

In-Vivo Antidiabetic Activity and Safety of The Aqueous Stem Bark Extract of *Kleinia squarrosa*

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Abstract

Kleinia squarrosa has been used traditionally to manage several diseases including diabetes, however, its efficacy and safety is not well evaluated. The aim of this study was to determine *in-vivo* hypoglycemic activity and safety of the aqueous stem bark extracts of this plant in male swiss white albino mice. The antidiabetic activity was screened in alloxan induced diabetic mice using oral and intraperitoneal routes. The safety of the extract was studied in mice that were orally and intraperitoneally administered with 1 g/kg body weight daily for 28 days by recording changes in body and organ weights, hematological and biochemical parameters and histology. Mineral composition was estimated using total reflection X-ray fluorescence system (TRXF) and atomic absorption spectrometry (AAS). Phytochemical composition was assessed using standard procedures. The extract showed hypoglycemic activity at dose levels of 50, 100, 200, 300 mg/kg body weight. Administration of 1 g/kg body weight of the extract decreased the body weight gain using both routes, and altered the organ to body weight percentage of the liver and lungs for intraperitoneal route while oral route only altered the liver. Oral administration of the same dose caused a change in levels of RBC, ALP, AST, LDH CK and Creatinine while the same intraperitoneal dose caused a change in RBC, WBC, Hb, PCV, PLT, MCH, MCHC, neutrophils, lymphocytes, eosinophils, monocytes and biochemical parameters: AST, ALT, GGT, LDH, T-BIL, D-BIL, Urea and Creatinine. Moreover, intraperitoneal administration caused significant histological lesions to the kidney, liver and spleen. The extracts contained tannins, phenols, flavonoids, saponins, and alkaloids. Sodium, Chlorine, Potassium, Calcium, Titanium, Vanadium, Chromium, Manganese, Iron, Copper, Zinc, Arsenic, Cadmium, Magnesium, Nickel and Lead were present in the extracts at levels below the recommended daily allowance. The observed hypoglycemic activity and slight toxicity could be associated with the phytochemicals present in this plant extract.

Keywords: Diabetes mellitus; *Kleinia squarrosa*; Hypoglycemic activity; Antidiabetic; Mineral composition; Phytochemicals; Toxicity

Introduction

The number of people living with diabetes is estimated at 382 million people worldwide as of 2014 and this number is expected to increase to over 592 million people in less than 25 years [1]. In 2012, 1.5 million deaths were reported to be directly caused by diabetes [2]. The disease has several pathogenic processes ranging from autoimmune destruction of pancreatic β -cell resulting in absolute insulin deficiency (Type I) to multiple abnormalities leading to the resistance to insulin action by body cells (Type II) [3].

Symptoms of diabetes mellitus include excessive excretion of urine (polyuria), thirst (polydipsia), constant hunger (polyphagia), weight loss, vision changes and fatigue [2].

Chronic hyperglycemia of diabetes leads to long term microvascular and macrovascular complications [4]. These complications include retinopathy, nephropathy, neuropathy and cardiovascular disorders [3].

Normal fasting plasma glucose levels range between 3.5-6.7 mmol/l (63-120.6 mg/dl). After a meal the blood glucose level rises to approximately 8mmoles/L and rarely exceeds this level. Repeated fasting blood glucose levels \geq 7.0 mmoles/L (126 mg/dl) or 2-hour postprandial glucose values \geq 11.1 mmol/L (200 mg/L) is considered to be diagnostic criteria for diabetes and correlates with Hb A1c threshold of 6.5% [5].

Type I diabetes patients always require exogenous insulin. For type II patients therapy options start with life style modifications but

as disease progresses oral hypoglycemic drugs or insulin or both are required to obtain glycemic target for diabetes management [6].

Although insulin is essential drug for diabetes management its continuous access is a major problem in many developing countries especially in sub-Saharan Africa [7]. Due to the many side effects associated with conventional antidiabetic drugs there is a growing interest in the herbal sources [8]. According to World Health Organization (WHO), up to 80% of the world's population in developing countries relies on traditional medicine practices for their primary health care needs [9]. Plants contain a great diversity of bioactive compounds which makes them a possible source for different types of drugs [10]. For example the widely used hypoglycemic drug Metformin is originally derived from the medicinal plant *Galega officinalis* [11].

More than 400 traditional plants have been reported to have

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antidiabetic effect [12]. Some of these herbs are proven to provide symptomatic relief and assist in the prevention of the secondary complication of the disease, while others were reported to help in regeneration of β -cells and in overcoming insulin resistance [13].

Kleinia Squarrosa (locally known as Mung'endyanthenge) has been in use by traditional health practitioners to treat different human diseases such as jaundice, stomach pain, asthma, syphilis, edema, malaria, and for women with menstruation problems [9]. The plant has also been in use successfully in the management of diabetes mellitus in some parts of Kenya. Its aqueous stem bark extract given through intraperitoneal route exhibited remarkable hypoglycemic properties in alloxan induced diabetic mice [14]. This study therefore contributes additional knowledge to the use of *Kleinia Squarrosa* in the management of diabetes and its safety from samples collected from Machakos county Kenya.

Materials and Methods

Study site

This study was undertaken at the Department of Biochemistry and Biotechnology, School of Pure and Applied Sciences, Kenyatta University from November 2012 to August 2014. Kenyatta University is 23 km from Nairobi off Thika Road.

Collection of the plant materials

The plant used in this study was collected from its natural habitat in Machakos County, Kenya. An acknowledged authority in taxonomy authenticated the botanical identity of the plant and a voucher specimen was deposited at the National Museums of Kenya Herbarium, Nairobi. A traditional medical practitioner provided the information on which plant to collect, what part to collect, and the precise locality where it grows.

Initial processing of the plant

The collected plant materials were chopped into small pieces, dried under shed at room temperature for 1 month, and then ground when completely dry using an electric mill. The powdered plant materials were kept at room temperature away from direct sunlight in closed dry plastic bags.

Preparation of the aqueous extracts

Each one hundred grams of the powdered plant material was extracted in 1 liter distilled water at 60°C for 6 hour. The mixture was left to cool at room temperature and then decanted into dry clean conical flask through folded cotton gauze stuffed into a funnel. The decanted extract was then filtered using filter papers under vacuum pump. The filtrate was then freeze-dried for 72 hour. The freeze-dried powder was then weighed and stored in airtight container at -20°C until used for bioassay.

Experimental animals

The study used male Swiss White Albino mice (3-4 weeks old) that weighed 23-27 g with a mean weight of 25 g. These were bred in the Animal house at the Department of Biochemistry and Biotechnology of Kenyatta University. The mice were housed at a temperature of 25°C with 12 hours/12 hours darkness photoperiod and fed on rodent pellets and water *ad libitum*. The experimental protocols and procedures used in this study were approved by the Ethics Committee for the Care and Use of Laboratory Animals of Kenyatta University, Kenya.

Induction of hyperglycemia

Hyperglycemia was induced experimentally by a single intraperitoneal administration of 186.9 mg/kg body weight of a freshly prepared 10% alloxan monohydrate (2,4,5,6 tetraoxypyrimidine; 5-6-dioxyuracil) obtained from Sigma (Steinhein, Switzerland) [15].

Forty-eight hours after alloxan administration, blood glucose level was measured using a glucometer. Mice with blood glucose levels above 2000 mg/L were considered diabetic and used in this study. Prior to initiation of this experiment, the animals were fasted for 8-12 hours [16] but allowed free access to water until the end of this experiment.

Experimental design

For either intraperitoneal or oral route of drug administration, the experimental mice were randomly divided into seven groups of five animals each. Group I consisted of normal mice either intraperitoneally or orally administered with 0.1 ml physiological saline; Group II consisted of alloxan induced diabetic mice either intraperitoneally or orally administered with 0.1 ml physiological saline; Group IIIa consisted of alloxan induced diabetic mice intraperitoneally administered with 0.025 insulin units (0.25 insulin units in 1 ml) (1 IU/kg body weight) in 0.1 ml physiological saline; Group IIIb consisted of alloxan induced diabetic mice orally administered with 0.075 mg glibenclamide (0.75 mg in 1 ml) (3 mg/kg body weight) in 0.1 ml physiological saline; Group IV consisted of alloxan induced diabetic mice either intraperitoneally or orally administered with 1.25 mg extract (12.5 mg in 1ml physiological saline) (50 mg/kg body weight) in 0.1 ml physiological saline; Group V consisted of alloxan induced diabetic mice either intraperitoneally or orally administered with 2.5mg extract (25 mg extract in 1 ml physiological saline) (100 mg/kg body weight) in 0.1 ml physiological saline; Group VI consisted of alloxan induced diabetic mice either intraperitoneally or orally administered with 5 mg extract (50 mg extract in 1 ml physiological saline) (200 mg/kg body weight) in 0.1 ml physiological saline; Group VII consisted of alloxan induced diabetic mice either intraperitoneally or orally administered with 7.5 mg extract (75 mg extract in 1 ml physiological saline) (300 mg/kg body weight) in 1 ml physiological saline. 0.1 ml of either insulin or glibenclamide or the plant extract solution was administered either intraperitoneally or orally to each experimental mouse.

Blood sampling and blood glucose determination

Blood sampling was done by sterilizing the tail with 10% alcohol and then nipping the tail at the start of the experiment and repeated after 1, 2, 3, 4, 6 and 24 hours. Bleeding was enhanced by gently "milking" the tail from the body towards the tip. After the operation, the tips of the tail were sterilized by swabbing with 70% ethanol. The blood glucose levels were determined with a glucose analyser model (Hypogaurd, Woodbridge, England).

In-vivo single dose toxicity test

The mice were randomly divided into four different groups of five mice each. Group I and II consisted of untreated control mice intraperitoneally and orally, respectively, administered daily for 28 days with 0.1 ml physiological saline. Group III and IV consisted of normal mice intraperitoneally and orally administered daily for 28 days with the extract at 1 g/kg body weight in 0.1 ml physiological saline. During this period, mice were allowed free access to mice pellet and water and observed for any signs of general illness, change in behaviour and mortality. At the end of 28 days, the mice were sacrificed.

Determination of body and organ weight

The body weight of each mouse was assessed after every seven days during the dosing period up to and including the 28th day and the day of sacrifice (day zero, 7, 14, 21, 28). On the day of sacrifice, all the animals were euthanized using chloroform as an inhalant anaesthesia and blood samples were drawn from the heart of each sacrificed mouse. The blood samples were collected in plastic test tubes and divided into two portions. One portion was used for determination of hematological parameters. The other portion was allowed to stand for 3 hours to ensure complete clotting. The clotted blood samples were centrifuged at 3000 rpm for 10 min and clear serum samples were aspirated off and stored frozen at -20°C for metabolite and enzyme assays. The liver, kidney, heart, lungs, spleen, intestine, brain and testis were carefully dissected out, weighed and preserved in 10% neutral buffered formalin for histological analysis.

Determination of hematological parameters

Blood parameters and indices were determined using standard protocols [17]. Red blood cells count, white blood cells count, hemoglobin, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and mean corpuscular volume were determined in whole blood with EDTA anticoagulant using the Coulter Counter System (Beckman Coulter®, ThermoFisher, UK).

Differential white blood cell count for neutrophils, lymphocytes, eosinophils, basophils and monocytes were determined from giemsa stained blood films using a hemocytometer [17]. Air-dried thin blood films stained with giemsa stain were examined microscopically using magnification 400 and 1000 for differential WBC counts and cell morphology, respectively.

Determination of biochemical parameters

The biochemical parameters determined on the sera specimen using the Olympus 640 Chemistry AutoAnalyser were Aspartate aminotransferase (AST), Alanine transaminase (ALT), Alkaline phosphatase (ALP), Gamma-glutamyl transpeptidase (GGT), Lactate dehydrogenase (LDH), Creatine Kinase (CK), α -Amylase (α -AMYL), Total bilirubin (T-BIL), Direct bilirubin (D-BIL), Urea and Creatinine. All reagents for the machine were commercially prepared to fit the required volumes and concentrations. The reagents were in specific containers referred to as reagent cartridges. The reagent cartridges were bar coded for the identification by the machine. The machine was programmed for the selected tests for each sample. The sample sectors were then placed into the autoloader assembly. A number of events that occurred simultaneously were performed automatically under the direct control of the instrument microprocessor. All the assays were performed based on the standard operating procedures (SOPs) written and maintained in the Department of Laboratory Medicine, Kenyatta National Hospital.

Histopathological analysis

The formalin fixed tissues were processed using standard histopathology protocol and stained with haematoxylin and eosin for microscopy. The stained tissues were cover slipped with DPX, dried and examined microscopically for any pathological changes [18].

Qualitative phytochemical screening

The extract was screened for the presence of five major classes of phytochemicals using the recommended procedures. Alkaloids [19], saponins [20], Flavonoids [21], phenolis [20], and Tanins [21].

Quantitative phytochemical screening

The phytochemicals present were quantified using standard procedures: Alkaloids [22], Saponins [23], Flavonoids [24], Phenols [25] and Tannins [26].

Mineral elements analysis

Mineral composition of the plant extract was analyzed using total reflection X-ray fluorescence system (TRXF) and atomic absorption spectrometry (AAS). TRXF system was used to determine the content of Sodium (Na), Magnesium (Mg), chlorine (Cl), potassium (K), Calcium (Ca), Titanium (Ti), Vanadium (V), manganese (Mn), iron (Fe), Copper (Cu), Zinc (Zn), Gallium (Ga), Arsenic (As), selenium (Se), Bromine (Br), Rubidium (Rb), Strontium (Sr), Nickel (Ni), Lead (Pb), and Uranium (U) in the lyophilized plant samples as described by Hagen [27]. Atomic absorption spectrometry (AAS) was used for the analysis of Magnesium, Chromium and Cadmium [28]. All the analysis was processed following the instructions from the manufacturer.

Data management and statistical analysis

The Data was entered in the Microsoft Excel Spread Sheet, cleaned and then exported to Statistical Package of Social Sciences (SPSS) Software for analysis. Results were expressed as Mean \pm Standard Deviation (SD) of the number of animals used per every study point. Statistical analysis were done using ANOVA and post-ANOVA to compare the means of untreated normal control mice with diabetic mice treated with saline, diabetic mice treated with the conventional drug, and diabetic mice treated with plant extract at doses of 50 mg/kg body weight, 100mg/kg body weight, 200 mg/kg body weight, and 300 mg/kg body weight. For *in-vivo* toxicity test student unpaired t-test was used to compare the data of normal control group with the group treated with the extract. $p \leq 0.05$ was considered statistically significant.

Results

Effect of oral and intraperitoneal administration of aqueous stem bark extracts of *Kleinia squarrosa* on blood glucose levels in alloxan induced diabetic mice

The dry powder of *Kleinia squarrosa* yielded 3.98% (w/w) aqueous stem bark extract. Treatment with the aqueous stem bark extracts showed hypoglycemic properties in alloxan induced diabetic mice (Table 1, Figures 1 and 2). Oral administration of the aqueous stem bark extracts of *Kleinia squarrosa* decreased the blood glucose levels at all the four tested dose levels (50, 100, 200 and 300 mg/kg body weight). As shown in Figure 1, during the 1st hour the percent reductions of the four therapeutic doses in the blood glucose levels were 41.32%, 45.60%, -0.92%, and 14.17%, respectively, compared to the reference drug glibenclamide which lowered blood glucose levels by 9.51% within the same hour. The aqueous stem bark extracts of *Kleinia squarrosa* decreased the blood glucose levels appreciably by the four therapeutic doses in the 4th and 6th hour. In the 4th hour the percent blood glucose reductions were 53.63%, 50.06%, 46.15%, and 59.46%, respectively, compared to glibenclamide which lowered blood glucose levels by 59.37% within the same hour. In the 6th hour the percent blood glucose reductions were 54.78%, 49.50%, 56.00% and 70.42%, respectively, compared to glibenclamide which lowered blood sugar levels by 76.18% within the same hour. After this, there was a gradual increase up to the twenty fourth hours.

Intraperitoneal administration of aqueous stem bark extracts of the four therapeutic doses (50, 100, 200 and 300 mg/kg body weight) of

Treatment	Route	Glucose levels at varying times in (mmole/L)						
		0hr	1hr	2hr	3hr	4hr	6hr	24hr
Control	Oral	5.18 ± 0.25	5.00 ± 0.21	5.04 ± 0.21	5.32 ± 0.33	5.34 ± 0.41	5.08 ± 0.19	5.66 ± 0.50 ^a
	IP	5.14 ± 0.17	5.04 ± 0.17	5.02 ± 0.27	5.24 ± 0.23	5.30 ± 0.28	5.30 ± 0.41	5.52 ± 0.41
Diabetic control	Oral	18.92 ± 2.19 ^A	20.98 ± 2.38 ^C	23.34 ± 1.80 ^{Ba}	24.92 ± 1.57 ^{Bb}	27.18 ± 1.71 ^{Bc}	28.70 ± 1.18 ^{Ad}	31.34 ± 1.21 ^{Be}
	IP	19.90 ± 1.02 ^A	22.22 ± 1.74 ^B	23.74 ± 1.77 ^{Ba}	25.24 ± 1.22 ^{Bb}	26.74 ± 1.39 ^{Ac}	27.78 ± 0.94 ^{Ac}	28.74 ± 0.83 ^{Bd}
Diabetic/Glib	Oral	20.82 ± 2.17 ^{Ad}	18.84 ± 2.29 ^{Bd}	15.48 ± 1.64 ^{Ac}	11.28 ± 1.46 ^b	8.46 ± 0.94 ^a	4.96 ± 0.47	6.86 ± 1.07
Diabetic/Insulin	IP	21.60 ± 1.53 ^{Bb}	6.46 ± 0.81	6.04 ± 0.47	6.48 ± 0.50	6.48 ± 0.49	5.72 ± 0.49	7.48 ± 0.79 ^a
Extract dose (mg/kg body weight)								
50	Oral	19.02 ± 1.30 ^A	11.16 ± 8.28	11.64 ± 7.53	11.10 ± 6.22	8.82 ± 4.04	8.60 ± 4.08	9.68 ± 4.18
	IP	14.30 ± 3.05 ^{Aa}	8.26 ± 1.50	7.40 ± 1.94	7.72 ± 1.85	4.88 ± 0.63	5.00 ± 1.05	8.84 ± 3.49
100	Oral	17.94 ± 1.44 ^{Aa}	9.76 ± 4.48	10.18 ± 3.81	10.34 ± 1.99	8.96 ± 1.94	9.06 ± 2.90	11.98 ± 4.80
	IP	19.58 ± 2.12 ^{Aa}	8.44 ± 1.62	7.86 ± 1.23	7.58 ± 1.90	5.36 ± 1.15	5.32 ± 1.14	7.00 ± 1.90
200	Oral	19.50 ± 0.93 ^{Ab}	19.68 ± 3.22 ^{Cb}	15.58 ± 3.76 ^{Aa}	15.86 ± 5.05 ^{Aa}	10.50 ± 1.79 ^A	8.58 ± 1.15	14.00 ± 4.75 ^A
	IP	19.62 ± 5.58 ^{Ac}	15.60 ± 5.44 ^{Ab}	12.96 ± 3.38 ^{Aa}	9.02 ± 1.36 ^A	6.42 ± 0.78	5.58 ± 0.68	10.42 ± 0.90 ^A
300	Oral	19.34 ± 1.52 ^{Ac}	16.60 ± 3.04 ^{Ab}	12.44 ± 3.00 ^a	9.84 ± 0.79	7.84 ± 3.25	5.72 ± 2.26	8.86 ± 2.45
	IP	14.28 ± 5.28 ^{Aa}	10.40 ± 4.01	9.10 ± 3.03	8.38 ± 2.42 ^A	6.88 ± 1.61	5.90 ± 1.08	6.80 ± 1.57

Values are expressed as Means ± SD for five animals per group. Means within respective columns followed by similar upper case letters are not significantly different at $p \leq 0.05$ by ANOVA and post ANOVA; means within respective rows followed by similar lower case letters are not significantly different at $p \leq 0.05$ by ANOVA and post ANOVA; and post ANOVA.

Table 1: Effect of oral and intraperitoneal administration of *Kleinia squarrosa* stem bark extracts on blood glucose levels in alloxan induced diabetic mice.

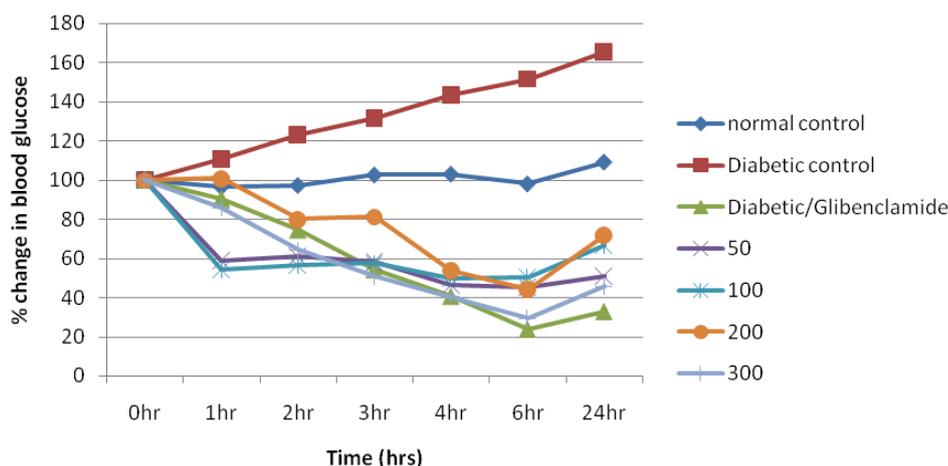


Figure 1: The mean percentage change in blood glucose levels of aqueous stem bark extract of *Kleinia squarrosa* administered orally in alloxan induced diabetic mice.

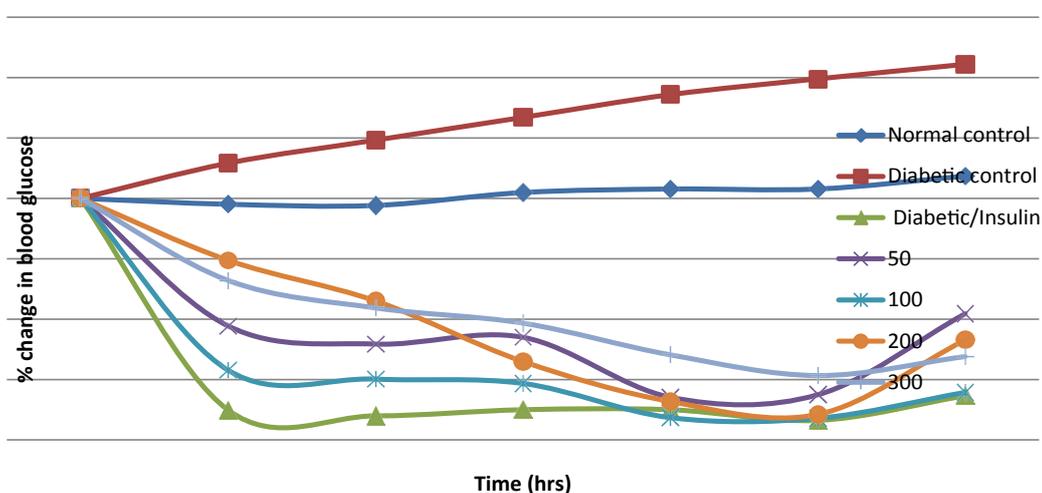


Figure 2: The mean percentage change in blood glucose levels of aqueous stem bark extract of *Kleinia squarrosa* administered intraperitoneally in alloxan induced diabetic mice.

Kleinia squarrosa also lowered blood glucose levels from the 1st hour to the 6th hour (Table 1 and Figure 2). By the 1st hour the four therapeutic doses of the extract had lowered the blood glucose levels by 42.24%, 56.89%, 20.49%, and 27.17%, respectively, compared to insulin which had lowered blood sugar levels by 70.09% within the same hour. In the 6th hour the percent blood glucose reductions by the four therapeutic doses of the extract were 65.03%, 72.83%, 71.56% and 58.68%, respectively, compared to insulin which had lowered blood sugar levels by 73.52% within the same hour. After this, a gradual increase was recorded up to the twenty fourth hour.

Effect of oral and intraperitoneal administration of 1 g/kg body weight of aqueous stem bark extracts of *Kleinia squarrosa* on body and organ weights in mice

Results are shown in Table 2. Oral and intraperitoneal administration of aqueous stem bark extracts of *Kleinia squarrosa* at 1 g/kg body weight to mice for one month significantly decreased the weekly body weight gain relative to that of the normal control mice (Table 2). Oral administration of 1 g/kg body weight of aqueous stem bark extracts of *Kleinia squarrosa* to mice for one month significantly decreased the percent organ to body weight of liver but did not significantly alter the percent organ to body weight of the brain, kidney, lungs, spleen, heart and testes relative to those of the normal control mice (Table 2). In addition, administration of the same intraperitoneal dose of aqueous stem bark extracts of *Kleinia squarrosa* to mice significantly increased the percent organ to body weight of liver and lungs while not altering the percent organ to body weight of the brain, kidney, spleen, heart, and testes relative to those of the normal control mice (Table 2).

Effect of oral and intraperitoneal administration of 1 g/kg body weight of aqueous stem bark extracts of *Kleinia squarrosa* on hematological parameters in mice

Table 3 shows the effect of oral and intraperitoneal administration of the extract for one month on some hematological parameters in mice. Oral administration of 1 g/kg body weight of *Kleinia squarrosa* aqueous stem bark extracts to mice for one month significantly decreased the level of RBC but did not significantly change the levels of Hb, PCV, MCV, MCH, MCHC, and PLT relative to those of the normal control mice (Table 3). In addition, administration of the same intraperitoneal dose of *Kleinia squarrosa* aqueous stem bark extract to mice significantly decreased the levels of RBC, Hb, PCV, and PLT and increased the levels of MCH and MCHC but did not significantly affect the level of MCV relative to those of the normal control mice (Table 3).

Effect of oral and intraperitoneal administration of 1 g/kg body weight of aqueous stem bark extracts of *Kleinia squarrosa* on white blood cell count in mice

Table 4 shows the effect of oral and intraperitoneal administration of the extract at 1 g/kg body weight to mice for one month on differential white blood cell count. Oral administration of the aqueous stem bark extract of *Kleinia squarrosa* at 1 g/kg body weight to mice for one month did not significantly change the differential white blood cell counts (Table 4). In addition, administration of the same intraperitoneal dose of stem bark extract of *Kleinia squarrosa* to mice caused a significant decrease in the levels of WBC, neutrophils, lymphocytes, eosinophils, and monocytes without significantly altering the level of basophils relative to those of the normal control mice (Table 4).

Treatment	Weekly body weight change (g) and Percent organ to body weight (%)							
	Δweight/week	Liver	Brain	Kidney	Lungs	Spleen	Heart	Testes
Control Oral	1.705 ± 0.522	7.27 ± 1.12	2.74 ± 0.27	1.78 ± 0.24	1.79 ± 0.21	1.12 ± 0.32	0.53 ± 0.09	0.93 ± 0.16
<i>Kleinia squarrosa</i> Oral	-0.125 ± 0.265*	5.18 ± 1.49*	3.22 ± 0.52	1.88 ± 0.22	1.92 ± 0.36	1.28 ± 0.29	0.57 ± 0.11	0.98 ± 0.22
Control IP	2.155 ± 0.089	5.01 ± 1.26	1.75 ± 0.31	1.39 ± 0.42	1.38 ± 0.54	0.61 ± 0.13	0.51 ± 0.21	0.91 ± 0.22
<i>Kleinia squarrosa</i> IP	0.090 ± 0.134*	7.75 ± 1.37*	1.74 ± 0.78	2.39 ± 1.08	2.45 ± 0.43*	0.75 ± 0.27	0.78 ± 0.18	0.77 ± 0.13

Results are expressed as Mean ± Standard Deviation (SD) for five animals for each parameter; *p<0.05 is considered statistically significant when the mean of the experiment group is compared to its relevant control group by T-Test.

Table 2: The effects of oral and intraperitoneal administration of aqueous stem bark extract of *Kleinia squarrosa* at 1 g/kg body weight on body and organ weights in mice.

Treatment	Hematological parameters						
	RBC (×10 ⁶ /μL)	Hb (g/dL)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	PLT (×10 ³ /μL)
Control Oral	7.61 ± 0.74	9.58 ± 0.73	32.74 ± 2.49	43.12 ± 2.84	12.64 ± 0.96	29.30 ± 0.54	607.40 ± 116.07
<i>Kleinia squarrosa</i> Oral	6.32 ± 0.88*	8.04 ± 1.38	28.22 ± 4.30	44.14 ± 1.38	12.66 ± 0.42	28.62 ± 0.58	482.40 ± 43.62
Control IP	6.38 ± 0.67	8.98 ± 0.80	28.66 ± 2.64	45.02 ± 1.44	14.10 ± 0.51	31.36 ± 0.66	352.80 ± 73.32
<i>Kleinia squarrosa</i> IP	1.91 ± 0.15*	3.64 ± 0.11*	8.96 ± 0.35*	46.50 ± 0.96	18.88 ± 0.34*	40.42 ± 1.28*	22.20 ± 0.88*

Results are expressed as Mean ± standard deviation (SD) for five animals in each treatment; *p<0.05 is considered significant when the mean of the control animals is significantly different from that of the extract treated animals by T-Test.

Table 3: The effects of oral and intraperitoneal administration of 1g/kg body weight of aqueous stem bark extracts of *Kleinia Squarrosa* on hematological parameters in mice.

Treatment	White blood cell and differential white blood cell count					
	WBC (×10 ³ /μL)	Neutrophils (×10 ³ /μL)	Lymphocytes (×10 ³ /μL)	Eosinophils (×10 ³ /μL)	Monocytes (×10 ³ /μL)	Basophils (×10 ³ /μL)
Control Oral	14.34 ± 3.48	4.46 ± 1.26	7.27 ± 1.68	1.35 ± 0.41	1.15 ± 0.21	0.09 ± 0.08
<i>Kleinia squarrosa</i> Oral	11.76 ± 1.93	3.30 ± 0.65	6.36 ± 0.92	1.14 ± 0.12	0.90 ± 0.20	0.10 ± 0.12
Control IP	6.87 ± 0.71	2.18 ± 0.25	3.51 ± 0.33	0.59 ± 0.14	0.54 ± 0.13	0.04 ± 0.04
<i>Kleinia squarrosa</i> IP	3.58 ± 0.56*	1.01 ± 0.16*	1.92 ± 0.30*	0.34 ± 0.08*	0.29 ± 0.05*	0.03 ± 0.02

Results are expressed as Mean ± Standard Deviation (SD) for five animals in each treatment; *p<0.05 is considered significant when the mean of the control animals is significantly different from that of the extract treated animals by T-Test.

Table 4: The effects of oral and intraperitoneal administration of 1 g/kg body weight of aqueous stem bark extracts of *Kleinia Squarrosa* on white blood cell count (WBC) in mice.

Effects of oral and intraperitoneal administration of 1 g/kg body weight of aqueous stem bark extracts of *Kleinia squarrosa* on biochemical parameters in mice

Oral administration of 1 g/kg body weight of aqueous stem bark extracts of *Kleinia squarrosa* to mice for one month caused an increase in the levels of Creatinine and decreased the levels of AST, LDH, CK, and ALP but did not significantly alter the levels of Urea, ALT, GGT, AMY, T-BIL and D-BIL relative to that of the normal control mice (Tables 5 and 6). Intraperitoneal administration of the same dose of aqueous stem bark extract of *Kleinia squarrosa* to mice caused significant increase in the levels of Urea, ALT, AST, GGT, LDH, T-BIL, creatinine and D-BIL but did not significantly alter the levels of CK, AMY and ALP relative to that of the normal control mice (Tables 5 and 6).

Histopathology

Oral administration of 1 g/kg body weight of the aqueous stem bark extracts of *Kleinia squarrosa* did not cause any significant histopathological lesion on the liver, brain, kidney, lung, spleen, heart and testes when compared to those of the normal control mice. Intraperitoneal administration of same dose of the aqueous stem bark extracts of *Kleinia squarrosa* to mice caused significant lesions to the kidney, liver and spleen relative to those of the normal control mice (Plates 1-3).

The kidney parenchyma of the mice intraperitoneally treated with aqueous stem bark extract of *Kleinia squarrosa* at 1g/kg body weight demonstrated changed appearance in renal cells, with some cells staining pale compared to normal pink staining cells of the normal control mice (Plate 1). The renal parenchyma was disorganized with loss of structure. This reflects degeneration of the kidney tubular epithelial cells.

The liver of the mice intraperitoneally treated with aqueous stem bark extract of *Kleinia squarrosa* at 1 g/kg body weight demonstrated abnormalities such as hepatocytes without their normal columnar arrangement when compared to the liver cells of the normal control mice. There was individualization of hepatocytes and the cytoplasm was pale staining and many hepatocytes were clumped together (Plate 2).

The spleen of the mice intraperitoneally treated with aqueous stem bark extract of *Kleinia squarrosa* at 1 g/kg body weight demonstrated depopulation of the lymphoid follicles when compared to the spleen of the normal control mice (Plate 3).

Quantitative analysis of the phytochemical composition of aqueous stem bark extracts of *Kleinia Squarrosa*

The phytochemical screening of the aqueous stem bark extracts of *Kleinia squarrosa* indicated the presence of Alkaloids, Saponins, Total Phenols, Flavonoids and Tannins. The quantity of each of these phytochemicals per gram of the extract is shown in Table 7.

Mineral elements analysis

Table 8 shows the mineral composition of the aqueous stem bark extracts of *Kleinia squarrosa*. Sodium (Na), chlorine (Cl), potassium (K), Calcium (Ca), Titanium (Ti), Vanadium (V), chromium (Cr), manganese (Mn), iron (Fe), Copper (Cu), Zinc (Zn), Arsenic (As), Cadmium (Cd) Magnesium (Mg), Nickel (Ni), and Lead (Pb) were present in the aqueous extract of the studied plant. The levels of these measured minerals and trace element were all below the recommended daily allowance.

Discussion

This study was carried out to investigate the *in-vivo* antidiabetic effect and safety of the aqueous stem bark extracts of *Kleinia Squarrosa* in alloxan induced diabetic mice and normal mice respectively. The alloxan-induced diabetic mice had 3 to 4 times increase in blood glucose levels compared to normal control group. Both oral and intraperitoneal administration of the aqueous extract of the studied plant showed hypoglycemic activity at the four tested dose levels (50 mg/kg body weight 100 mg/kg body weight, 200 mg/kg body weight, and 300 mg/kg body weight). However, intraperitoneal administration was more effective in lowering the blood glucose levels than the oral route. The hypoglycemic activity the aqueous stem bark extracts of *Kleinia squarrosa* confirmed the findings made by [14] who reported antidiabetic activity of the aqueous extracts of the same plant collected from Embu County, Kenya using the intraperitoneal route at doses of 50-150mg/kg body weight.

This hypoglycemic activity could be due to the presence of alkaloids,

Treatment	Enzyme Activities						
	ALT (U/L)	AST (U/L)	GGT (U/L)	LDH (U/L)	CK (U/L)	AMY (U/L)	ALP (U/L)
Control Oral	132.6 ± 20.6	692.3 ± 51.4	1.8 ± 0.2	1972.9 ± 158.7	953.4 ± 74.7	2940.2 ± 174.7	103.2 ± 9.1
<i>Kleinia squarrosa</i> Oral	132.7 ± 14.1	493.5 ± 77.0*	2.0 ± 0.4	1576.3 ± 189.7*	728.5 ± 98.2*	2664.4 ± 216.5	84.8 ± 9.5*
Control IP	80.3 ± 7.0	523.2 ± 94.7	2.0 ± 1.0	2137.2 ± 159.4	351.0 ± 59.1	1676.4 ± 230.2	46.6 ± 10.4
<i>Kleinia squarrosa</i> IP	367.0 ± 31.3*	1030.2 ± 70.1*	8.6 ± 1.1*	5397.6 ± 161.1*	436.4 ± 40.1	1443.0 ± 106.8	49.6 ± 3.9

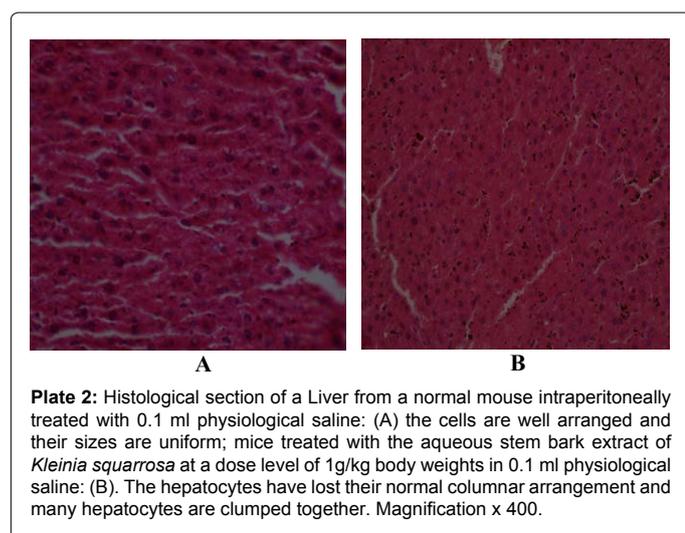
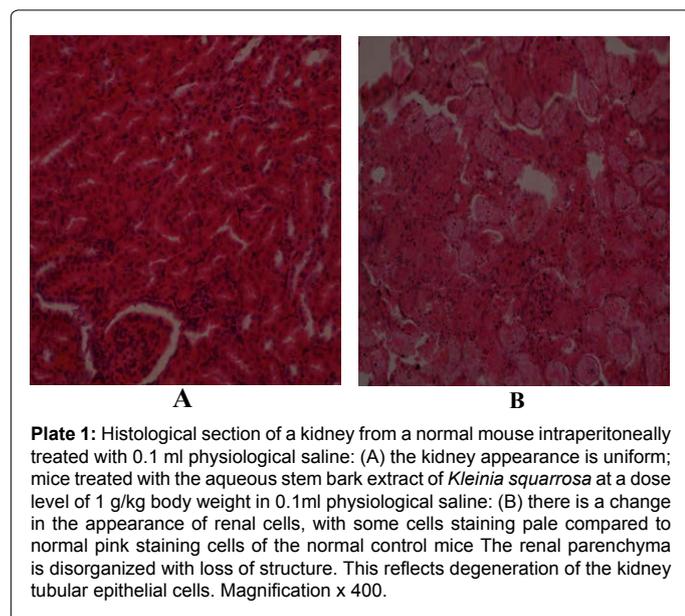
Results are expressed as Mean ± Standard Deviation (SD) for five animals in each treatment; *p<0.05 is considered significant when the mean of the control animals is significantly different from that of the extract treated animals by T-Test.

Table 5: The effects of oral and intraperitoneal administration of 1g/kg body weight of aqueous stem bark extracts of *Kleinia Squarrosa* on organ functions in mice.

Treatment	Metabolite Levels			
	UREA (mmole/L)	CREAT (µmole/L)	T-BIL (µmole/L)	D-BIL (µmole/L)
Control Oral	9.6 ± 0.7	75.0 ± 8.5	0.8 ± 0.1	0.4 ± 0.1
<i>Kleinia squarrosa</i> Oral	10.1 ± 0.9	93.9 ± 11.6*	0.9 ± 0.2	0.3 ± 0.1
Control IP	7.2 ± 0.8	43.2 ± 6.1	4.5 ± 1.2	2.6 ± 0.7
<i>Kleinia squarrosa</i> IP	22.4 ± 0.9*	62.8 ± 3.3*	18.2 ± 1.9*	9.7 ± 1.1*

Results are expressed as Mean ± Standard Deviation (SD) for five animals in each treatment; *p<0.05 is considered significant when the mean of the control animals is significantly different from that of the extract treated animals by T-Test.

Table 6: The effects of oral and intraperitoneal administration of 1g/kg body weight of aqueous stem bark extracts of *Kleinia Squarrosa* on the levels of selected metabolites in mice.



saponins, tannins and total phenols in the plant extract (Table 7). Saponin fraction isolated from *Momordica charantia* reduced blood glucose levels and increased insulin secretion and glycogen synthesis in alloxan induced diabetic mice [29]. As reported by [30] saponins were shown to reduce serum glucose levels in elderly diabetic patients. Total saponins from the seeds of *Entada phaseoloides* significantly decreased fasted blood glucose and attenuated hyperglycemia associated oxidative stress in type 2 diabetic rats [31].

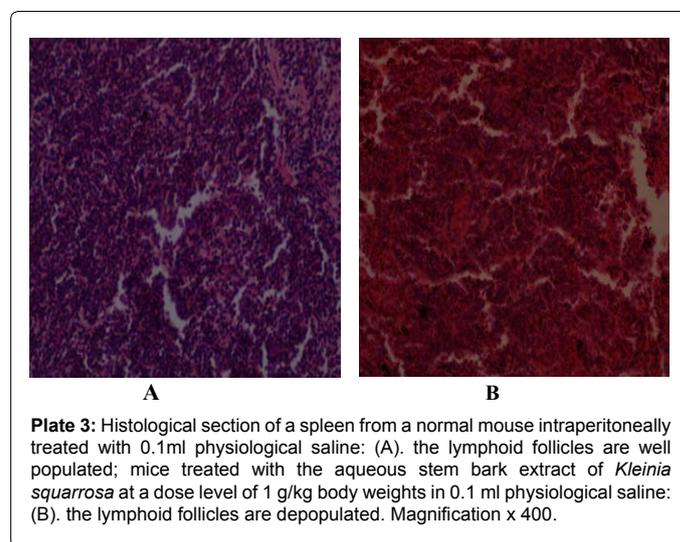
The presence of alkaloids in this extract could also be responsible for the hypoglycemic activity. Intraperitoneally administered alkaloids isolated from leaves of *Acanthus montanus* at doses of 100, 200 and 400 mg/kg body weight showed hypoglycemic action in alloxan-induced diabetic rats [32].

Four indole Alkaloids (vindoline I, vindolidine II, vindolicine III, and vindolinine IV) isolated from the leaves of *Catharanthus roseus* improved glucose uptake in pancreatic (β -TC6) and muscle (C2C12) cells. In addition these alkaloids inhibited protein tyrosine phosphatase PTP-1B which is a down regulator in the insulin signaling pathway

[33]. Alkaloids from *Ephedra distachya* herbs induced increased insulin secretion by causing regeneration and restoration of atrophied pancreatic islets [34].

Tannins are classified into two major groups; condensed tannins and hydrolysable tannins. The hydrolysable tannins are further divided into two groups; Tannic acid (gallotannins) and ellagitannins [35]. Condensed tannins extracted from some kenyan foods showed antihyperglycemic action due to inhibition of α -amylase and α -glucosidase enzymes [36]. Commercially available tannic acids induced phosphorylation of the insulin receptor (IR) and act, as well as translocation of glucose transporter 4 (GLUT 4), the protein factors involved in the signaling pathway of insulin-mediated glucose transport [35].

Commercially available four flavonoids: Boswellic acid, Ellagic acid, Quercetin, Rutin given orally at dose levels of 50 mg/kg & 100 mg/kg body weight decreased blood glucose levels in Normoglycemic and STZ-nicotinamide induced diabetic rats [37]. Flavonoids act on various molecular targets and regulate different signaling pathways in pancreatic β -cells, hepatocytes, adipocytes and skeletal myofibers [38]. Flavonoids were reported to act as insulin secretagogues or insulin mimetics, stimulate glucose uptake in peripheral tissues, and regulate the activity and/or expression of the rate-limiting enzymes involved in carbohydrate metabolism pathways [39]. For example The genistein derivatives isolated from *Tetracera scandens* possessed promising activities on Type-2 diabetes mellitus treatment by significantly stimulating glucose uptake, adenosine monophosphate-activated kinase (AMPK), glucose transport protein-4 (GLUT4) and GLUT1 mRNA expressions and protein tyrosine phosphatase 1B (PTP1B) inhibition in L6 myotubes [40]. Flavonoid fraction from *Pterocarpus*



Phytochemicals Present	Quantities (mg/g)
Tannins	0.6975 ± 0.094
Total Phenols	1.3823 ± 0.292
Flavonoids	4.5998 ± 0.339
Saponins	45.067 ± 3.489
Alkaloids	93.260 ± 18.074

Results are expressed as Mean ± Standard Deviation (SD). Tannins and Total Phenols are expressed as mg/g gallic acid equivalent (GAE), flavonoids are expressed as mg/g quercetin equivalent (QE), alkaloids and saponins are in mg/g

Table 7: Quantitative analysis of the phytochemical composition of aqueous stem bark extracts of *Kleinia Squarrosa*.

Element	<i>Kleinia squarrosa</i> (µg/g)	<i>Kleinia squarrosa</i> (µg)	RDA for mice (µg/day)*
Na*	2263.6 ± 119.6	56.59	5 × 10 ⁵ (178.6)
Mg*	212.2 ± 36.9	5.305	4.2 × 10 ⁵ (150)
Cl*	81.8 ± 1.2	2.045	7.5 × 10 ⁵ (267.9)
K*	695.7 ± 6.3	17.3925	3.5 × 10 ⁶ (1250)
Ca*	177.3 ± 1.8	4.4325	1.0 × 10 ⁶ (357.1)
Ti	3.06 ± 0.08	0.0765	
V	0.73 ± 0.04	0.01825	< 1.8 × 10 ³ < (0.64)
Cr*	0.009 ± 0.003	0.000225	3.5 × 10 (12.5)
Mn*	2.26 ± 0.05	0.0565	2.3 × 10 ³ (0.82)
Fe*	10.18 ± 0.12	0.2545	8.0 × 10 ³ (2.9)
Ni	0.27 ± 0.01	0.00675	< 1 × 10 ³ < (0.36)
Cu*	0.22 ± 0.01	0.0055	1.5 × 10 ³ (0.54)
Zn*	0.92 ± 0.02	0.023	1.1 × 10 ⁴ (3.9)
As	0.02 ± 0.01	0.0005	
Se	<0.030	<0.00075	3.5 × 10 (0.0125)
Hg	<0.050	<0.00125	
Pb	0.03 ± 0.01	0.00075	
Cd	4 ± 0.5	0.1	

Results on the concentration of each mineral are expressed as µg/g of dry powder of the aqueous stem bark extracts of *Kleinia Squarrosa* of three determinations and are in the second column; the amount of each mineral administered in µg based on its concentration in the plant's extract is in the third column. This is compared with the recommended daily allowance shown in the last column. This is expressed per the average weight of each mouse. *Recommended daily allowance estimated from that of human beings stated in Strain and Cashman (2009) [63].

Table 8: Mineral levels and amount given to each mouse from the aqueous stem bark extracts of *Kleinia Squarrosa*.

marsupium has been shown to cause pancreatic beta cell regranulation. Epicatechin, its active principle, has been found to be insulinogenic thus enhancing insulin release and conversion of proinsulin to insulin *in vitro* [41].

In addition to phytochemical components, the anti-diabetic action of the plant extract could result from the presence of mineral and trace elements like but not limited to: zinc (Zn), Chromium (Cr), manganese (Mn), selenium (Se), Potassium (K), Vanadium (V) and Calcium. These elements possess anti-diabetic activity. Zinc plays significant role in insulin metabolism: synthesis, secretion and utilization [42]. It was demonstrated that zinc has a protective role against β-cell destruction by viral infection. In Type 1 diabetics zinc supplements have been shown to decrease blood glucose levels [43]. The trivalent Cr is required in the maintenance of normal glucose metabolism [44]. It potentiates the insulin action by increasing the number of insulin receptors and enhancing receptor binding [42,44].

A deficiency of manganese is common amongst diabetics [43]. Experimental studies on guinea pigs demonstrated that manganese supplementation corrected impaired glucose tolerance initially induced by manganese deficiency [45]. Selenium possesses insulin-like activity such as promoting glucose uptake and regulating metabolic pathways

like glycolysis, pentose phosphate pathway, gluconeogenesis and fatty acid synthesis. Although the mechanism is not well understood, but reports showed that selenium activates several key proteins involved in the insulin signaling pathways [46].

Hypokalemia decreases the pancreatic capacity to secrete insulin, diminished β-cell response to glucose and hence caused glucose intolerance [47]. Potassium supplementation improves insulin sensitivity, responsiveness and secretion [48,49].

Vanadium acts as an insulin mimetic agent due to the structural similarity to phosphorus. It may act as a phosphate analog and affect several points in the insulin signaling pathway including but not limited to; insulin receptor auto-phosphorylation, increased protein tyrosine and serine threonine kinase activity, inhibition of phosphotyrosine phosphatase activity, inhibition of hepatic gluconeogenesis, and increased glycogen synthesis [44]. In animal models, vanadium has been reported to enhance insulin sensitivity and increase glucose uptake [50,51]. Calcium improves insulin sensitivity in some type 2 diabetic populations [52].

The oral and intraperitoneal administration of the aqueous stem bark extracts of *Kleinia squarrosa* causes a decrease in growth rate. This decrease in growth rate may have been caused by the presence of alkaloids, saponins, flavanoids, and tannins. Synephrines and p-octopamine (Alkaloids) decrease body weight through adrenergic agonist activity. Synephrines increases resting energy expenditure, thermal effect of feeding, and energy expenditure of physical activity by 70%, 10% and 20% respectively and decrease food intake and gastric motility [53]. Nicotine another alkaloid alters feeding patterns and reduces appetite and causes weight loss by modulating several pathways that regulate the aspects of food intake in the central nervous system. Cathinone (monoamine alkaloid) affect appetite by acting on the hypothalamus and enhances sympathomimetic activity leading to a delay in gastric emptying [53].

Flavonoids decrease body weight by reducing glucose absorption (energy source) leading to an increasing oxidation of the fat reserves. For example Catechins (flavanoids) are associated with inhibition of small-intestine micelle formation and the inhibition of α-glucosidase activity which would lead to a decrease in carbohydrate absorption. There are reports that have shown that catechins increase sympathetic nervous system activity, thermogenesis, and fat oxidation and hence accounting for the reduced body weight [53].

Tannins may reduce feed intake by decreasing palatability and by reducing feed digestion. Palatability is reduced because tannins are astringent. Astringency is the sensation caused by the formation of complexes between tannins and salivary glycoproteins. Low palatability depresses feed intake. Digestibility reduction negatively influences intake because of the filling effect associated with undigested feedstuff [54]. Hydrolysable tannins are converted by microbial metabolism and gastric digestion into absorbable low molecular weight metabolites such as tannic acid which are toxic. The major lesions associated with hydrolysable tannins poisoning are hemorrhagic gastroenteritis which decreases absorption of nutrients and necrosis of the liver and kidney [54].

Proanthocyanidins (PAs) (condensed tannins) which are not absorbed by the digestive tract, damage the mucosa of the gastrointestinal tract, decreasing the absorption of nutrients such as proteins, carbohydrates and essential amino acids such as methionine and lysine. They also increase excretion of proteins and essential amino acids and alter the excretion of certain cations [55].

The reduced growth rates of the experimental mice administered with 1 g/kg body weight of the extract relative to the control mice correlates with the reduced percent organ to body weight of liver (orally administered). Normalization of organ weight to body weight helps eliminate variations due to body weight differences, as some apparent difference in organ weight might have been the result of differences in body weight which are unrelated to the treatment [56]. The increased percent organ to body weight of liver and Lungs of mice intraperitoneally administered with the aqueous stem bark extracts of *Kleinia squarrosa* at 1 g/kg body weight daily for one month could not be explained in this study. It is possible that the extract promoted higher metabolic activity in these organs.

The investigated hematological parameters in this study are useful in the assessment of the toxic potentials of the plant extracts in mammals and human beings. They provide information about the status of bone marrow activity and hemolysis [57]. The dramatic reduction of red blood cell count, packed cell volume and hemoglobin concentration observed in mice administration with aqueous stem bark extracts of *K squarrosa* at 1 g/kg body weight relative to the control mice indicates induction of anemia by this extract. This anemic state leads to tissue hypoxia. These abnormal blood conditions could be caused by toxic constituents in the plant extract including among others total phenols, alkaloids, saponins, flavanoids, and tannins present in this plant extract. Saponins hemolyse and cause cell death in many tissues [58,59]. Alkaloids have been shown to cause liver megalocytosis, proliferation of biliary tract epithelium, liver cirrhosis and nodular hyperplasia [60]. These extract constituents could be reducing the erythron parameters [61]. This toxicity may not have been due to the presence of trace elements/minerals since the amounts administered into each mouse daily at a dose of 1 g/kg body weight were below the recommended daily allowance.

Tissue hypoxia causes most tissues to initially enlarge and as the swollen cells continue rupturing, the organ size reduces (organ atrophy) [62]. During tissue hypoxia, cells which rely only on glycolysis for ATP production rapidly deplete the store of phosphocreatine (a source of rapid ATP production) and glycogen. As the rate of ATP production decreases below the level required by membrane ion pumps for the maintenance of proper intracellular ionic concentrations, the osmotic balance of the cell is disrupted so that the cell and its membrane enveloped organelles swell. The overstretched membrane becomes permeable thereby leaking their enclosed contents. The decreased intracellular pH that accompanies anaerobic glycolysis because of lactic acid production permits the released lysosomal enzymes which are only active at acidic pH to degrade the cell contents. Among the degraded components are the initially elevated serum enzymes which are later reduced to values below the control values. The reduced metabolic activity results in irreversible cell damage [62,63].

Injury of organs resulting from tissue hypoxia was demonstrated by the enlargement of the liver; histologically it was demonstrated by observations that the spleen, liver, and kidney of mice intraperitoneally treated with aqueous extracts of *K squarrosa* at 1 g/kg body weight daily for one month demonstrated lymphoid follicles depopulation, and loss kidney and liver architecture; and biochemically it was demonstrated by increased levels of serum alanine aminotransferase (liver), aspartate aminotransferase (liver, kidneys, heart and pancreas), γ -glutamyltransferase (liver), lactate dehydrogenase (liver, kidney and heart), urea, creatinine, total bilirubin and direct bilirubin.

The decreased levels of platelet count (thrombocytopenia) in mice intraperitoneally administered with 1g of aqueous extract of *K*

squarrosa per kg body weight daily for 28 days may either be associated with (the immune system that causes antibodies produced by the spleen to kill platelets) bone marrow injury caused by toxic phytochemical substances in the extracts.

The decreased levels of white blood cell count and decreased neutrophil, lymphocytes, eosinophils and monocytes observed in mice intraperitoneally administered with 1g of aqueous stem bark extracts of *K squarrosa* per kg body weight daily for 28 days indicate a reduced ability of the body to respond to infection either due to liver or spleen or bone marrow injury caused by toxic phytochemicals contained in this extract

The observation that orally administered aqueous extracts of *K. squarrosa* at 1 g/kg body weight in mice for 28 days caused no histopathological abnormalities compared to the same dose administered intraperitoneally; confirms the known fact of the less toxic effects of drugs administered orally due to poor absorption, protein binding or metabolism in the gastrointestinal tract.

Conclusion

The aqueous stem bark extracts of *Kleinia squarrosa* had antidiabetic activity. The intraperitoneal route was more effective in reducing the blood glucose levels than the oral route. The aqueous extract of the studied plant at high dose of 1 g/kg body weights which is far from the therapeutic dose tends to cause toxicological effects. This was well demonstrated in the body and organ weight changes, hematological, and biochemical parameters together with the histopathological analysis. In the toxicological studies the oral administration of a dose (1 g/kg body weight) was found to have less toxic effects than the intraperitoneal administration of the same dose. This explains why the oral route is the most preferred route by the traditional health practitioners. The antidiabetic and toxic action of the studied plants may have resulted from its phytochemical and mineral constituents.

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