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The Therapeutic Potential of Humic Acid in Streptozotocin-Induced Hyperglycemic White Rats (*Rattus norvegicus* L.)

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ABSTRACT

Humic acid, an organic compound from peat soil, rich in antioxidants such as quinone and phenol, shows potential as an antidiabetic agent. This study aims to identify the optimal dose of humic acid extract for increasing body weight, lowering blood glucose levels, reducing malondialdehyde (MDA) levels, and improving the microanatomical structure of the pancreas in streptozotocin-induced diabetic rats. The humic acid was extracted from peat soil at Tanjungpura University. The study utilized 25 male Wistar rats (*Rattus norvegicus*), aged 8-12 weeks, organized in a group randomized design (GRD). Interventions included a single dose of streptozotocin (40 mg/kg b.w.) and varying doses of humic acid extract administered over 14 days post-induction. Results indicated that a dose of 375 mg/kg b.w. humic acid extract resulted in an increased final body weight by 11.86%, a reduction in fasting blood glucose levels by 79.23%, an AUC_{0-120} value of 19.311, and a final MDA level of 1.09 ±0.27 nmol/ml. These findings indicate the potential of humic acid as a therapeutic agent for diabetes.

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease with increasing incidence globally, including in Indonesia, due to unhealthy lifestyles such as consuming high-carbohydrate foods, a lack of physical activity, smoking, and stress (Sami et al., 2017; Unnikrishnan et al., 2017). This lifestyle leads to hyperglycaemia, a condition where blood glucose levels rise significantly (Ayuwardani & Susilowati, 2018). Hyperglycaemia can induce inflammatory responses and oxidative stress and increase the free radicals in the body. Hyperglycemia resulting from insufficient insulin production or ineffective insulin use by the body, leads to metabolic dysfunctions in carbohydrates, proteins, and fats and triggers oxidative stress and inflammation (Santoso et al., 2018; Yasin et al., 2015).

Hyperglycemia accelerates the formation of excessive reactive oxygen species (ROS), which can damage cells through lipid peroxidation, leading to increased levels of malondialdehyde (MDA) in the body (Ayuwardani & Susilowati, 2018). High MDA levels in diabetic patients indicate excessive oxidation processes due to free radicals, particularly in type 2 diabetes or those with complications (Jiang et al., 2023; Suryawanshi et al., 2006). Diabetes Mellitus management typically involves pharmacological and non-pharmacological therapies. However, drug therapies often come with undesirable side effects, prompting the search for safer natural alternatives to control blood glucose levels (Hussain et al., 2016).

One promising alternative is using humic acid, an organic compound found in peat soils, particularly in West Kalimantan (Yustiawati et al., 2014). Humic acid contains chemical components such as quinones and phenols, which have biological activities, including antioxidant and anti-inflammatory properties (Agazzi et al., 2007; Kodama & Denso, 2007; Rusliandi et al., 2020; Szot et al., 2019). Humic acid fraction contains the most phenolic moieties (300-470mg GAE/g) and shows antioxidant properties (Csicsor et al., 2023). The antioxidant content in the humic acid fraction has

the potential to neutralise oxidative stress and restore pancreatic beta cell function, which plays an important role in blood glucose regulation. the potential of humic acid as an antidiabetic agent merits further investigation, especially through well-designed in-vivo studies. Such research is vital to elucidate the mechanisms behind its effects and establish its feasibility as a therapeutic option in managing Diabetus milletus and its complications. This exploration addresses a critical health concern and aligns with the growing interest in natural compounds as beneficial adjuncts in chronic disease management.

MATERIALS AND METHODS

This research was conducted at the Biology Laboratory and Zoology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Tanjungpura University, Pontianak. Tools and materials used in this study include a glucometer, personal protective equipment (PPE), rat cages, microtome, light microscope, mortar, analytical balance sheet, set of glassware, micropipette, dropper pipette, centrifuge, UV-visible spectrophotometer, magnetic stirrer, syringe 1 mL, sonde, vortex, and microtube. The chemicals used in this study were streptozotocin, distilled water, citrate buffer, chloroform, entellan, eosin, ethanol, HCl, haematoxylin, H_2SO4, NaCl 0.9%, Na-thiobarbiturate acid (TBA), paraffin, phosphate buffer saline (PBS), trichloroacetic (TCA), and xylol.

Peat soil samples were taken from Sylva Arboretum, Tanjungpura University, Pontianak City. The extraction method of humic acid from peat soil uses the IHSS (International Humic Substance Society) procedure, which is based on precipitation in strong acids and solubility in weak bases. The study utilized a group randomized design (GRD) to assess the impact of humic acid on hyperglycemia induced by streptozotocin in male Wistar rats (*Rattus norvegicus* L.). The experiment was conducted with 25 male rats, aged 8–12 weeks, housed under controlled conditions with a temperature of 22 ± 2 °C, humidity of 55 ± 10%, and a 12-hour light/dark cycle.

Hyperglicemic Induction

Diabetes was induced by administering a single intraperitoneal injection of streptozotocin (STZ) at a dose of 40 mg/kg b.w and dissolved in a citrate buffer with a pH of 4.5. After 72 hours, diabetes was confirmed in the rats by measuring fasting blood glucose levels, and rats with levels above 200 mg/dL were included in the study. The rats were randomly assigned to five groups, each comprising five rats. The Normal Control (NC) group received only distilled water as the baseline reference. The Negative Control (NC-STZ) group was administered STZ but did not receive any further treatment, allowing for the observation of the effects of induced diabetes without intervention. The Positive Control (PC-GL) group was treated with glibenclamide at a dose of 5 mg/kg b.w., a standard antidiabetic medication used as a comparative treatment. The first experimental group, Treatment Group 1 (HA-375), received humic acid at a dose of 375 mg/kg b.w., while the second experimental group, Treatment Group 2 (HA-625), was administered humic acid at a dose of 625 mg/kg b.w. The humic acid, extracted from peat soil at Tanjungpura University, was administered orally once daily for 14 days post-STZ induction to evaluate its potential antidiabetic effects.

Measurements and Data Collection

Body weights were recorded at the start and end of the study. Mice were fasted for 12 hours before blood sampling. Fasting blood glucose levels (FBGL) were measured on days 0, 7, and 14 using a glucometer with blood samples from the tail vein. On day 14, an oral glucose tolerance test (OGTT) was conducted, and the area under the curve (AUC0-120) for glucose was calculated. Fasting rats performed an Oral glucose tolerance test for 12 hours and then gave glucose solution 1 gram/kg BB. Blood glucose levels were checked 30, 60, and 90 minutes after intake.

Cardiac blood samples were collected to measure malondialdehyde (MDA) levels using the thiobarbituric acid reactive substances (TBARS) method. Blood plasma was put into a test tube as much as 50 µL, and then 1 mL of distilled water was added. The 20% TCA reagent was taken as 100 µL, 1N HCl as 250 µL, and 1% Na-TBA as 100 µL; the homogeneous mixture was heated in a water bath at 100°C for 30 minutes. It was then allowed to cool at room temperature, and centrifuged at 500 rpm for 10 minutes. The supernatant was taken and transferred to a new microtube. Samples were measured for absorbance at a wavelength of 540 nm and plotted on an MDA standard curve to calculate sample concentrations (Herawati et al., 2012).

The histological description was used to determine differences in the structure of pancreatic

tissue in each treatment. The histological description of pancreatic tissue was done using the Hematoxylin-Eosin (HE) staining method. The histological features of pancreatic islets were observed using a microscope. The number of pancreatic islets was observed and calculated as a percentage. All data were analyzed using SPSS software version 25.0. They were tested for normality and homogeneity using Levene's test. To assess statistical significance among groups, a one-way analysis of variance (ANOVA) followed by the Duncan Multiple Range Test (DMRT) was used. A p-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The blood glucose parameter used in this study is fasting blood glucose levels (FBGL). The inclusion criteria for this study were healthy rats with normal fasting blood glucose levels (70–126 mg/dL). Test animals were considered hyperglycemic if their fasting blood glucose levels were more than 200 mg/dL (Rehman *et al.*, 2023). Fasting blood glucose levels were measured incrementally along with the measurement of the rats' body weight. The average FBGL of the rats for each treatment group can be seen in Table 1.

Fasting blood glucose levels (FBGL) were measured four times during the study. Day 0 served as the baseline data for the study. Day 3 marked 72 hours post-streptozotocin induction, as the cytotoxic effects of streptozotocin became observable after this period. Day 10 represented the first-week post-induction, and Day 17 represented the second-week post-induction (Table 1).

The mean FBGL on Day 3 post-induction showed a significant difference between the groups induced with streptozotocin and the non-induced group (normal control) (Table 3). The highest final FBGL was found in the negative control group and differed significantly from all other groups. The final FBGL in the group receiving 625 mg/kg b.w. humic acid extract was also found to be high and classified as hyperglycemic. However, the mean final FBGL in the group receiving 375 mg/kg b.w. humic acid extract and the positive control group fell within the normal range.

The average body weight of rats in each treatment group is shown in Table 2. Based on the statistical test results, the highest average body weight was observed in the normal control group, while the lowest average body weight was found in the negative control group. The final body weight measurements in the groups treated were 375 mg/kg b.w. and 625 mg/kg b.w. humic acid extract was significantly different from all control groups ($P<0.05$).

The body weight data on Day 0 represents the initial measurements taken before streptozotocin induction. This data was used to determine the appropriate dosage and volume of the administered solution. The Day 3 body weight data, collected post-streptozotocin induction, was used to confirm hyperglycemia in the rats, as the cytotoxic effects on pancreatic β-cells became noticeable after 72 hours (Eleazu, 2013). The Day 10 data, taken 7 days after hyperglycemia confirmation, evaluated any changes in body weight following the intervention. The Day 17 data was the final measurement of the study. These incremental measurements were conducted to assess the impact of the treatment over time.

The area under the glucose curve (AUC) value is an index of glucose excursion following oral glucose administration. AUC measurement is used to determine the glycemic index and to evaluate the effectiveness of drugs for postprandial hyperglycemia. The AUC value is essential for screening for glucose intolerance in oral glucose tolerance tests (OGTT) (Sakaguchi et al., 2016).

Table 1. Average of the Fasting Blood Glucose Rate (FBGR) in Rat (*Rattus norvegicus* L.)

Treatment	Fasting Blood Glucose Levels (mg/dl)			
	Day 0	Day 3	Day 10	Day 17
Normal Control	81.80±7.86^{a}	87.40±5.90^{a}	89.00±9.93^{a}	75.20±15.99^{a}
Negative Control	85.00±9.80^{a}	464.40±8.68^{bc}	545.40±12.50^{d}	570.20±17.10^{d}
Positive Control	80.80±5.59^{a}	457.60±11.80^{b}	141.80±16.02^{b}	82.40±2.70^{ab}
Humic Acid 375 mg/kg b.w	90.60±3.51^{a}	465.20±10.18^{c}	149.80±8.96^{b}	96.60±7.98^{b}
Humic Acid 625 mg/kg b.w	89.40±7.93^{a}	468.00±15.31^{bc}	227.60±12.16^{c}	193.20±10.84^{c}

Note: The data are presented as mean ± standard deviation. Numbers followed by different superscript letters indicate significant differences ($p<0.05$) by Duncan analysis

Table 2. Humic Acid Increased Body Weight of White Rat (*Rattus norvegicus* L.)

Treatments	Body Weight Levels (gram)			
	Day 1	Day 3	Day 10	Day 17
Normal Control	212.80±7.05^{a}	221.00±4.74^{b}	226.40±7.70^{d}	247.20±7.66^{d}
Negative Control	213.20±3.27^{a}	207.00±4.30^{a}	182.00±8.80^{a}	178.20±5.89^{a}
Positive Control	215.00±4.36^{a}	209.40±3.51^{a}	220.80±8.08^{cd}	243.40±6.69^{cd}
Humic Acid 375 mg/kg b.w	210.80±4.44^{a}	204.40±5.81^{a}	212.60±8.59^{bc}	235.80±5.63^{c}
Humic Acid 625 mg/kg b.w	211.40±2.79^{a}	205.80±9.34^{a}	206.60±3.05^{b}	216.00±3.39^{b}

Note: The data are presented as mean ± standard deviation. Numbers followed by different superscript letters indicate significant differences ($p<0.05$) by Duncan analysis

Table 3. Humic Acid Lowers Blood Glucose Levels in The Oral Glucose Tolerance Test

Treatments	Time (minute)			
	0	30	60	90
Normal Control	89.00±9.93^{a}	224.00±37.50^{a}	148.20±14.29^{ab}	92.20±18.620^{a}
Negative Control	419.20±94.16^{a}	546.40±84.78^{b}	556.40±60.41^{c}	568.20±48.36^{c}
Positive Control	129.20±27.42^{a}	215.60± 21.10^{a}	116.40±24.16^{a}	105.20± 18.62^{a}
Humic Acid 375 mg/kg b.w	129.40±21.92^{a}	282.80±67.32^{a}	190.00±57.74^{b}	105.00±28.77^{a}
Humic Acid 625 mg/kg b.w	148.80±35.82^{a}	284.80±74.88^{a}	196.00±45.41^{b}	154.40± 11.06^{b}

Note: The data are presented as mean ± standard deviation. Numbers followed by different superscript letters indicate significant differences ($p<0.05$) by Duncan analysis

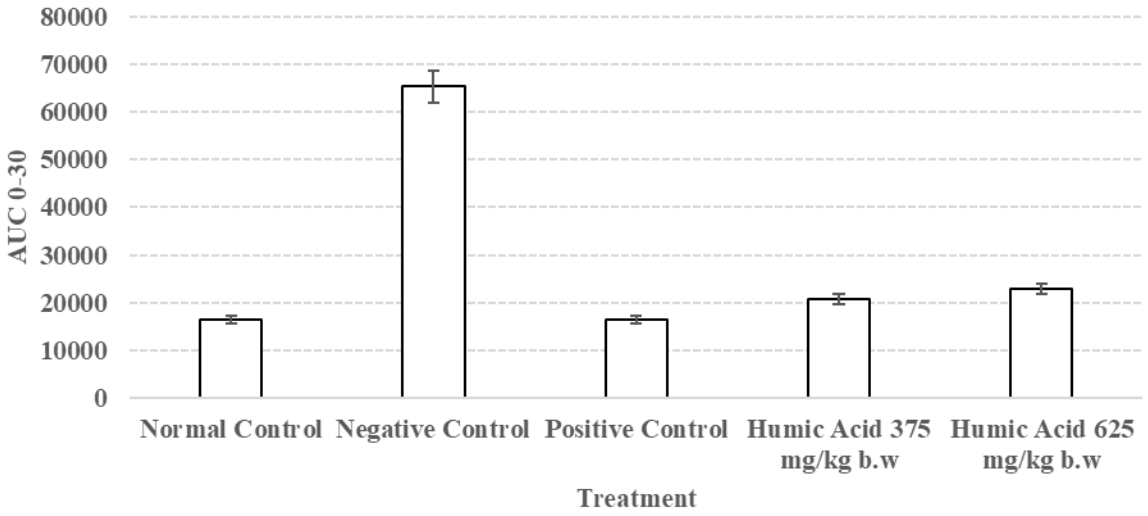

Figure 1. The glucose area under the curve (AUC) values were calculated from minute 0 to minute 120 for all treatment groups. The measurement data are presented as mean ± standard deviation. Different superscript letters in the same column indicate significant differences ($P<0.05$)

The magnitude of the AUC reflects the amount of the drug that reaches systemic circulation. A low AUC value indicates that the drug has been optimally absorbed to reduce blood glucose levels. Therefore, there is an inverse relationship between the AUC value and antidiabetic activity: the lower the AUC value, the more effective the drug lowers blood glucose levels (Amriani et al., 2021). Based on the graph above, the AUC values from lowest to highest are normal control at 17.868, positive control at 18.252, 375 mg/kg b.w. humic acid at 19.311 and 625 mg/kg b.w. humic acid at 29.716. The highest AUC value was found in the negative control group at 69.417.

Based on the graph below, the initial plasma MDA levels before the intervention (Day 0) (Figure 1) did not show any significant difference among all treatments ($p > 0.05$). The average initial plasma MDA levels ranged from 0.79 to 0.89 nmol/mL. On Day 3, MDA levels increased in all treatment groups except the normal control group. The group receiving 625 mg/kgBW humic acid extract showed the highest MDA level at 5.2±1.24 nmol/ml, but this was not significantly ḅerent from all groups induced with streptozotocın ($p > 0.05$). The highest plasma MDA level was observed on day 17 in the negative control group at 6.24±0.79 nmol/ml, while the lowest final plasma MDA level was found in the normal control group at 0.71±0.28 nmol/ml. The MDA levels in the 375 mg/kg b.w. humic acid extract group on Day 17 decreased from the previous measurement to 1.09±0.27 nmol/ml, closely approaching the MDA levels of the positive control group at 0.93±0.7 nmol/ml. The average final plasma MDA level in the 625 mg/kg b.w. humic acid extract group also decreased to 3.23±0.22 nmol/ml, although not as optimally as in the 375 mg/kg b.w. humic acid extract group (Table 3).

The normal control group had the highest percentage of normal Langerhans islet cells (100%). The negative control group had the lowest average percentage of normal pancreatic islet cells, measuring 15.00±5.59%. The positive control group had an average proportion of normal Langerhans islet cells (77.50±5.59%). Humic acid extract administered at 375 mg/kg/w resulted in 7500±883% of normal islet cells, comparable to the positive control. The group that received 625 mg/kg b.w. of humic acid extract had an average percentage of normal Langerhans islet cells of 4750±559%.

There were no degenerating Langerhans islet cells in the normal control group. The negative control group had the highest average percentage of degenerating Langerhans islet cells (45.00 ± 11.18%). The average percentage of degenerating Langerhans islet cells in the 375 mg/kg b.w. humic acid group was 15.00±7.13%, similar to the positive control group (13.75±5.23%). The 625 mg/kg b.w. humic acid extract treatment group had a greater average percentage of degenerating Langerhans islet cells than the 375 mg/kg b.w. humic acid group. Observed necrosis comprised of karyolysis, karyorrhexis, and pyknosis. There were no necrotic Langerhans cells in the normal control group. The negative control group had the highest average percentage of necrotic Langerhans islet cells (37.50% ± 8.84%). The average percentage of necrotic Langerhans islet cells in the 375 mg/kg BW humic acid group was 10.00±3.42%, comparable to the positive control group.

Streptozotocin was utilized as the diabetogenic substance in this research. Streptozotocin damages pancreatic β-cells, which synthesize insulin. It penetrates β-cells via the GLUT-2 glucose transporter. Streptozotocin is metabolized inside the cells and acts as a donor in nitric oxide synthesis. Increasing reactive oxygen in mitochondria disrupts metabolism, inhibiting the Krebs cycle and decreasing ATP synthesis in pancreatic β-cells. The presence of free radicals causes oxidative stress and metabolic abnormalities, resulting in elevated blood glucose levels or hyperglycemia (Ghasemi & Jeddi, 2023).

The positive changes from administering 375 mg/kg BW humic acid extract, with increased body weight after streptozotocin induction, are attributed to the antioxidant compounds in humic acid. Antioxidants such as quinones and phenols are believed to aid in regenerating pancreatic β-cells (Aeschbacher et al., 2012). As a result, energy needs from glucose can be met, and glucose storage in the liver and muscles can be properly managed, allowing the rats' body weight to gradually increase (Angeles et al., 2022). The positive effects of humic acid supplementation on growth performance have been confirmed in pigs, rabbits, and broiler chickens, showing increased body weight and more efficient feed intake (Hafsa et al., 2021; Mohmed et al., 2020; Trckova et al., 2018). This is in line with the research reported by Wang et al. (2022), where humic acid supplementation in dairy calves' feed modulated gut microflora, enhancing growth performance, immune status, and antioxidant capacity.

The findings from this study highlight the significant impact of the humic acid extract on key metabolic parameters in streptozotocin-induced diabetic mice. The observed increase in body weight among the treatment groups, particularly at the 375 mg/kg b.w. dose, suggests an amelioration of the catabolic state commonly seen in diabetes. This improvement in body weight can be attributed to the antioxidant properties of humic acid, which may enhance metabolic efficiency and reduce the muscle. Furthermore, there was a marked reduction in fasting blood glucose levels in the 375 mg/kg b.w.treatment group underscores the potential of acid as a glycemic control agent.

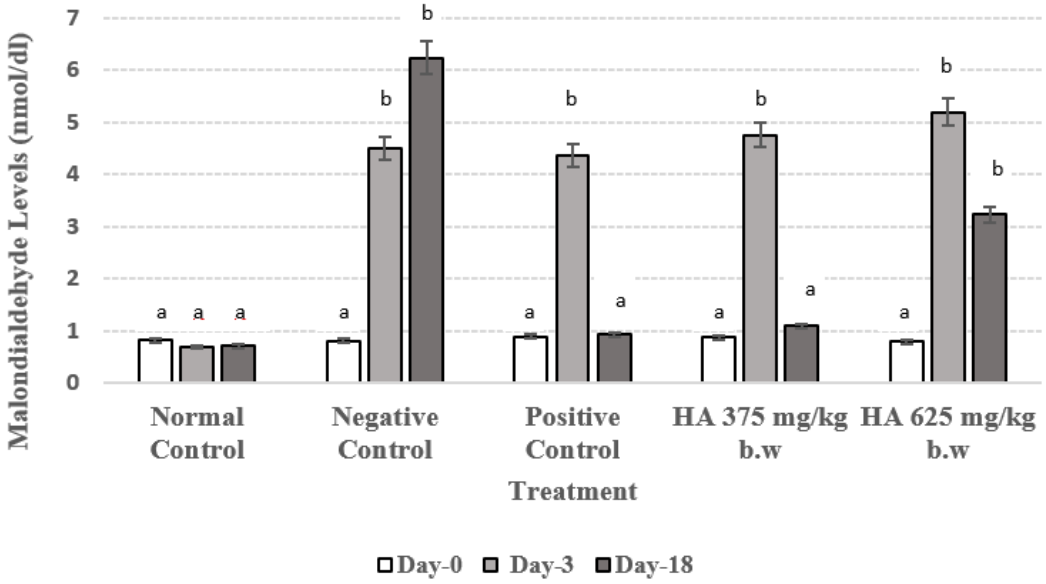

Figure 2. Malondialdehyde Levels of White Rats (*Rattus norvegicus*) in Each Treatment were measured three times, on days 0, 3, and 18. The data are presented as the mean, with different superscript letters indicating significant differences ($p<0.05$)

Table 4. Percentage of Damage to the Langerhans Islands in Rats (*Rattus norvegicus*)

Treatments	Day 1	Day 3	Day 10
Normal Control	100.00±0.00^{d}	0.00±0.00^{a}	0.00±0.00^{a}
Negative Control	15.00±5.59^{a}	45.00±11.18^{d}	37.50±8.84^{c}
Positive Control	77.50±5.59^{c}	13.75±5.23^{b}	8.75±3.42^{ab}
Humic Acid 375 mg/kg b.w	75.00±8.83^{c}	15.00±7.13^{b}	10.00±3.42^{b}
Humic Acid 625 mg/kg b.w	47.50±5.59^{b}	27.50±5.59^{c}	30.00±11.18^{c}

Compared to the control, the reduction of 79.23% in fasting blood glucose levels indicates that humic acid may restore pancreatic beta cell function due to oxidative damage and promote insulin secretion. This is consistent with previous studies suggesting that antioxidants can protect β-cells from oxidative stress-induced damage, thereby preserving their functionality.

The area under the curve (AUC0-120) value of 19.311 in the 375 mg/kg b.w. group reflects improved glucose tolerance, demonstrating that humic acid facilitates better glucose utilization. This is a critical finding, as it indicates that the compound lowers fasting glucose levels but also enhances the overall glucose metabolic profile over time. A notable outcome of this study is the significant decrease in malondialdehyde (MDA) levels in the treatment groups, with the 375 mg/kg b.w. group showing an MDA level of 1.09 ±0.27 nmol/ml. MDA is a key indicator of lipid peroxidation and oxidative stress (Abiola et al., 2021). The reduction in MDA levels suggests that humic acid effectively mitigates oxidative stress, a major contributing factor in the pathogenesis of diabetes and its complications. By lowering oxidative stress, humic acid may help protect cellular structures and maintain the integrity of the pancreatic tissue.

The therapeutic potential of humic acid as a natural antioxidant has been reported by Spilioti et al (2017), that humic acid has a buffering effect which means that this compound can produce and bind Reactive Oxygen Species (ROS). Giving humic acid compounds to rats can increase total antioxidant status and reduce oxidative stress levels (Vetvicka et al., 2014). Other results reported related to oral administration of humic acid extract in mice can increase endogenous antioxidant activities such as glutathione (GSH) and superoxide dismutase (SOD) and reduce biological biomarkers of lipid peroxidation, namely MDA (Vetvicka et al., 2015).

Histological examination of the pancreatic tissues further supports these biochemical findings. The treatment with humic acid at the optimal dose resulted in noticeable improvements in the pancreatic microanatomy. The preservation of islet cells and the reduction of inflammatory infiltrates were evident in the treated groups compared to the untreated diabetic control group. These histopathological improvements are indicative of the protective effects of humic acid against streptozotocin-induced pancreatic damage.

Interestingly, while the 625 mg/kg b.w. The dose of humic acid also produced beneficial effects; they were not significantly greater than those observed with the 375 mg/kg b.w. dose. This suggests a potential dose ceiling effect, where increasing the dose beyond a certain point does not yield proportional benefits. Future studies must explore the dose-response relationship further and determine the most effective and safe dosage range for humic acid.

CONCLUSION

In conclusion, the findings of this study provide solid evidence for the anti-diabetic advantages of humic acid. The improvements in body weight, fasting glucose levels, glucose tolerance, oxidative stress indicators, and the preservation of pancreatic microanatomy demonstrate humic acid's therapeutic potential. These findings call for further research in bigger, more diverse populations and clinical settings to fully understand the therapeutic potential of humic acid in treating diabetes.

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