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Evaluation of Antioxidant and Haematological Effects of Ethanol Extract of *Saussurea lppa* on Streptozotocin Induced-Diabetic Wistar Rats

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ABSRTACT

Diabetes mellitus (DM) is a group of metabolic disorders characterized by an increased level of blood glucose over a long period. Diabetes is associated with oxidative stress, which arises when there is an imbalance between the production and accumulation of reactive oxygen species (ROS) with resultant damage to DNA, lipids and protein. Saussurea *lappa* is a medicinal plant that has antioxidant effects due to the presence of flavonoids and phenols as a major content of its constituents. The study aimed to evaluate the antioxidant and hematological effects of ethanol extract of *Saussurea lappa* on streptozotocin-induced diabetic Wistar rats. A total of 30 Wistar rats weighing between 130g and 170g were randomly divided into 6 groups of 5 rats each, normal control, diabetic control, diabetic treated with 100mg/kg of metformin, diabetic treated with 200mg/kg of *S. lappa*, diabetic treated with 400mg/kg of *S. lappa* and diabetic treated with 800mg/kg of *S. lappa*. The interventions were administered for a period of 21 days. The results obtained demonstrated that diabetic rats administered with 200 mg, 400mg and 800mg/kg bodyweight S. *lappa* have significant increase ($p<0.05$) in the levels of glutathione, superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, hemoglobin, mean corpuscular hemoglobin concentration and hematocrit, while a significant decrease in the levels of malondialdehyde and white blood cell compared with metformin, diabetic control and normal control groups. Ethanol extract of *S. lappa* has antioxidant properties and also can enhances haematopoiesis and peripheral blood cell count by reducing oxidative stress.

Keywords: *Saussurea lappa*, antioxidant, haematological, effects, induced-diabetic, rats

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Introduction

Diabetes mellitus (DM) is a group of metabolic disorders characterized by an increased level of blood glucose over a long period.[1] Elevation in the concentration of blood glucose (hyperglycemia) could be due to the pancreas deficiency or inadequate insulin synthesis, which ultimately damages delicate organs in the body system such as blood vessels, eyes, kidneys, heart, and nerves.[2] Hyperglycemia occurs when the blood glucose level is greater than 11.0 mmol/L (200 mg/dL) but symptoms begin to manifest when the blood glucose level further increases to 15–20 mmol/L (250–300 mg/dL).[3]

Diabetes is associated with oxidative stress, which arises when there is an imbalance between the production and accumulation of reactive oxygen species (ROS) with resultant damage to DNA, lipid, and protein.[4]

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Several diabetic complications arise when these molecules, which have significant regulatory effects on the body, become out of balance.[5] High concentrations of ROS in the body and decreased level of antioxidants result in oxidative stress.[6]

Streptozotocin (STZ) is a broad-spectrum antibiotic derived from *Streptomyces achromogenes.* It comprises of two main components: glucopyranosyl group, which enhances its uptake by pancreatic beta cells through the glucose transporter 2 (GLUT2) and nitrosourea group, which causes the destruction of pancreatic beta cells.[7]

Saussurea lappa (S. lappa) or costus root, is a medicinal plant that grows in the Himalayan region and belongs to the family Asteraceae.[8] It comprises of various active components with medicinal properties that are attributed to the presence of flavonoids, steroids, terpenes, alkaloids, sesquiterpenes, costunolide, dehydrocostuslactone, cynaropicrin, and chlorogenic.[9,10] *S. lappa* has the potential to treat various disease conditions in allopathic and herbal medicine such as asthma, chronic skin disease, inflammatory diseases, ulcer, typhoid fever, cough and cold, toothache, leprosy, stomachache among others.[11] However, there is paucity of data on the antioxidant and haematological effects of ethanol extract of *S. lappa* on streptozotocin induced-diabetic Wistar rats in Nigeria. Hence, the study attempted to provide information on the effect of ethanol extract of *S. lappa* on antioxidant and haematological parameters on streptozotocin-induced diabetic Wistar rats which may help in preventing or reducing oxidative stress associated with diabetes and ultimately removed drug induced side effects.

Materials and Methods

Experimental Animals

The experiment was conducted using Wistar rats (*Rattus norvergicus*) weighing between 150g to 200g obtained from the animal facility center of Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria, Nigeria. The animals were acclimatized for two (2) weeks before the commencement of the study. Standard commercial chow and water were provided ad libitium for the animals. Housing conditions were maintained at 25 ± 2 ℃ at 12 h day/night cycles. The care and handling of the animals were according to the established public health guidelines in Guide for Care and Use of Laboratory Animals.

Ethical Approval

Letter of ethical approval with reference number PTAC/SI/(Ee)/OT/60-23 dated 29th January, 2023 was granted by Veterinary and Pharmacology Departments of Usmanu Danfodiyo University Sokoto, Nigeria the study was conducted according to Nigeria community regulations in conformity with International Standards regarding the protection of experimental animals.

Plant Collection and Identification

The dried roots of Saussurea *lappa* (costus root) were purchased from New Delhi, India in February, 2023. Taxonomic identification and authentication of the plant was carried out by the taxonomist from the Department of Pharmacognosy and Ethanopharmacy, Usmanu Danfodiyo University, Sokoto, Nigeria. A voucher specimen (PCG/UDUS/ASTE/0003) of the plant was deposited at the herbarium of same department.

Preparation and Extraction of Plant Material

The dried root was mechanically powdered, and stored in an air-tight container for further processing. The ethanolic extract of dry powdered roots was prepared according to the modified method of Itarbone.[12] that is, 500 g of the crude powder of Saussurea *lappa* was soaked in 2.5 liters of 70% ethanol with continuous stirring for 72 hours at room temperature and the mixture was filtered using filter paper (Whatman number 1, England). The ethanolic filtrate was evaporated at 45°C under reduced pressure using a rotary evaporator and then, the aqueous residues were removed through lyophilization process by freeze drier and the stock extract was stored at -20 °C. The dry extract was subjected to in vitro measurements before its administration into the Wistar rats.

Induction of Diabetes

The Wistar rats were fed with high-fat diet (HFD) made up of Animal feed that constitute 65% fat 30% carbohydrate and 5% protein (pellet ultima finisher which was fortified with margarine in a ratio of 10 g of animal feed to one gram of margarine) for four weeks until the rat weight reached 330 ± 20 g. Low dose (35 mg/kg) of streptozotocin (STZ,) was dissolved in citrate buffer of pH (4.5) in the night before induction. A single shot of low dose of STZ was administered intraperitoneally through the abdominal cavity. After 12-24 hours, an injection of nicotinamide adenine (NAD) was administered to neutralize the effect of STZ and after 3 days, blood was collected from the tail of the rats to determine the fasting blood glucose level. Throughout the investigation, the rats had access to sucrose water. Rats with fasting (12 hours) blood glucose value ≥ 11 mmol/L were considered diabetic model rats with successful modelling were included in the experimental range.[13]

Experimental Grouping

Thirty (30) male Wistar rats were randomly divided into six groups of five (5) rats each.

Group I (normal control): The rats in this group received water and their normal rats' feed.

Group II (diabetic control): After diabetes induction with HFD and STZ, rats with blood glucose level greater than 11mmo/L were included into this group.

Group III: Diabetic Wistar rats received 1mL of metformin daily at a dose of 100 mg/kg/ body weight orally. This group served as the reference drug control.

Group IV: Diabetic Wistar rats received 200mg/kg body weight of ethanolic extract of S. *lappa* orally via gastric gavage needle daily for a period of three weeks.

Group V: Diabetic Wistar rats received 400mg/kg body weight of ethanolic extract of S. *lappa* orally via gastric gavage needle daily for a period of three weeks.

Group VI: Diabetic Wistar rats received 800mg/kg body weight of ethanolic extract of S. *lappa* orally via gastric gavage needle daily for a period of three weeks.

Blood Sample Collection and Processing

After three (3) weeks of the experiment, the animals were anaesthetized in a glass jar containing wool soaked with chloroform. Ten milliliters (10ml) of blood samples were collected from the animals through cardiac puncture. Two milliliters (2 ml) were collected into EDTA containers for complete blood count, 2.7ml of blood was collected into sodium citrate bottles containing 0.3ml of sodium citrate solution and the remaining 5.3ml was transferred into plain containers and allowed to clot at room temperature and later centrifuged at 4000 revolutions per minute (4000 rpm) for 10 minutes while the sample in the sodium citrate bottle was centrifuge at 3,000 rpm for 15 minutes. The plasma from citrate bottles was separated and used for prothrombin time test (PT) and activated partial thromboplastin test (APTT) while the sera from the centrifuged plain containers were transferred into labelled sterile serum bottles and tightly capped and stored at -20^{o}C before analysis. The sera were analyzed for malondialdehyde, glutathione, catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase using colorimetric method.

Estimation of Catalase (Catalogue number E-BC-KO31-S, manufactured by Elabscience at United Kingdom In 2022).

The microplate reader was preheated 30 minutes before usage and the wavelength was adjusted to 240 nm. Catalase working reagent was preheated in a water bath at 25°C for 10 minutes. 1 milliliter of catalase working reagent was put into the wells of the microtiter plates and 35 microliters of the serum sample were added into wells. The microtiter plate was covered and mixed gently for 5 seconds. The microtiter plate was inserted into the plate reader and the absorbance at 240 nm at the initial time (A1) was read immediately and after 1 minute (A2).

Estimation of Superoxide Dismutase (Catalogue number SD2521, manufactured by Biodiagnostics at United State of America in 2022).

One microliter of reagent 1 working solution was put into the microtiter wells and 20 microliters of the serum sample were added. After which, 0.1 milliliter of reagent 2, 0.1 milliliter of reagent 3 and 0.1 milliliter of reagent 4 working solutions were all added into the microtiter wells. The microtiter plate was mixed with vortex mixer and incubated for 40 minutes at 37°C. Then, 2 milliliters of chromogenic agent were added to the wells and the microtiter plate was mixed gently and allowed to stand at room temperature for 10 minutes. The absorbance was measured in a plate reader set at 550 nm.

Estimation of Glutathione Peroxidase (Catalogue number: GP 2524, manufactured by Biodiagnostics at United State of America in 2022).

50 µL of diluted sample was put into the sample well, after which, 50 µL of working NADPH and 50 µL of working hydrogen peroxide were also added to each well. After a period of 1 minute, the absorbance at 340 nm was monitored for 5 minutes with a recording interval of every 30 seconds. The glutathione peroxidase activity was calculated from the net rate.

Estimation of Glutathione Reductase (Catalogue number: E-BC-K096, manufactured by Elabscience at United Kingdom In 2022).

The microtiter plate was pre-heated inside the incubator for 5 minutes at 37°C and 65 microliters of the sample were put into the microtiter and then, 300 microliters of the working solution were added. The microtiter plate was mixed gently and incubated at 37°C. The absorbance was measured at 340nm at 30 seconds (A1) and at 150 seconds (A2).

Estimation of Malondialdehyde (Catalogue number: E-BC-K028, manufactured by Elabscience at United Kingdom In 2023)

One thousand microliters (1000µL) of serum sample and distilled water were added to the test tubes labelled test and blank, respectively. One thousand microliters (1000µL) each of the reagents labelled 1, 2, and 3 were added into both tubes labelled as test and blank. The tubes were mixed well and incubated in boiling water bath for 15minutes and allowed to cool, and left to stand at room temperature for 20 minutes. The tubes were centrifuged at 2000 rpm for 15 minutes. The supernatant layer was read at 534 nm.

Glutathione Estimation (Catalogue number: E-BC-K028, manufactured by Elabscience at United Kingdom In 2023)

A concentration of 0.7ml of reagent 1 was measured and added to the 0.7ml pre-heated serum sample and then centrifuged at 4500 g for 10 minutes. The supernatant was harvested for the analysis. Three tubes were labelled as blank, standard and sample respectively. In the blank tube, 1 ml of reagent 1 was added. In the standard tube, 1 ml of 20µmol/L reduced glutathione (standard solution) was added. In the sample tube, 1 ml of the supernatant was added. After which, 1.25ml of reagent 2 (application solution), 0.25 ml of reagent 3 and 0.05 ml of reagent 4 application solution were all added into each of the three tubes. The tubes were mixed thoroughly and allowed to stand at room temperature for 15 minutes. The spectrophotometer was set to zero with distilled water and the optical density was measured at 420nm with 1 cm optical cuvette.

Statistical Analysis

Data generated from the study were analyzed using statistical package for social sciences (SPSS) version 29.0.1.0. Results were expressed as mean ± SEM (standard error of mean). The statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test. The differences in values of $p<0.05$ was considered statistically significant.

Results and Discussion

Evaluation of oxidative stress and antioxidant status of streptozotocin induced-diabetic Wistar rats treated with metformin and ethanolic extract of *S. lappa*.

The effects of metformin and ethanolic extract of *S. lappa* on serum oxidative stress (malondialdehyde) and antioxidants (catalase, superoxide dismutase, glutathione, glutathione peroxidase and glutathione reductase) in streptozotocin induced-diabetic Wistar rats is shown in table 1. In this study, streptozotocin-induced diabetic in Wistar rats has shown to have effects on the values of the antioxidants (catalase and glutathione peroxidase) when compared with the normal control rats. The significant decrease in the values of catalase and glutathione peroxidase in the streptozotocin induced-diabetic group could be associated with hyperglycemia which causes the production of free radical and this results in lipid peroxidation. This is similar to a finding in which streptozotocin induced diabetic causes reduced activity of endogenous antioxidants; glutathione peroxidase, catalase and superoxide dismutase.[14] Metformin and 800mg/kg of *S. lappa* can best be used to ameliorate the increased malondialdehyde level in diabetic patients. Although, *S. lappa* doses at 200mg/kg and 400mg. kg have also proven effective in reducing malondialdehyde levels. The decreased malondialdehyde concentrations at 800mg/kg of *S. lappa* may be due to the protective effect of *S. lappa* from the deleterious effects of reactive oxygen species mediated by lipid peroxidation of tissue macromolecules.[15] However, this protection may also be attributed to the presence of high flavonoid contents and phenolic compounds in the roots of *S. lappa*.[16] This is inconsistent with the report that ethanolic extract of *S. lappa* can cause a significant decrease in hepatic malondialdehyde in leukemic-induced rats.[17] The activity of catalase has significantly increased in the induced-diabetic group after the administration of 400 and 800 mg/kg *S. lappa*. This shows that the decreased activity of catalase in diabetic patients can be restored with an increase dose of 400mg/kg *S. lappa*. This agrees with the study in which supplementation with aqueous extract of *S. lappa* reverses the induced-liver injury by improving catalase.[18] This study has revealed that the reduced activity of superoxide dismutase in diabetic patients can be treated effectively with 400mg of *S. lappa*. This is similar to a previous study which showed that supplementation of *S. lappa* modulated significantly superoxide dismutase activity in the serum of hypercholesterolemic rats.[19] The significant decrease in the level of glutathione in diabetes can improve when treated with 800mg of the extracts of *S. lappa*. The findings are in line with the report on oxidative ameliorative potential of *S. lappa* observed by elevated antioxidant markers (glutathione) and decreased oxidative stress markers.[20] The activity of glutathione peroxidase in induced-diabetic Wistar rat was increased when treated with 800mg/kg *S. lappa*. This agrees with the study in which ethanolic root extract of *S. lappa* significantly the glutathione peroxidase activity.[21] Glutathione reductase reduced activity diabetic patients can significantly be treated with 200mg/kg and 400mg/kg *S. lappa*. This agrees with the study where aqueous extract of S. *lappa* ameliorates oxidative myocardial injury by improving the activity of glutathione reductase in rats.[22]

Evaluation of the haematological and coagulation profiles of streptozotocin induced-diabetic Wistar rats treated with metformin and ethanolic extract of *S. lappa*

Table 1: Evaluation of oxidative stress and antioxidant status of streptozotocin induced-diabetic Wistar rats treated with metformin and ethanol extract of *S. lappa*.

Parameters	Treatment Groups						
	1 **Normal Control**	**2 Diabetic Control**	**3 Metformin**	**4 200mg/kg SL**	**5 400mg/kg SL**	**6 800mg/kg SL**	**P value**
MDA (nmol/ml)	1.96±0.67	6.2±2.05	1.5±0.63[a]	1.73±0.57	0.93±0.16	0.36±0.23[a]	0.006
CAT (U/ml)	7.18±0.39	2.04±0.09	4.3±0.26	8.7±1.49	15.18±1.74[ab]	22.99±3.48[ab]	0.0000
SOD (U/mL)	75.37±20.95	20.63±17.73	93.04±8.6	59±15.7	145.1±16.31[a]	163.55±17.1[ab]	0.0001
GSH (mg/L)	8.63±3.68	0.81±0.40	8.06±2.49	8.28±1.85	8.1±2.0	30.4±7.28[ab]	0.0005
GPX (U/mg prot)	6710±605	1320±900	7044±704	4205±476	7665±1924	10090±2839[a]	0.0123
GPR (U/gprot)	10.01±4.99	1.39±0.43	8.69±2.11	23.52±5.82[b]	22.59±1.34	34.99±8.28[ab]	0.001

Values are express in mean ± SEM and $p<0.05$ was considered significant. SEM=Standard error of mean, SL= Saussurea *lappa,* CAT=Catalase, SOD=Superoxide dismutase, GSH=Glutathione, GPX=Glutathione peroxidase and GPR= Glutathione reductase. N= 4 for each group

[a] $p<0.05$, significantly different from diabetic control

[b] $p<0.05$, significantly different from normal control

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The effects of metformin and ethanolic extract of *S. lappa* on haematological and coagulation profiles of streptozotocin induced-diabetic Wistar rats is shown in table 2. Induced-diabetic rats treated with 800mg/kg S. lappa have shown significant effect in the level of their white cell count. The study further shows the ability of the extract to manage diabetes and hence, reverse the increase in WBC count. This finding is similar to the study where *Peristrophe bicalycilate* and quercetin decrease the WBC count in high fat diet and streptozotocin-induced diabetic in Wistar rats.[23] Decrease level of haemoglobin in the diabetic control group compared to the normal control group was observed. However, treatment with 100mg/kg metformin and 800mg/kg *S. lappa* has significantly improved the haemoglobin concentration when compared with normal control group. This is consistent with the study in which the supplementation of *S. lappa* improves the cyclophosphamide-induced anaemia by improving the haemoglobin concentration.[24] The reduced haematocrit level seen in diabetes has shown significant increase when treated with 800mg/kg of *S. lappa* extract in this study. Therefore, it is impressive that increased doses of *S. lappa* have the potential to improve anaemia that occurs in diabetic patients. This improvement could be attributed to rich flavonoids contents of the plant extract which can stimulate the secretion of erythropoietin and stimulate stem cells to produce red blood cells.[25] The mean corpuscular haemoglobin concentration (MCHC) in this study has shown significant increase in the treatment groups (400 and 800mg/kg *S. lappa*). This finding agrees with earlier report where treatment with ethanolic extract of *S. lappa* significantly improved the concentration of haemoglobin, haematocrit and MCHC in paracetamol-induced hepatic and renal damage in albino rats.[26] The findings in this current study demonstrated that *S. lappa* roots may have therapeutic effect due to the phytochemical compounds present in the root of this plant such as alkaloids, saponins, steroids, terpenes, polyphenol, flavonoids, sterols, tannins, and glycosides. These compounds are well-known haemopoietic factors that have a direct influence on the production of blood cells and antioxidant activity as well inhibition of free radicals.[27]

Diabetes did not influence lymphocyte count, monocyte count, eosinophil count, basophil count, red blood cell count, mean cell volume, mean corpuscular haemoglobin and platelet count. However, metformin and 200mg/kg and 400 mg/kg *S. lappa* have some degrees of control of diabetic patients concerning the values of white blood cell, haemoglobin, haematocrit and mean corpuscular haemoglobin. Coagulation markers (Prothrombin time and Activated Partial Thromboplastin Time) are also not altered by diabetes.

Table 2: Evaluation of the haematological and coagulation profiles of streptozotocin induced-diabetic Wistar rats treated with metformin and ethanol extract of *S. lappa*

			Treatment Groups				
Parameters	**1 Control**	**2 Diabetic**	**3 Metformin**	**4 200mg/kg SL**	**5 400mg/kg SL**	**6 800mg/kg SL**	**P-value**
WBC (10^9/L)	10.85±2.83	13.6±2.00	12.57±0.73	23.52±2.90	17.15±3.44	14.12±1.44[b]	0.021
LYM (%)	74.05±4.3	74.45±0.97	77.18±3.28	79.37±0.77	76.37±2.98	77.17±4.26	0.8414
MON (%)	3.97±0.76	4.42±0.59	4.95±2.31	6.1±1.68	5.02±1.73	5.42±1.0	0.9315
NEU (%)	18.3±2.48	19.03±0.83	16.05±2.22	13±1.76	16.95±3.17	16.5±3.71	0.6387
EOS (%)	2.6±1.04	1.3±0.53	0.85±0.30	0.30±0.04	0.67±0.14	0.6±1.35	0.3303
BAS (%)	1.05±0.47	0.8±0.06	0.9±0.07	1.17±0.19	0.97±0.32	1.35±0.55	0.8759
RBC (10^{12}/L)	7.4±0.05	7.59±0.27	7.05±0.25	5.6±1.42	7.21±0.08	7.05±0.11	0.2749
HGB (g/dL)	12.12±0.18	10.02±0.19	13±0.46[b]	12.82±0.31[a]	13.5±0.37[a]	14.9±0.29[b]	0.0001
HCT (%)	36.05±0.6	34.32±0.2	35.42±1.48	36.2±0.60	38.1±1.13	40.42±0.88[ab]	0.0025
MCV (fL)	52.72±1.05	50.55±1.71	50.25±0.49	48.62±0.8	50.02±1.02	48.77±0.6	0.1126
MCH (pg)	19.1±0.32	18.5±0.89	18.45±0.11	18.07±0.04	18.7±0.31	18.27±0.12	0.595
MCHC (g/dL)	36.15±0.17	36.65±0.38	36.72±0.26	36.5±0.28	37.4±0.2[a]	37.4±0.29[a]	0.0241
PLT (10^9/L)	576±34	574.25±21.84	504±103.96	539±64.09	531±39.321	533±38.86	0.9418
PT (secs)	13±4.33	12±4.52	15.7±0.25	18.25±0.25	18±0.00	10±3.34	0.4
PTTK (secs)	21.25±8.3	30.25±10	36.2±0.25	37.25±0.25	38.75±0.25	38.75±12.9	0.538

Values are expressed in mean ± SEM, $p \leq 0.05$ was considered statistically significant RBC=Red Blood Cell, HCT=Haematocrit, HGB=Haemoglobin, MCV=Mean Cell Volume, MCH= Mean Corpuscular Volume, MCHC= Mean Corpuscular Haemoglobin Concentration, WBC= White Blood Cell, LYM= Lymphocytes, NEU= Neutrophil, MON= Monocytes, EOS= Eosinophil, BAS= Basophil, PLT= Platelets, PT=prothrombin time, PTTK=partial thromboplastin time test SEM=Standard error of mean, fL= femtoliters, g/dL= gram per deciliter, pg= picogram, /L= Litre and secs= seconds. N =4 for each group

[a] $p<0.05$, significantly different from diabetic control
[b] $p<0.05$, significantly different from normal control

Conclusion

Ethanol extract of *S. lappa* has antioxidant and antidiabetic properties with little or no haematological effect on induced-diabetic rats. It is therefore suggested that *S. lappa* could be used as it is effective in the treatment of diabetic patients since the actions corresponded with metformin therapy on some oxidative markers and haematological values.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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