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In Vivo Anti-diabetic Effects of Aqueous Leaf Extracts of *Rhoicissus tridentata* in Alloxan Induced Diabetic Mice

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Abstract

The drawbacks associated with conventional anti-diabetic agents have largely stimulated desire for alternative and complementary anti-diabetic agents, majority of which are plant-derived. The medicinal plant, *Rhoicissus tridentata* has been used extensively in traditional medical practice against various ailments. However, its ethno medicinal use against diabetes mellitus has not been scientifically evaluated and validated. This study was, therefore, designed to evaluate the anti-diabetic potential of aqueous leaf extracts of *Rhoicissus tridentata* in alloxan induced diabetic mice. As the results show, the aqueous leaf extracts of *Rhoicissus tridentata* showed anti-diabetic activity. The intraperitoneal route of herbal extract administration was found to be more effective than the oral route. Further, qualitative and quantitative phytochemical screening of aqueous leaf extracts of *Rhoicissus tridentata* indicated the presence of phenols, alkaloids, flavonoids, tannins and saponins. However, cardiac glycosides and phylobatanins were not detected.

Keywords: *Rhoicissus tridentate*; Diabetes mellitus; Aqueous extracts; Anti-diabetic activity; Phytochemicals

Introduction

Diabetes mellitus is considered as one of the five leading causes of deaths in the world [1]. Diabetes mellitus is a heterogeneous metabolic disorder, characterized by altered carbohydrate, lipid and protein metabolism. It has been estimated that, about 1.3% of the world population suffer from this disease. Most of the hypoglycemic agents and hypolipidemics used in allopathic practice to treat diabetes mellitus and hyperlipidemia are reported to have side effects in long term use [2,3]. Within the human body the pancreas controls blood glucose by producing and releasing the hormones insulin and glucagon, which stabilize blood glucose within the physiological range of 70-120 mg/dl. DM is characterized by a dysfunction of the pancreas, often in combination with reduced insulin sensitivity [4].

Orthodox treatment of diabetes mellitus includes modification of lifestyle, such as diet and exercise and the use of insulin or oral hypoglycemic drugs [5]. In recent years there has been an increased inclination towards the herbal formulations, the complexity, side effects and costly treatment associated with the allopathic medicines have caused both the health care practitioners and the majority of world populations to turn towards these alternative therapies since, they are are believed to be free from side effects and are affordable [6].

Traditional medicine appears to offer gentler means of managing DM when allopathic medicines fail to work, or to patients who cannot afford them [7]. Due to inadequate knowledge of the contents of medicinal plants, herbal medicines are not often regulated, lack of information on the pharmacological toxicity of their compounds is also a major concern [8].

Rhoicissus tridentata (Plate 1) is a deciduous shrubby creeper in the family Vitaceae, commonly known as wild grape. The leaves are trifoliate with wedge-shaped leaflets. Decoctions and infusions of *R. tridentata* roots or tubers are taken orally in many South African pregnancy related traditional herbal remedies [9]. Other documented uses include, ease of indigestion by use of juice from the roots extracted through chewing, care of abdominal pain during menstruation by use extracts, treatment of swollen glands through warming of the roots in fire and then pressing them against the glands, its sap is reported to have healing and anesthetic properties. Tuberous roots are boiled and fed to young animals especially those that have lost there mothers, it is reported to be highly nutritious [10]. *R. tridentata* is used by populations in South Africa for gynaecological purposes and diarrhea. In other studies, *R. tridentata* is reported to possess direct uterotonic activity. An interesting result was that seasonal effect on the potency of uterotonic activity of this plant was reported [11].

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 May 2012 to October 2014. Kenyatta University is 23km from Nairobi off Thika Road.

Collection and preparation of plant materials

Whole stems of *R. tridentata* were collected from Narok County, Loita division in July 2012, cross identification with vernacular names of the plants was done before validation by a qualified taxonomist at the East Africa herbarium, National Museums of Kenya (NMK). Voucher specimens were deposited at the NMK and voucher numbers assigned as JM05. Coordinates for location of collection point were taken and recorded as E.36 N. 0797050 UTM 9822955 ALT. 2107.

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Preparation of aqueous extracts

The collected plant materials were chopped into small pieces, dried under shade at room temperature for four weeks, and then ground into fine powder by a mechanical grinder, followed by sieving through a 40 mesh sieve. The powders were packed in clean dry plastic air tight bags. One hundred grams (100 g) powder of each plant extract was later extracted in 1 liter of distilled water at 60°C in a metallic shaker for 6 hours. The extracts were decanted into clean dry conical flasks and then filtered through Whatman filter paper number 1 by use of a Buchner funnel at the biochemistry laboratory of Kenyatta University. The filtrates were stored in a refrigerator at 4°C. Freeze drying was done in 200 ml portions in a Modulyo freeze dryer (Edward England) for 48 hours and yield of each extract determined, freeze dryed materials were stored in a freezer at -20°C until the time that they were used.

Laboratory animals

Healthy adult male Swiss albino mice, 3-5 weeks old and 20-30 g in weight were used in the study. The animals were allowed to acclimatize for a period of two weeks in the animal house at the Department of Biochemistry and Biotechnology Kenyatta University prior to the study.

The mice were housed in polypropylene cages, maintained under standard laboratory conditions of 12 hour light and dark sequence, at ambient temperature of 25 ± 2°C and 35-60% humidity. The animals were fed with standard mice pellets obtained from Unga Feeds Limited, Kenya, and water ad libitum.

Experimental design

The following groups of mice were used for the experiments, group I, normal untreated mice was administered with 0.1 ml physiological saline, group II diabetic untreated mice (the negative control) was administered with 0.1 ml physiological saline, group III, alloxan induced diabetic control mice was administered with 0.06 mg of glibenclamide (for oral based experiment) or insulin (for intraperitoneal based experiment) 3 mg/kg body weight (positive control group) in 0.1 ml physiological saline, group IV, (diabetic mice treated with 50 mg/kg body weight of plant extracts, Group VI, (diabetic mice treated with 100 mg/kg body weight) of plant extract, group V1 (diabetic mice treated with 200 mg/kg body weight of plant extracts), group VII (diabetic mice treated with 300 mg/kg body weight of plant extract. The extracts were first dissolved in 0.1 ml physiological saline, oral and intraperitoneal routes were used in separate groups as indicated above.

Induction of hyperglycemia

Diabetes was induced experimentally by administration of 10% alloxan-monohydrate (Sigma Chemicals, St. Louis, OH). The animals were fasted for 8-12 hours, but allowed free access to water. A dose of 186.9 mg/kg body weight alloxan monohydrate was administered by intraperitoneal injection while still fresh, the dose was found to be most optimum in inducing diabetes in a separate study [12]. Forty eight hours after injection, blood glucose was determined by use of glucose analyzer model (On call plus-ACON LAB Inc-U.S.A) with glucometer strips, lot number 2014-09. Mice with blood glucose levels above 2000 mg/L (>11.1 mmol/L), were considered diabetic and suitable for use in the study.

Blood sampling and *in vivo* hypoglycemic assays

Blood sampling was done by sterilizing the tail with 10% alcohol and then nipping the tail at the start of the experiment and after 1, 2, 3, 4, 12 and 24 hours. Blood glucose was determined by use of glucose analyzer model (On call plus-ACON LAB Inc-U.S.A) with glucometer strips, lot number 2014-09.

Qualitative phytochemical screening

Tannins were determined as follows; 2 ml of 5% $FeCL_3$ was added to 2 ml aqueous extract of each sample. Yellow brown precipitate indicated presence of tannins [13]. Alkaloids were determined as follows; 1.5 ml of 1% HCL was added to 2 ml methanolic filtrates of samples. The solution was heated and six drops of dragendroff reagent was added. Orange precipitate confirmed presence of alkaloids [13].

For saponins determination, aqueous extract of 2 g powder was made and subjected to frothing test. Frothing persistence indicated presence of saponins. Later the froth was mixed with few drops of olive oil. Formation of emulsion indicated presence of saponins [13]. For determination of flavonoids (shimodas test), 2 g material was extracted in 10 ml H_2O, few drops of HCL followed by 0.5 g of zinc turnings were added. Tubes were boiled for a few minutes formation of pink color indicated presence of flavonoids [13].

For phenolics determination, to 2 ml of aqueous extract, 1 ml of 1% ferric chloride solution were added. Blue colour indicated presence of phenols [13]. In determination of phylobatannin, 10 ml of aqueous extract of each plant sample was boiled with 1% HCl acid in a test tube or conical flask. A deposition of a red precipitate indicated the presence of phlobatannins.

Quantitative determination of phytochemicals

Alkaloids: Two and a half grams (2.5 g) of the powder was extracted using 100 ml of 20% acetic acid in ethanol. The solution was covered for 4 hours, the filtrate was concentrated to 25 ml. Concentrated ammonium hydroxide was added stepwise to attain precipitation. The whole solution was kept as such so that precipitate will settle. Collected precipitate was washed with dilute ammonium hydroxide and finally filtered. Filtrate was discarded and pellet obtained was dried and weighed [13].

Saponins: Ten grams (10 g) of sample was mixed with 100 ml of 20% aqueous ethanol. The mixture was kept for 4 hours on water bath shaker at 55°C filtrate was again extracted in same manner. The combined extracts were concentrated to 40 ml over water bath at 90°C. Concentrate obtained was transferred into separating funnel and 10 ml of diethyl ether was added to it. After shaking vigorously aqueous layer was recovered and ether layer was discarded. The process was repeated. To the aqueous layer n-butanol was added. The whole mixture was washed in separating funnel twice with 10 ml 5% of aqueous NaCl. Upper part was retained and heated in water bath until evaporation. Later it was dried in oven to a constant weight [13].

Phenolics: One gram (1 g) of sample powder was extracted with 80% ethanol. Filtrate obtained was evaporated to dryness and again re-dissolved in water. Different aliquots (0.1-1 ml) were pippeted out and volume was made to 3 ml by distilled water 0.5 ml of Folins reagent followed by 2 ml 20% Na_2CO_3 solution was added. Tubes were vortexed, heated in boiling water for 1 min and finally cooled. Absorbance was measured at 650 mm against blank. A standard curve using different concentrations of 2 mg% catechol was prepared [13].

Tannins: Two grams (2 g) of plant powder from each sample was extracted thrice in 70% acetone. Samples were centrifuged and supernatant was collected. Different aliquots were prepared and final volume was made to 3 ml by distilled water and vortexed, 1 ml of 0.0016M $K_3(Fe(CN_6)$ 1 ml of 0.02M $FeCL_3$ in 0.1M HCL were added.

Plate 1: Photograph showing plant specimen of *Rhoicissus tridentata*, taken in July 2012, at Naikara Narok county.

Tubes were shaken and then kept as such for 15 minutes, 5 ml of stabilizer (3:1:1 ratio of water H_3PO_4 and 1% gum Arabic) was added and tubes were again revortexed. Absorbance was taken at 700 nm; standard curve was plotted using different concentrations of 1.9 mg% gallic acid [13].

Flavonoids: Ten grams (10 g) of the sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper No 42. The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to constant weight [14].

Data analysis

The data collected was entered into data base designed using Microsoft excel sheet. It was cleaned and organized into the SPSS software for statistical analysis. Data was expressed as mean ± standard deviation (SD). Differences between the means of the various groups of animals in the efficacy study was done using ANOVA and Post ANOVA statistical test, while the differences between the means of the two groups used in toxicity study was done using unpaired students t-test. Level of significance for all the analysis was set at $P<0.05$.

Results

In vivo hypoglycemic activity of *Rhoicissus tridentata*

Whole stem extracts yielded a 6% brown powder. Intraperitonealy administered aqueous whole stem extracts of *R. tridentata* decreased the blood glucose levels at all the four doses of 50, 100, 200 and 300 mg/kg body weight (Table 1). In the first four hours, the extract caused a steep decline in blood glucose levels, followed by a steady decline in the fifth to sixth hour. In the twenty fourth hour, there was a gradual increase in blood glucose levels. However, the sugar levels were not reduced in a dose dependent manner. In the first hour, the extracts lowered blood glucose levels by 7%, 15%, 8% and 6% for 50, 100, 200 and 300 mg/kg body weight doses, respectively, compared to insulin treated diabetic mice whose blood sugar levels was lowered by 58% within the first hour. By the fourth hour, all the four doses (50, 100, 200 and 300 mg/kg body weight) had lowered blood sugar levels by 36%, 29%, 40% and 34%, respectively, compared to insulin treated diabetic mice whose sugar levels was lowered by 70% within the same hour (Figure 1).

Orally administered aqueous leaf extracts of *R. tridentata* also lowered blood glucose levels at all the four doses of 50, 100, 200 and 300 mg/kg body weight (Table 2), from the first hour to the sixth hour in a dose-independent manner. By the second hour the extract had lowered the blood glucose levels by 55%, 33%, 55%, and 40% respectively for the four doses, compared to 28% for the conventional oral drug, glibenclamide (Figure 2). The reduction in blood glucose levels when compared to the negative control was statistically significant ($P \leq 0.05$).

Qualitative and quantitative phytochemical screening

As Table 3 shows, qualitative screening of aqueous extracts of *R. tridentata* indicated the presence of phenols, alkaloids, flavonoids, tannins, saponins, and phylobatannins and the absence of cardiac glycosides.

Discussion

In this study, the alloxan-induced diabetic mice had a three to four fold increase in blood glucose (5 mg/dl to 20 mg/dl) relative to the normal control mice. Studies show that alloxan causes a glucose oxidation and reduction in insulin release by the destruction of β-cells of the islets of Langerhans [15]. The aqueous leaf extract of *R. tridentata* showed blood glucose lowering effect when administered intraperitonially and orally.

Studies involving other plants have similarly been reported to have glucose lowering effect, for example the results of anti-diabetic test done on *Bougainvillea glabra* showed that 100 mg/kg and 400 mg/kg extract

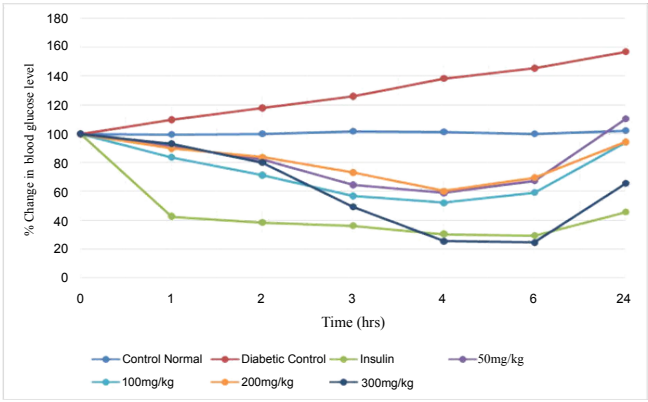

Figure 1: Mean percentage change in blood glucose levels of *Rhoicissus tridentate* intra-peritoneally administered in alloxan induced diabetic mice.

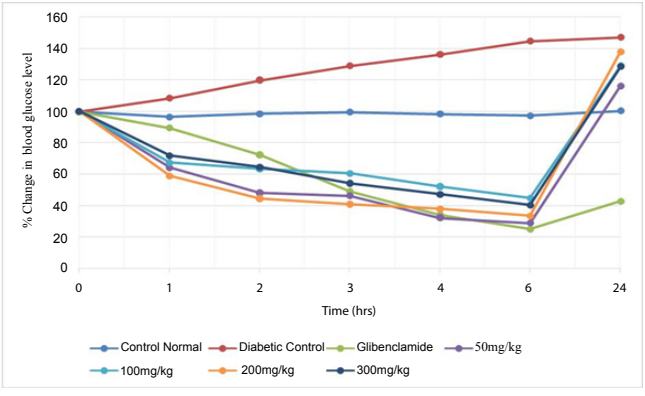

Figure 2: Mean percentage change in blood glucose levels of *Rhoicissus tridentata* orally administered in alloxan induced diabetic mice.

Treatment	Glucose levels at varying times (mM)						
	0 hr	1 hr	2 hr	3 hr	4 hr	6 hr	24 hr
Control/Saline	5.14 ± 0.09	5.12 ± 0.05	5.14 ± 0.15	5.24 ± 0.09	5.22 ± 0.10	5.14 ± 0.15	5.26 ± 0.09
Diabetic/Saline	14.98 ± 1.48	16.48 ± 1.60	17.54 ± 1.35	18.76 ± 1.51	20.40 ± 1.37	21.46 ± 1.34	23.12 ± 1.50
Diabetic/insulin	17.26 ± 1.65	7.22 ± 0.65^{a}	6.50 ± 0.45^{a}	6.10 ± 0.58^{a}	5.12 ± 0.13^{a}	4.94 ± 0.13^{a}	7.70 ± 0.72^{a}
50 mg/kgbw	22.54 ± 1.81	20.94 ± 2.31^{bc}	19.22 ± 2.55^{bc}	15.22 ± 2.79^{bc}	14.08 ± 2.58^{b}	16.0 ± 2.46^{b}	24.94 ± 1.96^{b}
100 mg/kgbw	15.80 ± 1.58	13.50 ± 1.99^{b}	11.68 ± 2.10^{b}	9.48 ± 2.20^{bd}	8.72 ± 2.08^{bc}	9.60 ± 2.13^{bc}	15.60 ± 2.11^{bc}
200 mg/kgbw	14.98 ± 2.28	13.82 ± 2.55^{b}	13.06 ± 2.61^{b}	11.60 ± 2.58^{b}	9.96 ± 2.02^{bc}	11.28 ± 2.11^{bc}	14.32 ± 1.81^{bc}
300 mg/kgbw	15.88 ± 1.27	14.88 ± 0.68^{b}	12.74 ± 0.57^{b}	7.70 ± 1.13^{ad}	4.02 ± 0.54^{ad}	3.92 ± 0.70^{acd}	10.32 ± 1.29^{bcd}

Results are expressed as means ± SD for five animals per group. Values followed by the same superscript are not statistically different (P ≤ 0.05; analysed by ANOVA followed by Tukey's post hoc test).

*a,b,c=non-significant which is indicated by same superscript notations.

Table 1: Effects of intra-peritoneally administered aqueous leaf extracts of *Rhoicissus tridentata* on blood glucose levels in alloxan induced diabetic mice.

Treatment	Glucose levels at varying times (mM)						
	0 hr	1 hr	2 hr	3 hr	4 hr	6 hr	24 hr
Control/Saline	5.24 ± 0.11	5.06 ± 0.13	5.16 ± 0.18	5.22 ± 0.13	5.16 ± 0.15	5.10 ± 0.12	5.26 ± 0.11
Diabetic/Saline	19.86 ± 0.95	21.52 ± 1.10	23.76 ± 0.90	25.54 ± 0.91	26.94 ± 0.94	28.62 ± 0.64	28.98 ± 0.84
Diabetic/Glen	18.76 ± 1.07	16.84 ± 1.19^{a}	13.56 ± 0.79^{a}	9.24 ± 0.81^{a}	6.38 ± 0.87^{a}	4.66 ± 0.36^{a}	7.96 ± 0.60^{a}
50 mg/kgbw	16.76 ± 2.43	10.80 ± 1.82^{bc}	7.46 ± 1.45^{b}	6.94 ± 1.52^{ac}	4.52 ± 0.78^{a}	4.06 ± 0.48^{a}	19.24 ± 1.85^{bc}
100 mg/kgbw	13.78 ± 1.43	9.84 ± 1.81^{bc}	9.24 ± 1.66^{bc}	8.88 ± 1.67^{ac}	7.56 ± 1.29^{a}	6.60 ± 1.27^{a}	18.24 ± 2.03^{bc}
200 mg/kgbw	10.58 ± 0.77	6.36 ± 0.91^{b}	4.78 ± 0.48^{b}	4.38 ± 0.47^{bc}	4.06 ± 0.47^{a}	3.58 ± 0.36^{a}	14.36 ± 2.15^{bd}
300 mg/kgbw	13.42 ± 1.27	8.92 ± 1.08^{bc}	8.08 ± 1.20^{bc}	6.76 ± 1.06^{ac}	5.92 ± 0.73^{a}	4.96 ± 0.72^{a}	14.00 ± 2.04^{bd}

Results are expressed as means ± SD for five animals per group. Values followed by the same superscript are not statistically different (P ≤ 0.05; analysed by ANOVA followed by Tukey's post hoc test).

*a,b,c=non-significant which is indicated by same superscript notations.

Table 2: Effects of orally administered aqueous leaf extracts of *Rhoicissus tridentata* on blood glucose levels in alloxan induced diabetic mice.

Phytochemicals present	Quantity
Phenols (mg/g)	1.33
Alkaloids (g/100g)	2.90
Flavonoids (mg/g)	0.14
Saponins (g/100g)	8.53
Tannins (mg/g)	0.97
Cardiac glycosides (mg/g)	ND
Phylobatannins (mg/g)	ND

Key: ND - Not Detected

Table 3: Phytochemical composition of the aqueous leaf extracts of *A. nilotica*.

significantly reduced the blood glucose levels in diabetic rats induced with alloxan. The reduction of diabetes in the alloxan induced animals by the extract groups was comparable with the normal control and non-diabetic extract fed groups [16]. It was observed that the G Mice induced with diabetes using alloxan and treated with aqueous extract of root of *Panax ginseng* showed a remarkable hypoglycemic activity on administration [17]. The extract of *Scoparia dulcisin* administered in Albino mice significantly lowered blood glucose level at doses of 100 mg/kg and 200 mg/kg body weight they showed maximum reduction; at 100 mg/kg [18]. The plant *Memecylon malabaricum* was found to possess anti-diabetic activity, and the results were comparable to that of Gliclazide. The extract at 400 mg/kg p.o, showed a maximum reduction of raised blood glucose level as that of 100 and 200 mg/kg. The results obtained indicated that the extract had a significant anti-diabetic activity in rats [19].

The possible mechanism through which the extract might have brought about blood glucose lowering effect were either by increasing utilization of glucose or by direct stimulation of glucose uptake through increased insulin secretion [20]. It might also have been due to the extracts stimulating β cells in islet of Langerhans, increased serum insulin and reduced blood sugar [21]. The findings also suggest that plant extracts may regenerate β cells and has protective effect on β cells from glucose toxicity. As other studies showed, plant extracts might bring about its hypoglycemic effect through insulin secretion from the remaining β cells and insulin sensitivity [22,23]. A number of other plants have also been observed to exert hypoglycemic activity through insulin release stimulatory effects [24].

That the aqueous whole stem extracts of *Rhoicissus tridentata* at all dose levels did not significantly lower the blood glucose levels in the first hour, but significantly lowered the blood glucose levels in both oral and intra-peritoneal routes from the, third to sixth hours may have been due to the fact the extracts may not have been absorbed quickly or compound in the extract could have been a pro drug thus the active principles in the extracts required biotransformation so as to become anti-hyperglycemic.

The blood glucose lowering effect of these plant extracts may be attributed to the presence of phenols, flavonoids, alkaloids, tannins, phylobatanins, and saponins that have been associated with hypoglycemic activity [25]. Flavonoids are one of the most numerous and wide spread groups of phenolic compounds in higher plants. Some of them, due to their phenolic structure, are known to be involved in the healing process of free radical mediated diseases including diabetes. Oral administration of the flavonoids content (8%) of the seeds of *Cuminum nigrum* caused a significant blood glucose lowering at a dose range of 0.5 to 1.5 g/kg, both in normoglycaemic and alloxan-induced diabetic rabbits [26]. Flavonoid and terpenes isolated from the other antidiabetic medicinal plants has been found to stimulate secretion or possess an insulin like-effect [27]. Effect of the flavonoids quercetin and ferulic acid on pancreatic β-cells leading to their proliferation and secretion of more insulin have been proposed by [28,29] as the mechanism by which they reduced hyperglycaemia caused by streptozocin in diabetic rats. The flavonoids present in *c* may also be acting similarly.

Presence of saponins in this extract could also be responsible for

the hypogycemic activity. For instance ginseng and its saponins have been shown to lower blood glucose in alloxan-treated, genetically diabetic, and normal mice [30].

[31] Reported that all the forms of tannins may participate in managing glucose level in blood. Tannin stimulates the receptor cells to utilize carbohydrate. The presence of tannins in *R. tridentata* might have brought about blood glucose lowering effect.

Conclusion

From this study it can be concluded that the aqueous leaf extracts of *R. tridentata* showed anti-diabetic activity. The intra-peritoneal route of herbal extract administration was found to be more effective than the oral route. Further, qualitative and quantitative phytochemical screening of aqueous leaf extracts of *R. tridentata* indicated the presence of phenols, alkaloids, flavonoids, tannins and saponins. This study, therefore, recommends use of aqueous stem extracts of *R. tridentata* in management of diabetes mellitus.

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