Original Article

Phytochemical Studies and Blood Glucose Lowering Effect of Zambian Cultivars of *Piliostigma thonningii* in Alloxan - Induced Diabetic Rats

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ABSTRACT

Background: There is anecdotal evidence among some Zambian traditional medicine practitioners that *Piliostigma thonningii possesses antidiabetic effects. This study assessed the in-vivo antidiabetic effects of ethanol extract of the plant in alloxan-induced diabetic wistar rats.*

Study design*:* A laboratory-based experimental study involving 24 rats divided into 4 groups of 6 and administered the extract, glibenclamide and placebo.

Methods*:* Diabetes was induced by a single intra-peritoneal injection of 150mg/kg alloxan monohydrate. The *in-vivo* glucose lowering effect of ethanol extract of stem bark of *Piliostigma thonningii* was evaluated in comparison to normal saline as negative control and glibenclamide as positive control. Standard methods for identification of alkaloids, flavonoids, saponins, tannins, glycosides, terpenoids and anthraquinones were employed in the phytochemical analysis of the extract.

Results*: Piliostigma thonningii* ethanol stem bark extract showed presence of alkaloids, flavonoids, saponins, tannins, glycosides and terpenoids. The extract caused a 55.3% (P=0.002) reduction in blood glucose of the experimental animals over the treatment period from the initial 19.7±7.0 mmol/l to 8.8±2.5 mmol/l, compared with glibenclamide which caused 68.3 % (P=0.001) lowering blood glucose levels from 24.0±4.5 mmol/l to 7.6±5.9 mmol/l.

Conclusions*:* Stem bark extract of *Piliostigma thonningii* showed a significant glucose lowering effect in diabetic rats. The study also demonstrated presence of secondary plant metabolites which could be responsible for some of the medicinal properties of the plant. Further studies are required to purify the plant and identify active fractions responsible for the anti-diabetic effect observed.

INTRODUCTION

Diabetes mellitus remains a serious threat to global health that respects neither socioeconomic status nor national boundaries. It has plagued mankind with a global epidemiology of 463 million people in 2019, and a projected figure of 700 million people by 2045[1]. In Africa, 19 million people suffered from diabetes in 2019. This figure is expected to increase to 47 million people by the year 2045, an increase of about 143%[1]. In Zambia, diabetes with a prevalence of 3.4% is estimated to have claimed about 8,000 lives in 2019[1].

Current antidiabetic agents have limited efficacy and undesirable side effects[2]. Apart from the side effects, availability of diabetes medicines is globally variable, with poorer populations having less access as these drugs require life-long treatment and are not affordable for most of the people, especially those from developing countries like Zambia[1,3].

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World Health Organisation recognises the importance of traditional medicines in the development of economic and effective treatments especially for diseases without a cure[4]. The increasing interest in plant-derived products as alternatives to the present conventional oral drugs in the treatment of diabetes, is emerging and receiving much attention by many researchers[5,6].

Piliostigma thonningii (Schumach) known locally as *Musekese* (Tonga) *is widely used in Africa as a source of folklore medicine[7,8]. In Zambia, its r*oots, bark, seed, and fruit have been reported to be used for various medicinal purposes including dysentery, fever, leprosy, respiratory ailments, snake bites, tooth ache, wounds, diarrhea. *Piliostigma thonningii* is also used traditionally to treat HIV/AIDS related coughs, skin rashes, gonorrhea and syphilis in Zambia[9-11].

This study aimed at investigating the blood glucose lowering effect of the Zambian variety of Piliostigma thonningii extract following anecdotal evidence which shows that the plant has been used traditionally for the purpose of treating diabetes. It is hoped that this study will contribute to the growing knowledge of scientifically tested indigenous plants used locally for the treatment of diabetes.

MATERIALS AND METHODS

Study design: An *in vivo* randomised controlled trial study design was used on wistar rats.

Plant Material: The fresh stem bark of *Piliostigma thonningii* was collected from Mumbwa District of Central Zambia during the rainy season (December). The plant was identified by a Botanist from The University of Zambia, Department of Biological Sciences and a Voucher Specimen (No. 22,208) was deposited in the herbarium.

Preparation of samples: The collected fresh stem bark of *Piliostigma thonningii* was washed using sterile water, pruned and shade dried for 14 days at room temperature. It was then cut into small pieces and pulverized until powdered. 75g of powdered stem bark was Soxhlet extracted with 300ml of 99% ethanol at room temperature for 8 hours to obtain the crude extract[12,13]. The filtered crude extract was evaporated to dryness in a rotary evaporator at 40 - 50°C under reduced pressure.

Determination of percent (%) yield: Percentage yield of the extract was determined using the weight of the extract residue obtained after solvent removal (w_1) and weight of the plant powder (w_2).

$$\% \text{ yield} = (w_1/w_2) \times 100$$

Qualitative Phytochemical Studies: The phytochemical constituents of the stem bark of *Piliostigma thonningii* were determined using standard methods as follows[14-16]: (i) Flavonoids by reacting with metallic magnesium and hydrochloric acid, (ii) Saponins using the foam test, (iii) Alkaloids with Dragendroff's reagent, (iv) Tannins with ferric chloride reagent, (v) Terpenoids with chloroform and sulfuric acid, (vi) Glycosides with chloroform and sulphuric acid and (vii) Anthraquinones with benzene and ammonia solution.

Animals: Twenty-four healthy adult wistar rats weighing between 120g and 350g of both sexes were obtained from the animal housing unit at the Department of Physiological Sciences, University of Zambia, School of Medicine. The rats were housed in clean cages, maintained at normal room temperature (21°C-25°C) and natural daylight/night conditions. The rats were given free access to standard commercial pelleted feed and clean drinking water. They were allowed to acclimatise to the laboratory conditions for 7 days before starting the experiment.

Induction of diabetes: The rats were fasted overnight in which they only received water. 1g of alloxan monohydrate (Sigma Aldrich, Germany) was dissolved in 10ml normal saline to make a solution of 100mg/ml concentration. Specific volumes of the solution were administered by a single intraperitoneal injection to ensure that each rat received 150mg/kg body weight[12,17], after which the rats were maintained on an ad libitum standard diet. The animals were monitored for 3 days for elevation of Fasting Blood Sugar (FBS) levels using the Accu-Check Active Glucometer (Germany). The diabetic state was confirmed by the FBS level higher or equal to 7.0 mmol/l[18].

Administration of test substances: The 24 rats were randomly assigned to 4 groups of 6 each namely A, B, C and D and were treated orally using a 22G feeding tube as follows[12,19,17]:

Group A (Diabetic control): 5ml Normal Saline

Group B (Normal control): 5ml Normal Saline

Group C: 2mg/kg Glibenclamide

Group D: 500mg/kg *Piliostigma thonningii* stem bark *extract*

Measurement of Fasting Blood Sugar: The rats were fasted for 12 hours before FBS levels were checked once a day for the duration of the experiment. Blood samples were obtained by piercing the tip of lignocaine-anaesthetised tails of the rats and FBS level estimated by the colorimetric glucose oxidase method using a glucometer[47].

Data Analysis: The phytochemical analyses data were qualitative and were presented as present or absent. Data on blood glucose were analysed with Stata statistical software version 13.0 and presented as mean ± SEM. Differences between groups were analysed using one-way analysis of variance (ANOVA) with a $P<0.05$ indicating significant difference.

Ethical Considerations: Ethics approval was sought from the University of Zambia Biomedical and Research Ethics Committee (No. 013-11-16). The study complied with guidelines relating to humane handling and use of laboratory animals for research including minimisation of pain, ensuring a conducive environment, minimizing the number of animals used, and euthanasia[46].

RESULTS

Percent yield: The result of the Soxhlet extraction showed that the ethanol extract yielded 3.63g (4.84%) by mass.

Phytochemical screening: The ethanol extract of *Piliostigma thonningii* carried out to identify phytoconstituents showed presence of alkaloids, flavonoids, tannins, saponins, terpenoids and glycosides, while anthraquinones were absent (Table 1). Some phytochemical constituents demonstrated in this experiment have been shown in other studies to possess hypoglycemic effects.

Table 1: Phytochemistry of Piliostigma thonningii crude ethanol extract

Chemical compound	Reagents	Positive indicator	Observation	Result*
Flavonoids	Magnesium Hydrochloric acid	Orange-Pink colour	Pink colour	+
Saponins	Frothing	Persistent froth after vigorous shaking	Persistent froth	+
Alkaloids	Dragendroff's	Orange-brown precipitate	Orange precipitate	+
Tannins	Ferric chloride	Black-green precipitate	Dark-green precipitate	+
Terpenoids	Chloroform Sulfuric acid	Reddish-brown interface	Brown colour	+
Glycosides	Chloroform Sulfuric acid	Reddish-brown ring (steroid ring)	Reddish-brown ring	+
Anthraquinones	Benzene Ammonia	Pink, red or violet colour	Brown colour	-

**Key: (+) positive test, (-) negative test*

Blood sugar lowering effect: Figure 1 shows fluctuations in fasting blood sugar levels for all groups. The group treated with *Piliostigma thonningii extract initially showed a rise in the fasting sugar levels. This was followed by a gradual decrease from day 4 until the end of the experiment on day 7.* The glibenclamide-treated group showed a sustained decrease in fasting blood sugar levels from day 1 until day 7 of the experiment. The diabetic control (Normal saline -treated diabetic group) maintained high levels of FBS while the normal control (Normal saline-treated non-diabetic group) consistently showed low levels of the FBS levels.

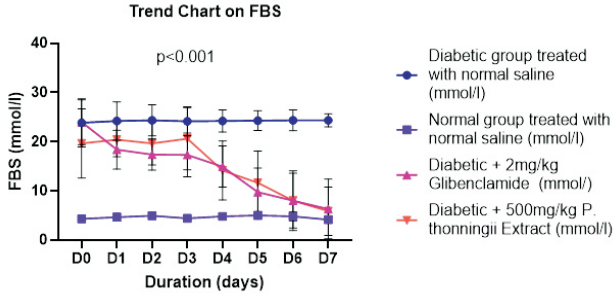

Figure 1: Effects of ethanolic extract of 500mg/kg Piliostigma thonningii stem bark, compared with standard drug, 2mg/kg glibenclamide and normal saline on fasting blood sugar levels of diabetic and normoglycemic rats. Each point is the mean ±SEM.

Table 2 shows percent (%) FBS level changes across all the groups (A, B, C and D) in the experiment. The group treated with glibenclamide showed a reduction in FBS levels of 68.3% compared to 55.3% observed in the group treated with ethanolic stem bark extract of *Piliostigma thonningii*. The untreated groups did not show any significant fluctuations in their FBS levels.

Table 2: Percentage (%) glycaemic change after administration of ethanolic stem bark extract of *Piliostigma thonningii* and glibenclamide in alloxan-induced diabetic rats (n=6) between Day 0 and Day 7

Group	Treatment	Day 0 – Baseline Blood Glucose (mmol/l)	Day 7 – Endpoint Blood Glucose (mmol/l)	°Glycaemic % difference
A	Diabetic + Normal Saline	–	24.3±1.3	-2.1
B	Non-diabetic + Normal Saline	4.3±0.6	4.1±0.6	4.6
C	Diabetic + 2mg/kg Body Weight Glibenclamide	24.0±4.5	7.6*±5.9	68.3
D	Diabetic + 500mg/kg *Piliostigma thonningii* stem bark extract	19.6±7.0	8.8*±2.5	55.3

**Superscripted items indicate statistically significant (P<0.05) difference exist between mean value of rats in the group on baseline (Day 0) and end point (Day 7). %°Difference between Day 0 and Day 7.*

Figure 2. To show the effect of *Piliostigma thonningii extract* on blood glucose level, rats with elevated glucose levels were treated for 7 days. In the diabetic group treated with normal saline, there was no reduction in blood glucose levels. In the diabetic group treated with 2mg/kg Glibenclamide – a drug used clinically to reduce blood glucose levels, there was significant reduction of glucose. Similarly, 500mg/kg *Piliostigma thonningii ethanol* extract showed significant reduction in blood glucose levels.

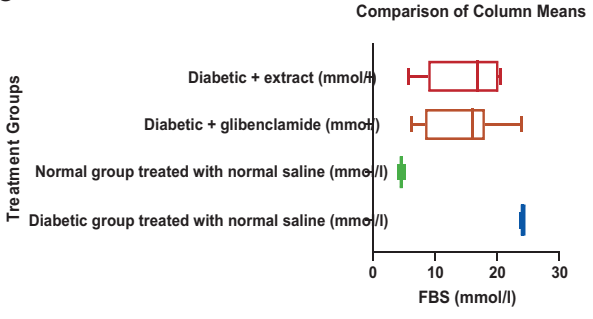

Figure 2: Comparison of means of FBS levels of rats at baseline (day 0) and endpoint (day 7). The normal and diabetic groups treated with normal saline did not show any significant fluctuations in the FBS levels while the diabetic groups treated with reference drug, glibenclamide and Piliostigma thonningii extract showed significant fluctuations in the FBS levels.

DISCUSSION

The aim of this research was to elucidate the phytochemical composition and antidiabetic properties and therefore, establish the scientific basis for the traditional use of extracts of *Piliostigma thonningii* in the management of DM.

Phytochemical screening of ethanol extract of *Piliostigma thonningii confirmed presence of alkaloids, flavonoids, tannins, saponins, terpenoids as well as glycosides while anthraquinones were absent (Table 1). Findings of this study are supported by authors from different geographical regions*[20-25]. It is well documented that the geographical location where a particular plant species is obtained, the part of the plant used as well as the method of extraction are some of the factors that can affect the phytochemical composition[26,27], and hence the pharmacological activity, of a plant extract.

Experimental evidence shows that flavonoids have anti-diabetic activities that are mediated by decreases in the level of glycosylated haemoglobin, increases in the activities of glutathione peroxidases[28], significant reductions in plasma glucose values, increases in hepatic hexokinases and glucose-6-phosphate dehydrogenases[29], increasing insulin levels and ameliorating oxidative stress as evidenced by assay of superoxide dismutase, catalase and glutathione peroxidase activities and by also increasing the levels of vitamin C and E[30]. Saponins have been reported to elevate serum insulin levels and alleviate hyperglycemia as well as decrease lipid levels[31]. The anti-diabetic mechanism of the alkaloids has been reported as a significant improvement in the activity of GLUT 4, glucokinase activity and PPAR-Y[32]. Tannins have exhibited anti-diabetic effects mainly by inhibiting the activation of α-amylase and α-glucosidase activities[33]. Tannic acid has also been reported to stimulate transportation of glucose and

inhibit differentiation in 3T3-L1 adipocytes[34]. Terpenes have demonstrated antidiabetic activity through translocation of GLUT 4[35] and potentiation of insulin action amongst other possible mechanisms[36]. Bioactive glycosides have been reported to possess anti-diabetic activity in streptozotocin-induced diabetic rats by decreasing the serum α-amylase and lactate dehydrogenase activities[37] and increasing insulin secretion[38].

Figure 1 shows that in the diabetic control group, the FBS levels remained very high confirming destruction of β-cells by alloxan and non-stimulation of insulin secretion. The glibenclamide-treated group showed a gradual and progressive decline in FBS levels over the period of the experiment. A significant decrease in blood glucose concentration was noticed by day 7, a reduction of 68.3% (P=0.001). Glibenclamide, classified as an insulin secretagogue, promotes insulin release from the β- cells of the pancreas by blocking the ATP-sensitive K^+ channels, resulting in depolarisation and Ca^{2+} influx as the main action. Other actions of glibenclamide include reduction in hepatic glucose production and increase in peripheral insulin sensitivity as long-term effects.

The *Piliostigma thonningii*-treated group showed an initial rise in FBS levels (Figure 1). This could likely be due to the crude nature of the extract and can be attributed to the carbohydrate content of the plant[22,24]. This effect was not observed in diabetic rats administered with normal saline. As a consequence, this initial rise in FBS levels seems to offset the early anti-hyperglycaemic effect of the crude extract and lead to a delayed response compared to glibenclamide. The greatest reduction in the FBS levels by the plant extract was seen on day 4 of the experiment with an overall reduction amounting to 55.3% (P=0.002) on the final day of the study. The pattern of blood sugar lowering effect by *Piliostigma thonningii* extract from day 3 looked similar to that of glibenclamide and can be postulated to act by the same mechanism[39,40].

In this study, the diabetic state was induced by injecting the rats with alloxan monohydrate. Alloxan induces diabetes by generation of free radicals which promotes selective necrosis of β-cells of the pancreas. There is increasing evidence that increased production and/or ineffective scavenging of free radicals may play a critical role in chronic diseases including diabetes[42]. Therefore, plants particularly those with high levels of antioxidant compounds have an important role in the improvement of disorders involving oxidative stress such as diabetes mellitus.

Phytochemicals with antioxidant effects include some cinnamic acids, coumarins, diterpenes, flavonoids, lignans, monoterpenes, phenylpropanoids, tannins and triterpenes[43]. In this study, the presence of flavonoids, tannins and terpenoids among other phytochemicals has been demonstrated. Several studies have reported that antioxidant vitamins and supplements can help lower the markers indicative of oxidant stress and lipid peroxidation in diabetic subjects and animals. A number of authors have demonstrated that vitamin C and E and beta-carotene are decreased in diabetic patients and experimental animals[44,45]. D-3-O-methylchiroinositol isolated from the stem bark of *Piliostigma thonningii has also been cited as having antidiabetic and antioxidant effects*[12].

It is noted that there was high variability of results on development of hyperglycemia. At baseline, initial group blood glucose levels were different for the diabetic groups (A, C and D). This variability was due to the unstable nature of chemical-induced diabetes and was difficult to control. However, we were able to determine the extent and rate of decrease in FBS levels which was more in the group treated with extract and glibenclamide compared to the diabetic group administered normal saline.

Biochemical processes that influence the diabetic state are diverse and involve not only insulin but also other chemical substances such as glucagon, somatostatin, gastrointestinal hormones and corticosteroids. The establishment of the precise mechanism of action by which plants such as Piliostigma thonningii extracts interact with these processes to lower blood sugar levels is of utmost importance and efforts to achieve this are encouraged.

In this study, the investigators are convinced that this is the first scientifically validated study of the Zambian species of this plant for the management of diabetes. Further, we recommend preclinical and safety toxicology studies that will help establish the safety of the plant extract for human use.

CONCLUSION

Piliostigma thonningii extract growing in Zambia has demonstrated blood sugar lowering properties in rats. The stem bark ethanol extract also revealed presence of *alkaloids, flavonoids, tannins, saponins, glycosides and terpenoids.* These findings suggest that the crude extract of Piliostigma thonningii could be useful for the control of blood glucose levels and supports the use of the plant in traditional medicine. Further studies are recommended to establish the active fractions and toxicity of the plant before consideration for clinical trials is made.

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