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Investigation of the *in vitro* antioxidant activity, *in vivo* antidiabetic efficacy and safety of *Capparis tomentosa* aqueous roots extracts in male alloxanized mice

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Abstract

The current study investigated the *in vitro* antioxidant activity, *in vivo* antidiabetic efficacy and safety of *Capparis tomentosa* aqueous root extracts. Antioxidant activity was determined using 1, 1-diphenyl-2-picrylhydrazyl (DPPH), phosphomolybdate and reducing power assay with ascorbic and gallic acid as references. Six groups of BALB/c mice each comprising of five were used in evaluating the antidiabetic activity. Diabetes mellitus was induced in five groups using 10% alloxan monohydrate at a dose of 186.9 mg/kg body weight. Non-diabetic control mice was orally administered with 0.1 ml physiological saline; diabetic mice with 0.075 mg of reference drug, glibenclamide at 3 mg/kg body weight; 1.25 mg, 2.5 mg, and 5 mg extracts in 0.1 ml physiological saline for 50, 100 and 200 mg/kg body weight, and the other group of diabetic mice was given 0.1 ml physiological saline. The blood glucose level was determined after 0, 2, 4, 6 and 8 hours. Safety was evaluated by daily administration of a single dose of 1000 mg/kg body weight extract to BALB/c male mice of comparable age and weight over a period of one month, while recording body weights every 7 days and organs weights after the 28th day. The antioxidant activity by DPPH was $35.50 \pm 0.02\%$, by phosphomolybdate assay was 41.22 ± 0.17 mg/kg ascorbic acid equivalent, and the reducing power increased with increase in concentration up to a maximum at 800 $\mu\text{g/ml}$. The antidiabetic activity was dose dependent and significantly higher. There was no significant change in body weights for treated and untreated mice in safety studies ($p = 0.69$), and the weight gain was normal for both experimental and control mice. Except kidneys, which changed significantly ($p = 0.009$), all the other organ weights were not affected. The study supports the claim that *C. tomentosa* is effective and safe in the management of diabetes mellitus.

Keywords: Antidiabetic, BALB/c mice, *Capparis tomentosa*, Safety, Efficacy

1. Introduction

Diabetes mellitus is a chronic metabolic disorder caused by inherited and/or acquired deficiency in production of insulin by the pancreas, or by the ineffectiveness of the insulin produced. This deficiency in insulin production results to increase in the concentration of blood glucose, which in turn causes damage to many of the body's systems particularly the blood vessels and nerves^[1]. Diabetes mellitus is a major health problem, affecting about 5% of the total population in the U.S and 3% of the world population. Epidemiological studies^[2] and clinical trials^[3], strongly support the premise that most of the complications are caused by hyperglycemia. Effective blood glucose control is the critical intervention measure in the management of diabetic complications and improving quality of life^[4]. Thus, sustained reduction of hyperglycemia lowers the risk of developing microvascular complications and reduces the risk of macrovascular complications^[5].

Capparis tomentosa belongs to the family Capparaceae commonly referred to as Woody Caper Bush in English^[6]. It is an indigenous South African plant that grows naturally in the savanna forest of Western, Eastern and Southern Africa^[7]. It is a scrambling shrub, sometimes maturing into a tree that can grow as high as 10 meters tall and is covered with scattered spines. It is well branched and the branches are normally covered with thick yellow hairs; even the robust, recurved spines are often hairy. The twigs and leaves are yellow-green in colour and are covered in soft, velvety hairs. The oblong leaves are approximately 50×20 mm, with

a pair of sharp, hook-like thorns at the junction of the stem and leaf base. The white and pink flowers have multiple stamens. The fruit is pink to orange in colour, round and stalked. The seeds are surrounded by fleshy, grey fruit pulp [8, 6].

In herbal and traditional medicine, *C. tomentosa* is used to treat rheumatism, madness, snakebite, chest pain, jaundice, malaria, headache, coughs, pneumonia, constipation, infertility and to prevent abortions. It is used to treat leprosy, tuberculosis and gonorrhea [1]. The roots are boiled in water and half a cup of this infusion is drunk three times per day to manage cough and chest pain [7, 9]. Despite the continued use of *C. tomentosa* in management and treatment of many ailments among many communities, it has been reported to be toxic to livestock [8, 10]. Data on its *in vitro* antioxidant activity, antidiabetic efficacy and safety in Kenya is scarce. Therefore, this study investigated the *in vitro* antioxidant activity, *in vivo* antidiabetic efficacy and safety of *C. tomentosa* in alloxan induced diabetic male BALB/c mice, to validate the folkloric claim among the herbalists and local communities.

2. Materials and Methods

2.1 Preparation of the aqueous root extracts

Fresh roots of *C. tomentosa* were collected from West Pokot and Marakwet parts of the North Rift Valley of Kenya in the month of December 2012. The plant is likened to ‘the biblical hyssop’ by Mr. Ndura Boniface, who has been using it for over six years in treatment and management of diabetes mellitus, HIV/AIDS, reproductive problems and cancer among many other diseases (Unpublished, personal communications). The fresh roots were washed with clean tap water, cut into small pieces and dried under shade at room temperature for 4 weeks. They were then milled into fine powder using electric mill, and the powder kept at room temperature away from direct sunlight in dry airtight plastic bags. 100 g of the root powder was extracted in 1 liter of distilled water at 60 °C in a metabolic shaker for 6 hours. The extract was decanted into a clean, dry conical flask and then filtered through cotton gauze into another dry clean conical flask. The filtrate was freeze dried in 200 ml portions in a Modulyo Freeze Dryer (Edward, England) for 48 hours. These extracts were weighed and stored in airtight, amber containers at the 4 °C ready for use.

The extracts for *in vivo* antidiabetic studies were prepared according to the protocol described elsewhere by Karau *et al* [12], with slight modifications. Briefly, 125 mg to make an oral dose of 50; 250 mg to make 100, and 500 mg to make 200 mg/kg body weight, respectively, were each dissolved in 10 ml of physiological saline, mixed and filtered using 0.45 µm membrane. Glibenclamide, a sulfonyl urea conventionally used in managing diabetes mellitus was prepared by dissolving 7.5 mg to make a dose of 3 mg/kg body weight was dissolved in 10 ml of physiological saline. To every experimental mice, 0.1 ml of the plant extracts and reference drug solution was orally administered to each mice according to the experimental group in the design.

2.2 Free radical scavenging activity by DPPH assay

The free radical scavenging activity of the root powder was determined by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) method [12]. In this method, a stock solution was prepared by dissolving 2.4 mg of DPPH free radical in 100 mL of methanol. The solution was kept at 20 °C until required. The working solution was prepared by diluting DPPH stock solution with methanol till the absorbance was 0.980 ± 0.02 at 517 nm. Then, 3 ml of the working solution was mixed with 100 µl of a methanol extract (1 mg/mL). After incubating the mixture in the dark for 30 min, absorbance was read at 517

nm. The blank contained all reagents except the roots extract. Ascorbic acid at a concentration of 1 mg/ml was used as reference. The scavenging activity was calculated by using the formula shown in equation 1.

Percent scavenging activity =

$$\text{Percent scavenging activity} = \frac{(\text{Absorbance of the blank} - \text{Absorbance of the sample})}{\text{Absorbance of the blank}} \times 100\%$$

Equation 1

2.3 Total antioxidant activity by phosphomolybdate assay

This was carried out according to the procedure described elsewhere [13]. The phosphomolybdate reagent was prepared by mixing equal volumes of 100 ml each of 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. Test samples were prepared by dissolving 1 mg of plant methanolic extract in 1 ml of methanol. Then, 0.1 ml of the test sample was dissolved in 1 ml of reagent solution in a test tube which was capped with silver foil and incubated in a water bath for 90 min at 95 °C. After cooling the sample to room temperature, the absorbance was read at 765 nm against a blank. Ascorbic acid was used as a standard antioxidant with a concentration ranging from 10 to 50 mg/L. The ascorbic acid absorbances were used in the construction of the standard curve. The results were expressed as µg of ascorbic acid equivalent (AAE) per mg of the dried weight of the root extracts of *C. tomentosa*. The AAE was determined according to the expression in equation 2.

$$\text{Ascorbic acid equivalent} = \frac{\text{Absorbance at } 765\text{nm}}{0.0034} \text{ (}\mu\text{g/mg of dried matter).}$$

Equation 2

2.4 Reducing power assay

The reducing power assay was carried out by the method of [14] with some modifications. The root extract or gallic acid solution ranging from 25 to 800 µg/ml, each 2.5 ml was mixed with 2.5 ml of 0.2 M sodium phosphate buffer and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min and 2.5 ml of trichloroacetic acid solution (100 mg/L) was added. The mixture was centrifuged at 650 rpm for 10 min, and 5 ml of the supernatant was mixed with 5 ml of distilled water and 1 ml of 0.1% ferric chloride solution. The absorbance was measured at 700 nm.

2.5 Experimental animals

The experiment was designed as previously described in our laboratory [11] with few modifications. This study employed 3-5 weeks old male BALB/c mice of weights 20-30 g bred in the animal house at the department of Biochemistry and Biotechnology of Kenyatta University. This study was conducted according to the ‘‘Principles of Laboratory Animal Care’’ [15] and all the experimental protocols were approved by the Ethics Committee for the Care and Use of Laboratory Animals of Kenyatta University. The mice were housed at a temperature of 25 °C with 12 hours light /12 hours darkness / photoperiod and fed on rodent pellets (Unga Feeds Limited, Kenya) and water *ad libitum*.

The BALB/c mice were randomly divided into six experimental groups consisting of five animals each. These groups included: the normal unmanipulated mice (the reference group of the experiment) orally administered with 0.1 ml physiological saline; the alloxan-induced diabetic mice

(the negative control group) orally administered with 0.1 ml physiological saline; alloxan induced diabetic control mice orally administered with 0.06mg of glibenclamide (3 mg/kg body weight, positive control group) in 0.1 ml physiological saline; and alloxan-induced diabetic mice orally administered with 1, 2, and 4 mg of extracts, respectively, in 0.1 ml physiological saline (50, 100, and 200 mg of plant extracts/kg body weight, respectively).

2.6 Induction of diabetes

Diabetes was experimentally induced in male BALB/c mice fasted for 8-12 hours, but allowed free access to water by a single intraperitoneal injection of 186.9 mg/kg body weight of a freshly prepared 10% alloxan monohydrate (Sigma Chemicals, St. Louis, OH) in physiological saline. This dose was found to be optimum in inducing stable diabetes in male BALB/c mice [16]. Forty eight hours after injection, blood glucose was determined by use of a glucometer (Contour RTS, Bayer Pty. Ltd; Healthcare Division, Japan), and mice with blood glucose levels above 2000 mg/L (>11.1 mmol/L), were considered diabetic and suitable for use in the study.

2.7 Blood glucose determination

Determination of blood glucose was carried out on blood drops obtained by tail bleeding of the mice at predetermined time points. Briefly, the tip of the tail was sterilized with 10% alcohol, and then nipped at the tip. A drop of blood was applied at the glucometer's sample pot. Blood glucose was determined at times 0, 2, 4, 6 and 8 hours after oral administration of aqueous roots extracts of *C. tomentosa*.

2.8 Single dose toxicity study

The mice were randomly divided into two groups of five mice each. Group I consisted of untreated control mice orally administered daily for 28 days with 0.1 ml physiological saline. Group II, consisted of normal control mice orally administered with aqueous roots extracts of *Capparis tomentosa* at 25 mg (1 g/kg body weight) in 0.1 ml physiological saline daily for 28 days. During this period, the

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. 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. On the day of sacrifice, all the animals were euthanized and organs removed and the weights determined.

2.9 Statistical analysis

All the data was recorded as mean \pm standard deviation (SD) of the blood glucose levels. One-way ANOVA and post-ANOVA (Bonferroni-Holm) test was used 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 extracts at doses of 50, 100 and 200mg per kg body weight. Also, the student T test was used to compare mean differences between organ weights, and animal weights in the single dose toxicity study. $P \leq 0.05$ was considered statistically significant.

3. Results

The *C. tomentosa* aqueous roots extracts was pink in colour, and on lyophilization this yielded a pink lyophilate that was used in the *in vivo* hypoglycemic efficacy and safety studies. As depicted in figure 1, the reducing power of the roots powder of *C. tomentosa* increased with increase in extracts concentrations with 800 $\mu\text{g/mL}$ possessing the highest reducing power. In a similar manner the reducing power of gallic acid used as the standard increased with increase in concentration. At the lower concentrations of the extracts and gallic acid, the reducing powers were low. At 400 $\mu\text{g/mL}$ the gallic acid had completely attained maximum reducing power when compared to the *C. tomentosa* roots powders and even at the maximum concentration tested still the roots powders had not attained the optimum reducing power. This is a significant free radical scavenging activity which accounts for the dose-dependent reducing potential observed in the reducing assay.

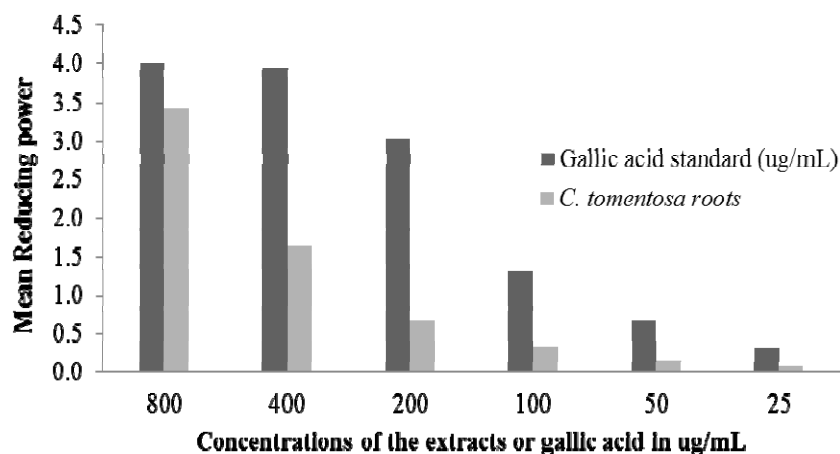


Fig 1: The concentration dependent reducing power of *C. tomentosa* roots compared with gallic acid standard.

The radical scavenging activity of the *C. tomentosa* roots, according to the DPPH method was found to be 35.50 ± 0.02 % compared to the ascorbic acid pure standard which had 96.50 ± 0.02 %. By the phosphomolybdate assay the reducing power was found to be 41.22 ± 0.17 mg/kg ascorbic acid equivalent. The extract was further found to have 35.50 ± 0.02 % free radical scavenging activity by DPPH assay, and this value was significantly different from that observed with

ascorbic acid standard at a concentration of 1 mg/ml. In this case ascorbic acid was used an external standard in a serial dilution ranging from 0.5 to 20 mg/ml.

Prior to the oral administration of the extracts, the body weights, age and blood sugars of all the mice were comparable ($p \leq 0.05$). As depicted in figure 2, the antidiabetic activity of the aqueous extract is dose-dependent with 200 mg/kg body weight displaying higher activity even after 8 hours. The

activities of the three doses are higher than that of the reference drug glibenclamide at 3 mg/kg body weight up to the 6th hour when their activities becomes comparable. As shown in table 1, the blood sugar of the negative group of mice (untreated) significantly increased within 8 hours, while groups treated with conventional drug (glibenclamide) and the

extracts at 50, 100 and 200 mg/kg body weight doses, the blood sugar declined significantly ($p \leq 0.05$). At the 2nd hour after treatment except the untreated, those treated with glibenclamide and 200 mg/kg body weight of the extract, the rest had a significant decline in blood sugar which persisted till the 8th hour.

Table 1: Hypoglycemic effects of oral administration of aqueous roots extracts of *Capparis tomentosa* in alloxan-induced diabetic BALB/c mice

Mice Group	Treatment	Blood glucose levels (mmol/L)				
		0 hr	2 hr	4 hr	6hr	8 hr
Normal control	Saline	5.0±0.1	5.2±0.1	4.6±0.2	5.0±0.3	5.2±0.3
Diabetic control	Saline	20.3±1.9	21.9±1.6 ^A	25.1±1.3 ^A	26.6±1.3 ^A	28.4±0.6 ^{Ad}
Diabetic control	Glibenclamide (3 mg/kg body weight)	23.1±1.6	20.1±1.5 ^A	16.3±1.2 ^{Bb}	7.8±0.5 ^{Bc}	4.9±0.2 ^{Bd}
Diabetic treated	50 mg/kg body weight	16.9±0.6	12.8±0.8 ^B	9.0±0.4 ^B	6.3±0.5 ^B	5.6±0.2 ^B
	100 mg/kg body weight	24.5±1.5 ^A	14.9±1.4 ^B	11.7±1.5 ^B	9.5±1.7 ^B	6.4±0.7 ^B
	200 mg/kg body weight	16.2±0.8 ^A	9.5±0.1 ^A	7.1±0.2 ^B	5.6±0.5 ^B	4.7±0.5 ^B

Results were expressed as mean ± standard error of mean (SEM) of five mice per group. Means followed by similar upper case letters in the same column are not significantly different at $P \leq 0.05$ by ANOVA and post ANOVA (Bonferroni-Holm) test. ^a $p \leq 0.05$ when blood glucose levels at 0 hour is compared to blood glucose at the 2nd hour; ^b $p \leq 0.05$ when blood glucose levels at 0 hour is compared to blood glucose at the 4th hour; ^c $p \leq 0.05$ when blood glucose levels at 0 hour is compared to blood glucose at the 6th hour; ^d $p \leq 0.05$ when blood glucose levels at 0 hour is compared to blood glucose at 8th hour is compared to blood glucose at the 8th hour by ANOVA and post ANOVA (Bonferroni-Holm) test.

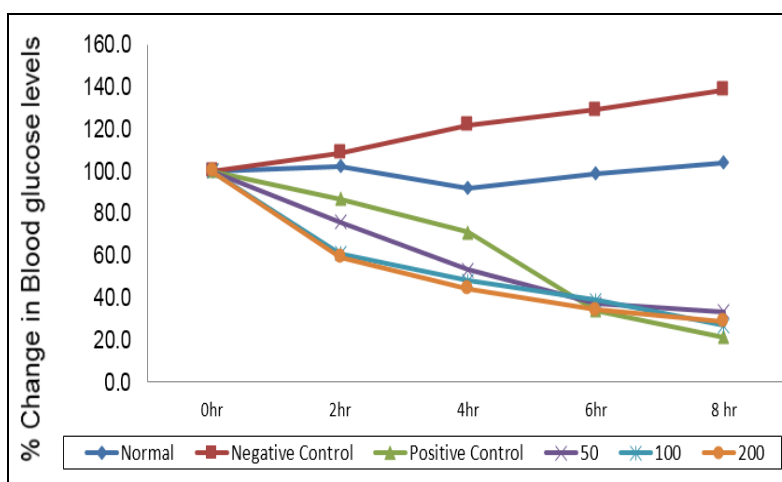


Fig 2: Mean percentage change in blood glucose levels after oral administration of aqueous roots extracts of *C. tomentosa* in alloxan-induced diabetic male BALB/c mice. Values are expressed as % Means ± SEM for five animals at each time point.

It was observed that there was no significant change in body weights ($p = 0.69$) for the animals under treatment compared to the controls over one month administration of the oral extracts at 1000 mg/kg body weight, and the mice gained weight normally. The organ weights were similarly found to

be comparable for both experimental and normal control mice ($p \leq 0.05$) as shown in figure 3. However, there was a significant reduction in kidney weight ($p = 0.001$), among the experimental mice compared to the normal control mice.

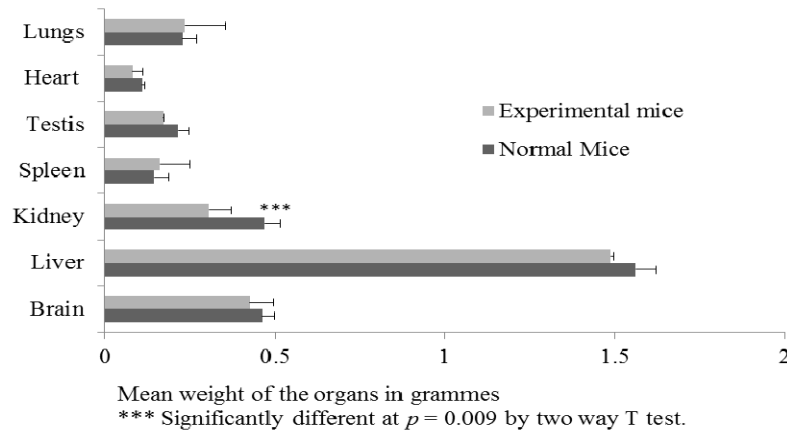


Fig 3: The mean weights of various organs in normal control mice and experimental mice in the single dose toxicity assay of *C. tomentosa* at 1000 mg/kg body weight.

4. Discussions and Conclusions

The observation that the reducing power of the aqueous roots extracts of *C. tomentosa* increased with concentration could be explained by the fact that the extracts contained chemical substances capable of reacting with potassium ferricyanide (Fe^{3+}) to form potassium ferricyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm [17]. The observation that the reducing power was linearly proportional to the concentration is explained by the fact that the amounts of antioxidants increased with an increase in the amount of crude extracts. Reducing power is associated with antioxidant activity and indicates that primary and secondary antioxidants with ability to donate electrons and reduce oxidized intermediates of lipid peroxidation processes are present in the extracts [17]. In the assay, the yellow colour of the test solutions changes to various shades of green and blue and this depend on the reducing power of the extracts concentrations. Similarly, the radical scavenging activity by DPPH and phosphomolybdate assay indicates that the roots extracts contains antioxidant compounds.

In the body, free radicals are constantly generated and they can cause extensive damage to tissues and biomolecules leading to various disease conditions, especially degenerative diseases, and extensive lyses. Many synthetic drugs protect against oxidative damage but they have adverse side effects. An alternative solution to the problem is to consume natural antioxidants from food supplements and traditional medicines [18]. These vitamins are involved in synthesis of enzymes that are essential to metabolic cell activity, synthesise of hormones, repairing genetic materials, and maintain normal functioning of the nervous system, processes critical in alleviating the effects of diabetes mellitus [19].

This study demonstrates that the aqueous roots extracts of *C. tomentosa* exhibits dose dependent hypoglycemic activity that is significantly higher than that of the reference drug glibenclamide. These findings are consistent to those of 30% extracts of *C. decidua* fruit powder orally administered to alloxanized rats for 3 weeks and found to significantly reduce the induced lipid peroxidation in erythrocytes, kidneys and heart and also to alter superoxide dismutase and catalases enzymes activities [20]. The observed hypoglycemic activity of *C. tomentosa* could be due to its strong antioxidant activity and anti-diabetic chemical compounds [21]. Also, the observed hypoglycemic activity of the *C. tomentosa* aqueous roots extracts could be due to the inhibition of carbohydrate absorption into the portal hepatic circulation, increased

glucose plants transport and uptake, increased glycogen storage, and modulation of insulin secretion.

Herbal medications are preferred in management of diabetes since they can target multiple mechanisms, including enhancement of insulin sensitivity, stimulation of insulin secretion, and reduction of carbohydrate absorption, inhibition of protein glycation and polyol pathway and inhibition of oxidative stress [11]. This contrasts with Western medicine which usually contains a single active ingredient that targets a specific mechanism [22].

Medicinal have been found to cause abnormal laboratory results in form of alteration in liver function tests, electrolyte disturbances, blood sugar level changes, heavy metal poisoning, and alteration in thyroid profiles. However, the single dose toxicity test of 1000 mg/kg body weight of aqueous roots extracts of *C. tomentosa* was found to allow normal growth of mice compared to the normal control over 30 days toxicity experiment. This could be explained by the fact that the extracts did not contain any toxic chemical compound or mineral element [23].

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