

In Vivo Antidiabetic Effect of Aqueous Leaf Extract of *Azadirachta indica*, *A. juss* in Alloxan Induced Diabetic Mice

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Abstract

There is growing interest in the potential of plant remedies to treat and manage many diseases owing to the adverse side effects, unavailability and unaffordability associated with the conventional therapy. Among the traditional plants that has been prescribed for clinical use for many ailments including diabetes mellitus is *Azadirachta indica*. Their continued use is largely based on their long-term therapeutic effects although this has not been authenticated scientifically. This study therefore, aims to evaluate the *in vivo* hypoglycemic effect of aqueous leaf extracts of *A. indica* in alloxan-induced white male albino mice. The blood glucose lowering effect of the extract was intraperitoneally and orally bioscreened in diabetic mice in serial dilutions of the extract at 25 mg/kgbw, 48.4 mg/kgbw, 93.5 mg/kgbw, 180.9 mg/kgbw and 350 mg/kgbw. Qualitative analysis of phytochemicals was done using standard procedures. In both routes, the extract lowered blood glucose at all dosages in a dose independent manner. The extracts contained flavonoids, tannins, sterols, saponins, anthraquinones and alkaloids. The antidiabetic activity may be attributable to these phytochemicals present in the plant extract. The study confirms the traditional use of this plant part in the treatment of diabetes mellitus. However, organic solvent extraction of the leaves of this plant should be done to compare effects of both organic and aqueous fractions.

Keywords: *Azadirachta indica*; Antidiabetic; Hyperglycemia; *In vivo*; Phytochemical; Insulin; Glibenclamide

Introduction

Diabetes mellitus is a metabolic disorder characterized by chronic elevated blood glucose levels that could lead to mortality and morbidity [1]. The pathophysiological processes involved in etiology of this disorder may be due to abnormalities that result in insulin resistance or molecular mimicry that results in autoimmune destruction of islet β -cells of Langerhans with consequent insulin deficiency, or both [2]. Insufficient action and deficiency of insulin on peripheral tissues disrupts the metabolism of dietary fats, proteins, and carbohydrates [3].

The clinical manifestation of hyperglycemia include polydipsia, polyuria, fatigue, weight loss and polyphagia [4]. Untreated high blood sugar levels increases the risk of microvascular damage such as nephropathy, retinopathy and neuropathy. It is associated with significant morbidity, reduced life expectancy, increased risk of macrovascular complications (stroke, ischaemic heart disease and peripheral vascular disease), diminished quality of life and death [5].

There is a tremendous increase of diabetics globally. It was found out that the number of diabetics by the year 2000 was 170 million and this figure is projected to rise to 366 million by the year 2030 [6]. This may be attributed to ineffectiveness and adverse side effects associated with allopathic antidiabetic drugs [7]. Moreover, the conventional drugs are unavailable and expensive to most people especially those from under developed and developing countries who therefore resort to alternative therapies from herbal medicines. These herbal

preparations are believed to be safe and effective [8]. However, most herbal medicines have not been scientifically evaluated [9].

Azadirachta indica (*A. juss*), (family, Meliaceae) popularly known as Neem (English) or Mwarobaini is one of the commonly used plants in Africa traditional medicine for the management of diabetes mellitus. It is the most versatile, evergreen, multifarious and popular tree of tropics, with immense potential. The therapeutic effect attributed to *A. indica* are as diverse as antiplasmodial, larvicidal, antiviral, antiulcer, fungicidal, spermicidal, antibacterial, immunocontraceptive [10,11], anti-inflammatory, antinociceptive, antipyretic [12] insecticidal, anthelmintic, anti-implantation, immunomodulating [13,14], molluscicidal, nematocidal, antioxidant [15, 16], antidiabetic [17], anticancer [18] and antitrypanosomal [19].

The phytoconstituents of herbal products and their respective potency depends on the age of the plant, the plant part used, the plant extract derivative, the season the plant part was harvested and the method of extraction employed [20]. The aim of this study was to investigate the hypoglycemic effect of aqueous leaf extract of *A. indica* in alloxan induced white albino male mice.

Materials and Methods

Collection of medicinal plant

A. indica leaves were collected from Kijauri village Nyamira County, Kenya. A traditional medical practitioner provided ethnobotanical information of the plant collected. Botanical identity of the plant was authenticated by a taxonomist and a voucher specimen deposited at the National Museums of Kenya Herbarium, Nairobi.

After collection, the leaves were dried at room temperature under a shade. The dry leaves were then ground by use of an electric mill into fine powder. The powder was kept in closed, dry plastic air tight bags at room temperature.

Processing and extraction of the plant

One hundred grams of the powdered plant material was weighed into a conical flask and extracted in 1 liter distilled water at 60°C for 6 hours. It was left to cool, decanted then filtered under vacuum pump. The filtrate was then lyophilized for 72 hours using a Modulyo-freeze drier (Edward England). The granules were then weighed and refrigerated at -20°C in an airtight container until used for bioassay [2,3].

Experimental animals

Male Swiss White Albino mice weighed 21-25 g with a mean weight of 23 g were used in this study. The mice were housed in polypropylene cages, maintained under standard laboratory conditions of 12 hour light and dark sequence, at temperature of 25°C. The mice fed on rodent pellets and water *ad libitum*. The Principles of Laboratory Animal Care were followed.

Induction of hyperglycemia

Diabetes was induced experimentally by intraperitoneal administration of a freshly prepared 10% alloxan monohydrate obtained from Sigma Aldrich (Steinhein, Switzerland) [21], at 186.9 mg/kg body weight [2]. The animals were fasted for 8-12 hours, but allowed free access to water prior to use in bioassay. Blood glucose level was measured using a glucometer forty-eight hours after alloxan administration. Mice with blood glucose levels above 200 mg/dL (>11.1 mmol/L), were considered diabetic and suitable for use in the study [2].

Experimental design

The activity of aqueous leaf extract of *A. indica* was studied in alloxan-induced diabetic mice. The mice were divided into two portions. The first portion was used for anti-diabetic assay through intraperitoneal administration of aqueous plant extract. It consisted of the following groups of five mice each; Group I consisted of normal un-manipulated mice (the reference group of the experiment treated with the vehicle alone, 0.1 ml); Group II consisted of alloxan-diabetic negative control mice (treated with 0.1 ml vehicle alone); Group III consisted of alloxan-diabetic positive control mice treated with insulin (at 1 IU/kg body weight); Group IV through VIII consisted of alloxan-diabetic experimental mice treated with 25, 48.4, 93.5, 180.9 and 350 mg/kg body weight of aqueous plant extract. The second portion was used for antidiabetic assay through oral administration of aqueous plant extracts. The experimental design was similar to the first portion except that the reference drug used was glibenclamide (at 3 mg/kg body weight).

Blood sampling and *in vivo* hypoglycemic assays

The blood was collected by gently "milking" the tail from the body towards the tip after sterilizing the tail with 10% alcohol and then nipping to initiate bleeding. Blood sampling was repeated after 1, 2, 3, 4, 7 and 24 hours and blood glucose levels determined using a glucose

analyzer model (Hypogaurd, Woodbridge, England). The tips of tail were sterilized by swabbing with 70% ethanol after the operation.

Phytochemical screening

Phytochemical screening of the *A. indica* was done qualitatively to determine the class of secondary metabolites present which included, saponins, tannins, anthroquinones, alkaloids, terpenoids, flavonoids and sterols using standard procedures [22,23].

Data analysis

The data collected was entered in the Microsoft Excel Spread Sheet where it was cleaned and then transferred to SAS statistical software version 9.1.3 for analysis. The results of statistical analysis were expressed as Mean \pm Standard Deviation (SD). One-way ANOVA and post-ANOVA (Tukey's post hoc test) were used to compare the means of untreated group of normal mice with diabetic groups of mice treated with saline, conventional drug and plant extract at various dosages. Statistical significance was set at $p \leq 0.05$.

Results

In vivo antidiabetic effect of *Azadirachta indica* (*A. juss*) in alloxan induced diabetic mice

Azadirachta indica aqueous leaf extract yielded 8% brown powder. The intraperitoneal administration of *A. indica* decreased the blood glucose levels at all the five doses of 25, 48.4, 93.5, 180.9 and 350 mg/kg body weight in a dose independent manner (Table 1). This occurred in three phases, whereby in the first hour, the extract caused a steep decline in blood glucose levels, followed by a steady decline from second to the seventh hour. A gradual increase was then observed in the twenty fourth hour. In the first hour, the extract decreased blood glucose levels to 59.1%, 70.6%, 56.8%, 61.2% and 71.4% for 25, 48.4, 93.5, 180.9 and 350 mg/kg body weight doses, respectively, compared to insulin treated diabetic mice whose blood sugar levels were lowered to 50.3% within the first hour. By the fourth hour, all the five doses (25, 48.4, 93.5, 180.9 and 350 mg/kg body weight) had lowered blood sugar levels to 28.2%, 34.4%, 25.6%, 24.3% and 30.4%, respectively, compared to insulin treated diabetic mice whose blood sugar levels was decreased to 35.6% within the same hour (Figure 1).

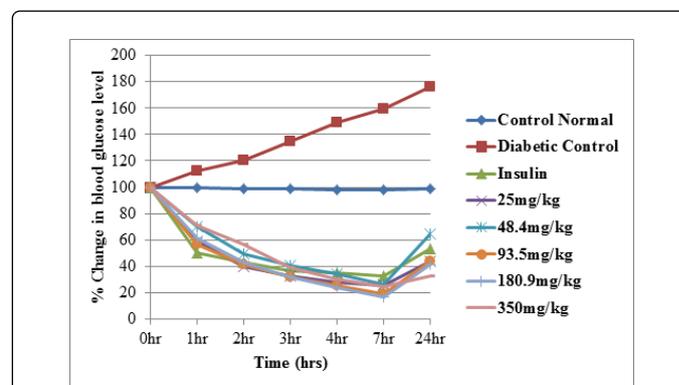


Figure 1: Mean percentage change in blood glucose levels of *Azadirachta indica* intraperitoneally administered in alloxan induced diabetic mice.

Blood Glucose Levels at Varying Times (mmoles/L)							
Treatment	0 hr	1 hr	2 hr	3 hr	4 hr	7 hr	24 hr
Control/Saline	5.24 ± 0.11 ^b	5.24 ± 0.54 ^c	5.20 ± 0.10 ^b	5.16 ± 0.05 ^b	5.12 ± 0.08 ^b	5.14 ± 0.09 ^b	5.16 ± 0.09 ^{cd}
Diabetic/Saline	15.1 ± 23.13 ^a	16.94 ± 3.32 ^a	18.20 ± 3.72 ^a	20.26 ± 3.44 ^a	22.40 ± 4.15 ^a	23.90 ± 4.24 ^a	26.08 ± 3.11 ^a
Diabetic/insulin	14.4 ± 3.12 ^a	7.06 ± 0.55 ^{cb}	5.94 ± 0.40 ^b	5.14 ± 0.11 ^b	4.92 ± 0.08 ^b	4.56 ± 0.21 ^b	7.40 ± 0.61 ^{cb}
25 (mg/kgbw)	17.8 ± 3.78 ^a	10.32 ± 1.59 ^b	7.00 ± 1.33 ^b	5.70 ± 0.97 ^b	4.94 ± 0.75 ^b	4.40 ± 0.58 ^b	7.52 ± 0.72 ^{cb}
48.4 (mg/kgbw)	13.4 ± 3.00 ^a	9.50 ± 2.33 ^b	6.60 ± 1.19 ^b	5.38 ± 0.86 ^b	4.54 ± 0.61 ^b	3.50 ± 0.50 ^b	8.32 ± 1.02 ^b
93.5 (mg/kgbw)	14.1 ± 2.18 ^a	7.90 ± 0.44 ^{cb}	5.78 ± 0.59 ^b	4.52 ± 0.54 ^b	3.60 ± 0.60 ^b	2.78 ± 0.49 ^b	6.22 ± 0.89 ^{cbd}
180.9 (mg/kgbw)	13.1 ± 3.79 ^a	7.82 ± 1.45 ^{cb}	5.54 ± 0.90 ^b	4.12 ± 0.75 ^b	3.12 ± 0.74 ^b	2.08 ± 0.43 ^b	5.16 ± 0.74 ^{cd}
350 (mg/kgbw)	12.9 ± 2.27 ^a	9.10 ± 1.28 ^b	7.24 ± 1.44 ^b	4.90 ± 0.93 ^b	3.86 ± 0.52 ^b	3.12 ± 0.66 ^b	4.18 ± 0.74 ^d

Results are expressed as Means ± SD for five mice 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).

Table 1: Effects of intraperitoneally administered aqueous leaf extracts of *Azadirachta indica* on blood glucose levels in alloxan induced diabetic mice.

Upon oral administration, the aqueous leaf extracts of *A. indica* lowered blood glucose levels appreciably from the first hour to the seventh hours at all the five doses of 25, 48.4, 93.5, 180.9 and 350 mg/kg body weight but not in a dose dependent manner (Table 2). By the second hour the extract had lowered the blood glucose levels to 40.2%, 48.2%, 47.7%, 44.4%, and 53.1% respectively for the five doses, compared to 56.6% for the conventional oral drug, glibenclamide (Figure 2).

Discussion

The alloxan-induced diabetic mice had a four to five fold elevation in blood glucose levels (5 mg/dl to 25 mg/dl) relative to the normal control mice. Studies show that chemical induction of diabetes by intraperitoneal administration of a diabetogenic agent, alloxan monohydrate induces Type I diabetes in experimental animals [24,25]. Alloxan monohydrate is derived from urea and induces diabetes by selective necrosis of pancreatic beta-cells of Langerhans [26]. This therefore, affects endogenous insulin synthesis and release making it biologically unavailable or insufficient and thus results in hyperglycemia [27]. The toxic alloxan confers its toxicological effect on pancreatic beta cells through inhibition of glucokinase enzyme, generation of free radicals, disturbances in intracellular calcium homeostasis and oxidation of essential sulphhydryl (-SH groups) [21,28,29]. The underlying mechanism of action involves the selective uptake of the compound due to its structural similarity to glucose as well as highly efficient uptake mechanism of the pancreatic beta-cells [30].

Intraperitoneal and oral administration of aqueous leaf extract of *A. indica* at 25, 48.4, 93.5, 180.9, and 350 mg/kg after 1, 2, 3, 4, 7 and 24 hours lowered the blood glucose levels in alloxan induced diabetic mice but not in a dose related manner. The plant extract at all doses and in both routes showed a comparable activity with the glibenclamide and insulin treated groups. Glibenclamide is an orally

administered standard drug that stimulates insulin secretion from beta cells of islets of Langerhans thereby reducing the glucose concentration. It promotes insulin secretion by closure of potassium-ATP channels, membrane depolarization and stimulation of calcium ion influx, an initial key step in insulin secretion [31].

Intraperitoneal administration of insulin as a standard antidiabetic drug enhances glucose uptake across the cell membrane by ATP-dependent translocation of glucose transporter GLUT4 to the plasma membrane [32]. Insulin causes cells in the liver, skeletal muscles, and fat tissue to absorb glucose from the blood. In the liver and skeletal muscles, glucose is stored as glycogen, and in fat cells (adipocytes) it is stored as triglycerides [32].

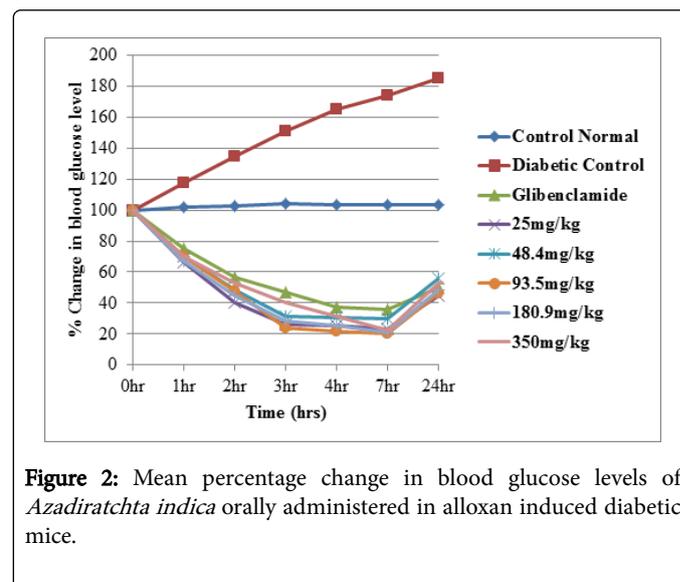


Figure 2: Mean percentage change in blood glucose levels of *Azadirachta indica* orally administered in alloxan induced diabetic mice.

Blood Glucose Levels at Varying Times (mmoles/L)							
Treatment	0 hr	1 hr	2 hr	3 hr	4 hr	7 hr	24 hr
Control/Saline	4.94 ± 0.18 ^b	5.04 ± 0.21 ^c	5.06 ± 0.11 ^c	5.14 ± 0.09 ^{cb}	5.12 ± 0.08 ^{cb}	5.12 ± 0.08 ^b	5.10 ± 0.10 ^b
Diabetic/Saline	14.92 ± 2.16 ^a	17.54 ± 2.39 ^a	20.02 ± 2.59 ^a	22.48 ± 2.61 ^a	24.42 ± 2.15 ^a	25.76 ± 2.15 ^a	27.30 ± 1.63 ^a
Diabetic/ Glen	14.44 ± 3.08 ^a	10.88 ± 2.94 ^b	8.12 ± 1.58 ^b	6.70 ± 1.18 ^b	5.14 ± 0.55 ^b	4.98 ± 0.34 ^{cb}	7.14 ± 1.11 ^b
25 (mg/kgbw)	14.56 ± 0.57 ^a	9.68 ± 1.19 ^b	5.86 ± 0.79 ^{cb}	3.88 ± 0.80 ^c	3.70 ± 0.46 ^{cb}	3.36 ± 0.40 ^{cbd}	6.44 ± 0.99 ^b
48.4 (mg/kgbw)	14.18 ± 2.33 ^a	9.86 ± 1.11 ^b	6.76 ± 0.73 ^{cb}	4.28 ± 0.43 ^{cb}	4.30 ± 0.45 ^{cb}	4.22 ± 0.79 ^{cbd}	7.96 ± 1.98 ^b
93.5 (mg/kgbw)	13.94 ± 0.74 ^a	9.78 ± 1.03 ^b	6.68 ± 1.54 ^{cb}	3.40 ± 0.79 ^c	3.06 ± 0.86 ^{cb}	2.80 ± 0.75 ^d	6.44 ± 1.61 ^b
180.9 (mg/kgbw)	15.20 ± 1.61 ^a	10.16 ± 1.36 ^b	6.74 ± 1.24 ^{cb}	4.24 ± 0.64 ^{cb}	3.84 ± 0.74 ^{cb}	3.18 ± 0.80 ^{cbd}	7.38 ± 1.60 ^b
350 (mg/kgbw)	14.88 ± 2.84 ^a	10.12 ± 0.97 ^b	7.48 ± 1.44 ^b	5.44 ± 2.20 ^{cb}	4.32 ± 1.25 ^{cb}	3.12 ± 0.70 ^{cd}	7.40 ± 1.73 ^b

Results are expressed as Means ± SD for five mice 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).

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

Qualitative phytochemical composition of *Azadirachta indica* A. Juss

As depicted in Table 3, *A. indica* contained free and bound anthraquinones, saponins alkaloids, tannins, terpenoids, sterols and flavonoids.

Phytochemicals	<i>Azadirachta indica</i>
Alkaloids	+
Sterols	+
Terpenoids	+
Saponins	+
Tannins	+
Flavanoids	+
Free and Bound Anthraquinones	+
Key: Present phytochemicals are denoted by (+) sign	

Table 3: Qualitative phytochemical screening of aqueous leaf extract of *Azadirachta indica*.

The probable mechanism for the hypoglycemic effect of the aqueous leaf extract of *A. indica* could be linked to potentiation of insulin release from pancreatic beta cells of islet or by elevating the peripheral glucose uptake and utilization [33,34]. The antidiabetic effect of the extract might be due to the increased release of insulin from remnant β-cells and/or regenerated β-cells [35,36], restored insulin sensitivity [37], malabsorption of dietary carbohydrates in the small intestine [38] or facilitated uptake of blood glucose by peripheral tissues mediated by an insulin dependent glucose transporter, GLUT-4 [39].

The hypoglycemic effect of this plant extract could be attributed to the presence of phytochemical constituents including, flavonoids, free and bound anthraquinones, tannins, terpenoids, sterols, saponins and

alkaloids that have been associated with antidiabetic activity [40]. As reported by [41], flavonoids enhances lipogenesis and glucose transport in the adipocytes hence lowering blood sugar [40]. The alkaloids promotes the regeneration of pancreas islets thereby restoring insulin secretion [40]. Tannins and saponins have also been shown to have hypoglycemic activity [42] and [43]. The plants contained terpenoids which are heart-friendly because they help to reduce diastolic blood pressure and lower the sugar level in blood [44]. Anthraquinones which have earlier been reported to lower blood glucose are used also in the treatment of peripheral neuropathy [43].

The observed dose independent hypoglycemic action of the plant extract in this study suggests that the extract may have been absorbed in the cell system through active transport, where a particular concentration saturation of the extract occurred resulting to the rest of extract being excreted [34]. A gradual increase of blood sugar levels observed from 7th-24th hrs following the oral and intraperitoneal administration of *A. indica* may have been due to the extract having a short half-life or it might have been prone to fast hepatic metabolism and renal clearance [45].

Conclusion

The results from this study indicated that *Azadirachta indica* had hypoglycemic effects in alloxan induced diabetic mice, thus scientifically verifying its folkloric use in the management of diabetes mellitus. These actions were exhibited due to cumulative effect of phytoconstituents present in the extract including free and bound anthraquinones, alkaloids, tannins, terpenoids, flavonoids, saponins and sterols. However, further investigation should be done in order to isolate the constituents responsible for the antidiabetic effect of this plant through bioassay guided fractionation. Moreover, the organic solvent extraction for this plant should also be done to compare the antidiabetic activities of both aqueous and organic fractions.

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