**e, f**) shows apparently normal myocardium (H, E).

The diabetes group (Figure 6 c, d) showed vacuolation in the myocardium. Some sections showed muscular edema manifested by dispersion of the muscle fibers by edematous fluid with the existence of mononuclear inflammatory cells. Heart muscles of the earthworm-treated group (Figure 6 e, f) appeared normal in almost all the examined individuals. Similarly, another study suggested that earthworm extract interrupted the apoptotic cascade, preventing cell death and cardiac fibrosis [78].

3.12. Oxidative stress markers.

Free radicals are highly reactive chemicals that occur naturally in the human body. The antioxidant system was created to counteract the harmful effects of these free radicals, such as proteins, lipids, and DNA oxidation [79]. Cell death and tissue malfunction can occur from an imbalance between the release of free radicals and the antioxidant system [80]. Diabetes-induced oxidative stress is caused by the excessive formation of ROS in tissues and a reduction in antioxidants, leading to damage to membranes, DNA, and protein structures [81].

The diabetic group recorded a significant decrease ($p<0.05$) in the liver and kidney GSH, GST, and CAT levels and a significant increase ($p<0.05$) in the liver and kidney MDA and NO concentrations compared to the control group (Table 1). In contrast, earthworm extract treatment caused a significant increase ($p<0.05$) in the liver and kidney GSH, GST, and CAT levels, although the rise in kidney GSH levels was insignificant ($p>0.05$). In addition, the earthworm extract-treated group showed a significant decrease ($p<0.05$) in the liver and kidney MDA and NO concentration compared to the diabetic group.

MDA is a toxic end product of lipid peroxidation and a significant indicator of oxidative tissue damage [82,83]. In the current study, the rise in MDA concentration in the liver and kidney tissues of untreated diabetic rats revealed oxidative damage and tissue injury, followed by a decrease in the endogenous antioxidant system, including GSH, GST, and CAT. In addition, auto-oxidation of glucose, alterations in redox balance, reduced GSH content, and impaired antioxidant enzyme activities were symptoms of oxidative stress caused by excessive ROS generation [84-86].

However, treatment with earthworm extract restored the enzyme activity and decreased MDA level with a concurrent rise in the antioxidant defense system, including GSH, GST, and CAT activities, compared to diabetic rats. These findings indicate the ROS scavenging activity of earthworm extract. There are several mechanisms for earthworm extract action, such as inhibiting the production of reactive oxygen groups, scavenging these groups, and regulating the antioxidant enzyme system [25,87,88].

NO is produced by various nitrous oxide systems (NOS) and is an essential messenger molecule that has both positive and negative effects on the human body. Excess NO interacts with superoxide to generate peroxynitrite, a powerful oxidant associated with various illnesses, including diabetes [89,90]. The present results showed that the NO concentration decreased significantly in diabetic rats. This drop might be attributed to hyperglycemia, which contributes to endothelial dysfunction and reduced NO bioavailability [91]. On the opposite, NO concentration decreased in earthworm extract-treated groups, which could be related to earthworm extract's anti-inflammatory properties, blocking the production of NOS and reducing NO levels [92].

4. Conclusions

This study indicated the efficacy of earthworm extract in improving the biochemical and histopathological changes in the rats' liver, kidney, testis, and pancreas following experimental induction of diabetes mellitus type 1 using streptozotocin. The therapeutic effect of earthworm extract against diabetic complications results from its hypoglycemic activity, antioxidant impact, and regeneration of damaged tissues.

Funding

No funding sources.

Acknowledgments

The authors extend their appreciation to the Deanship of Scientific Research at Cairo University, Egypt, for supporting the current work.

Conflicts of Interest

The authors declare no conflict of interest

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