

Hypoglycemic Property of Different Fractions of Crude Ethanol Extract of *Azadirachta indica* Leaves in Streptozotocin-induced Diabetic Rats

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Abstract

Background: *Azadirachta indica* (AI) is used in the traditional management of diabetes in Nigeria. This study investigated the hypoglycemic potential of *Azadirachta indica* leaf fractions (AILF) in streptozotocin-induced diabetic male rats.

Methods: Ethanol crude extract of AI leaf was fractionated with solvents of increasing order of polarity (n-hexane, chloroform, ethyl-acetate, and n-butanol). Phytochemical analysis was done using standard methods. Diabetes mellitus was induced in the rats intraperitoneally with 50mg/kg bodyweight of streptozotocin. Treatment with the AILF was done for a period of twenty-eight days to ascertain which of the AILF possess better hypoglycemic property. Fasting blood glucose levels were checked at one week intervals using One Touch Glucometer and Test Strips.

Results: Bioactive compounds detected in moderate and trace amounts in the AILF include alkaloids, cyanogenic glycosides, flavonoids, phenols, saponin and tannins. Induction of diabetes caused a significant ($p < 0.05$) increase in the fasting blood glucose levels of the experimental animals followed by observable weight loss. Treatment with the AILF and the standard drug caused a significant weight gain in the animals in week 4 compared with the diabetic-untreated control. The group that was treated with 400mg/kg bodyweight of ethyl acetate fraction showed a better weight gain which was observed from the 2nd week to the 4th week of treatment.

The n-hexane and ethyl acetate fraction decreased the fasting blood glucose levels more significantly within the four weeks of treatment compared with chloroform fraction, n-butanol fraction and the standard drug. However, a better and significant ($p < 0.05$) reduction was observed in the fasting blood glucose levels of the group treated with 400mg/kg bodyweight of ethyl acetate fraction from week 1 to week 4 of the treatment compared with the diabetic-untreated.

Conclusion: The data from this study suggest that ethyl acetate fraction of *A. indica* leaf has a better hypoglycemic property and can serve as a potential adjuvant for the development of an effective antidiabetic drug.

Keywords: Hypoglycemic, Bioactive compounds, Diabetes mellitus, *Azadirachta indica*, Fractions, Ethyl acetate fraction.

I. INTRODUCTION

Diabetes is a group of metabolic disorders characterized by hyperglycemia resulting from defects in insulin secretion, action or both. Ogbonnia *et al.*¹ reported that it is a major degenerative disease in the world today, affecting at least 15 million people and leading to secondary diseases which include atherosclerosis, hypertension and microcirculatory disorders. Research has shown that prevalence of diabetes is higher in developed countries than in the developing countries in the mid 90's as reported by King *et al.*². However, it has been estimated that the number of adults with diabetes will increase to 300 million by the year 2025² and the world health organization predicts that the number will reach 366 million or more by the year 2030³.

Although, there is paucity of data on the widespread of diabetes in Nigeria and other African countries, available data suggest that diabetes is emerging as a major health problem in Africa, including Nigeria⁴. It is the fourth leading cause of death in the most developed countries and there is significant evidence that it is epidemic in many developing and newly industrialized nations. Okutan *et al.*⁵ reported that patients with uncontrolled diabetes mellitus usually experience heart failure which indicates that hyperglycemia may be responsible for the disease. Hyperglycemia produces symptoms of polyuria, polydipsia and polyphagia. It is also associated with long term damage and failure of various organs such as eyes, kidney, liver, nerves, heart and blood vessels. It is also associated with alteration in the plasma lipid and lipoprotein profile⁶. Besides the use of insulin as a therapeutic approach to control diabetic hyperglycemia, various oral antidiabetic agents such as sulfonylurea, glinides, biguanides, α -glucosidase inhibitors, thiazolidinediones, dipeptidyl-peptidase-4 inhibitors are also used⁷. These drugs are potent in their mode of action but exert serious side effects in long term use. Hence, great interest had been developed worldwide for the search of plant based new therapeutic agents which may promise safety along with efficacy in the treatment of diabetes.

Azadirachta indica, has attracted much interest within the worldwide medical community in recent years, due to its wide range of medicinal properties. It has been used extensively in Ayurvedic, homeopathic, and folk medical traditions over thousands of years^{8,9}. A vast array of biologically active compounds has been isolated from this plant, many of which have been studied in laboratory conditions for their pharmacological properties. Modern scientific research has validated the traditional uses of *Azadirachta indica* for the maintenance of general health and especially for skin disorders including acne. It is also known to be effective in treating arthritis, blood disorders, bronchitis, cough, diabetes, drowsiness, jaundice, nausea, obesity, syphilis, parasites, rheumatism, skin diseases, malaria and tumors^{8,10,11}. The crude ethanol extract of *Azadirachta indica* leaf has earlier been used in the treatment of T2D¹². The present study is concerned with comparing different fractions of crude ethanol extract of *A. indica* leaf to know the one with better hypoglycemic potential which can serve as an adjuvant to the pharmaceutical companies in the preparation of antidiabetic drugs.

II. MATERIALS AND METHODS

2.1 Sample Collection and Identification

The leaves of *A. indica* were collected from Mgbakwu, Awka North L. G. A, Anambra State, Nigeria. The sample was identified by a taxonomist in the Department of Botany, Nnamdi Azikiwe University, Awka. The voucher number as deposited in the herbarium of Nnamdi Azikiwe University, Awka is 14.

2.2 Preparation of Ethanol Extract of *A. indica* Leaf

The leaves were properly washed and air dried at room temperature for two weeks. The dried leaves were pulverized into powder using corona manual grinding machine. Exactly 2kg of the pulverized leaf powder of *A. indica* was soaked in 8 litres of 80% ethanol for 24 hrs for ethanol extraction. The ethanol mixture was sieved using muslin cloth and filtered using Whatman no 1 filter paper. The filtrate was concentrated using rotary evaporator at 50°C. The percentage yield of the extract after extraction was 156g. The ethanol extract was stoppered in universal bottles and preserved in the refrigerator for use.

2.3 Fractionation of *Azadirachta indica* Leaf Extract

The crude ethanol leaf extract (156g) was fractionated by the method of Wu *et al.*¹³. The method involved successive extraction by increasing polarity with n-hexane, chloroform, ethyl acetate, n-butanol and water. Thirty grams (30g) of the ethanol extract was dissolved in 250ml of Methanol/Water (MeOH/H₂O) (9:1) mixture and shaken with n-hexane (3 x 100ml). Combined extract was left to dry on the bench to yield n-hexane fraction. Methanol (MeOH) was further fractionated by successive solvent extraction with chloroform (4 x 100ml), ethyl acetate (2 x 100ml) and n-butanol (3 x 100ml). Each fraction was left to evaporate to dryness on the

bench to yield n-hexane fraction (5.71g), chloroform fraction (6.43g), ethyl acetate fraction (11.52g), n-butanol fraction (2.9g) and water fraction (3.44g).

2.4 Phytochemical Analysis

Phytochemical tests were done on the *A. indica* leaf fractions using standard phytochemical methods as described by Harbone¹⁴, Sofowora¹⁵, Trease and Evans¹⁶. The phytochemicals that were tested include anthracine glycosides, alkaloids, cardiac glycosides, cyanogenic glycosides, flavonoids, phenols, saponins and tannins.

2.5 Experimental Animal and Grouping

A total of thirty (35) male albino rats of Wistar strain weighing between 120 and 125g were purchased from Chris Experimental Animal Farm and Research Laboratory, Awka, Anambra State, Nigeria and used for the study. The rats were randomized into seven (7) groups (n=5) and allowed to acclimatize for two weeks at the Department of Applied Biochemistry, Faculty of Biosciences, Nnamdi Azikiwe University, Awka before the commencement of the research. They were maintained in aluminium cages throughout the period of the experiment. The animal grouping is as follows:

Group A: Normal Control

Group B: Diabetic-untreated

Group C: Standard Drug (100mg/kg bodyweight Glucophage)

Group D: 400mg/kg bodyweight of n-hexane fraction

Group E: 400mg/kg bodyweight of chloroform fraction

Group F: 400mg/kg bodyweight ethyl acetate fraction

Group G: 400mg/kg bodyweight of n-butanol fraction

2.6 Investigation of Hypoglycemic Property of Different Fractions of *A. indica* Leaves

The blood glucose levels of the rats were checked using One Touch Glucometer and Test Strips. The weights of the rats were also recorded. The rats (Groups B-G) were then fasted for 16 hours, but with free access to water after which they received an intraperitoneal injection of streptozotocin 50mg/kg bodyweight¹⁷. The rats were orally given 5ml each of 5% glucose solution after 2 hours to prevent hypoglycemia. The animals were allowed free access to food and water after streptozotocin injection. After 48 hours of the streptozotocin administration, blood was collected *orbito rectally* and the glucose concentrations were determined using a One Touch Glucometer (Life Scan, USA) and Test Strips based on the method of Trinder¹⁸.

Diabetes was confirmed to have been induced when the fasting blood glucose level was observed to be far much higher than normal (between 60mg/dl to 110mg/dl or 70mg/dl to 120mg/dl) to above 200mg/dl. The *A. indica* leaf fractions that were investigated for antidiabetic property at the same dose of 400mg/kg bodyweight include n-hexane fraction,

chloroform fraction, ethyl acetate fraction and n-butanol fractions. Group A was left uninduced and used as the normoglycemic rats.

2.7 Data Analysis

Data obtained from the experiments were analyzed using the Statistical Package for Social Sciences (SPSS) software for windows version 25 (SPSS Inc., Chicago, Illinois, USA). All the data were expressed as Mean ± SEM. Statistical analysis of the results obtained were performed by using ANOVA tests to determine if significant difference exists between the mean of the test and control groups. The limit of significance was set at $p < 0.05$.

III. RESULTS

3.1 Result of the Phytochemical Analysis

The result of the phytochemical analysis showed that alkaloids were detected in substantial amount in the chloroform fraction while n-butanol fraction contains alkaloids in moderate amount. Alkaloids were detected in trace amount in the n-hexane and ethyl acetate fraction (Table 1). Cardiac glycosides were detected in trace amount in the ethyl acetate fraction only but were not detected in the other fractions. Cyanogenic glycosides were moderately detected in the chloroform fraction and in trace amount in the n-hexane fraction. Flavonoids were moderately detected in the chloroform and ethylacetate fraction while it was found in trace amount in the n-hexane and n-butanol fractions. Phenols were moderately detected in the n-hexane and n-butanol fractions while it was detected in trace amount in the chloroform and ethylacetate fractions. Saponin and tannins were detected only in the ethylacetate and n-butanol fractions (Table 1).

Table 1: Results of the phytochemical analysis of the fractions of crude ethanol extract of *A. indica* leaf.

S/no	Phytoconstituents	n-hexane Fraction	Chloroform Fraction	Ethyl acetate Fraction	n-butanol Fraction
1	Anthracine glycosides	-	-	-	-
2	Alkaloids	+	+++	+	++
3	Cardiac glycosides	-	-	+	-
4	Cyanogenic glycosides	+	++	-	-
5	Flavonoids	+	++	++	+
6	Phenols	++	+	+	++
7	Saponin	-	-	+	+
8	Tannins	-	-	+	+

+ Present ++ Moderately present +++ Present in substantial Amount - Not present

3.2 Results of the effect of the different fractions of *A. indica* leaf on Bodyweight.

Induction of diabetes which was visible at week 0 caused an observable decrease in the bodyweight of all the groups except the normal control group that was not induced (Table 2). The bodyweight of the rats started increasing from week 1 and continued to the fourth week in the course of the treatment. There was no significant increase in bodyweight during the week one of the treatments for all the fractions. The group that was treated with ethyl acetate fraction showed a significant ($p < 0.05$) increase in bodyweight from week 2 of

the treatment to week 4 when compared with the diabetic-untreated control. The group that was treated with n-hexane fraction showed a significant ($p < 0.05$) increase in weight from week 3 to week 4 when compared with the diabetic-untreated control. All the groups treated with the fractions and the standard drug showed a significant ($p < 0.05$) increase in bodyweight at week four of the treatment when compared with the diabetic-untreated. However, the group that was treated with ethyl acetate fraction showed a better weight gain in the course of treatment followed by the group treated with n-hexane fraction (Table 2).

Table 2: The effect of the different fractions of *A. indica* leaf on the weight of diabetic rats

Groups/Dose	Weight (g) Initial	Weight (g) Week 0	Weight (g) Week 1	Weight (g) Week 2	Weight (g) Week 3	Weight (g) Week 4
Normal Control	123.9±3.46	125.2±0.63	134.7±2.19 ^c	139.7±2.61 ^c	147.5±2.73 ^c	155.8±3.25 ^c
Diabetic-untreated	123.3±1.49	112.3±6.30	110.3±10.2	112.5±8.61	116.7±5.60	119.6±2.18
100mg/kg Glucophage	123.3±6.15	117.5±3.26	121.6±3.01	126.1±4.92	134.0±2.33	143.3±6.35 ^c
400mg/kg n-hexane	124.5±3.24	112.6±5.41	120.8±3.20	129.6±4.11	136.7±3.71 ^c	140.5±2.13 ^c
400mg/kg Chloroform	124.2±3.81	114.3±2.35	117.6±11.3	123.7±6.43	129.3±5.37	137.8±2.11 ^c
400mg/kg Ethyl acetate	121.5±0.95	110.6±4.62	123.7±8.52	131.5±3.54 ^c	142.6±2.06 ^c	148.1±6.77 ^c
400mg/kg n-butanol	123.1±1.34	117.2±2.13	120.2±6.12	124.9±2.32	131.6±3.26	135.4±5.05 ^c

^asignificant ($p < 0.05$) increase with respect to normal control; ^bsignificant ($p < 0.05$) reduction with respect to normal control; ^csignificant ($p < 0.05$) increase with respect to diabetic-untreated; ^dsignificant ($p < 0.05$) decrease with respect to diabetic-untreated.

3.3 Result of the Hypoglycemic Property of the different Fractions of *A. indica* leaf.

Induction of diabetes mellitus significantly ($p<0.05$) increased the fasting blood glucose level of all the groups except the normal control which was not induced. Treatment was done for four weeks using the AILF and standard drug to compare the hypoglycemic potential of the fractions and the standard drug. The fractions were evaluated for their potential to reduce blood glucose level on streptozotocin induced diabetic rats. The same dose (400mg/kg bw) of the fractions of the ethanol extract of *A. indica* was administered to the different groups of rats and the effect on the fasting blood glucose levels were recorded at weekly interval (Table 3). The group

of rats treated with the standard drug showed a significant ($p<0.05$) reduction in the fasting blood glucose levels starting from week 2 to week 4 compared with the diabetic-untreated control. The groups treated with the n-hexane and n-butanol fractions showed significant ($p<0.05$) decrease in their fasting blood glucose levels compared with the diabetic-untreated control but their decrease was not consistent. The group of rats treated with 400mg/kg bw of the ethyl acetate fraction showed significant ($p<0.05$) reduction in their fasting blood glucose levels from week 1 to week 4 compared with the diabetic-untreated group. This reduction observed in the fasting blood glucose level was observed to be consistent from week 1 to week 4 of the experiment (Table 3).

Table 3: The effect of the different fractions of *A. indica* leaf on the fasting blood glucose levels of diabetic rats

Groups/Dose	Glucose Level (mg/dl) Initial	Glucose Level (mg/dl) Week 0	Glucose Level (mg/dl) Week 1	Glucose Level (mg/dl) Week 2	Glucose Level (mg/dl) Week 3	Glucose Level (mg/dl) Week 4
Normal Control	82.5±2.43	79.3±0.64	76.8±1.29	85.2±2.76	73.7±0.16	75.5±1.27
Diabetic untreated	94.0±1.55	509.0±18.5 ^a	579.4±12.8	585.4±53.0	567.5±7.83	586.3±9.54
100mg/kg Glucophage	73.8±3.07	515.9±10.4 ^a	431.0±11.2	349.6±8.13 ^d	313.8±5.82 ^d	306.3±10.6 ^d
400mg/kg n-hexane	79.0±1.92	498.6±8.57 ^a	381.6±7.99 ^d	439.5±14.3	319.2±7.26 ^d	329.3±4.91 ^d
400mg/kg Chloroform	86.4±1.28	511.2±5.61 ^a	499.6±8.21	487.3±6.62	431.0±16.5	456.0±10.4
400mg/kg Ethyl acetate	84.2±2.66	573.8±7.13 ^a	330.1±9.75 ^d	342.5±8.65 ^d	305.0±7.01 ^d	273.4±5.67 ^d
400mg/kg n-butanol	72.9±3.90	532.4±9.21 ^a	516.9±11.0	317.2±6.81 ^d	413.6±7.12	419.0±16.2

^asignificant ($p<0.05$) increase with respect to normal control; ^bsignificant ($p<0.05$) reduction with respect to normal control; ^csignificant ($p<0.05$) increase with respect to diabetic-untreated; ^dsignificant ($p<0.05$) decrease with respect to diabetic-untreated.

IV. DISCUSSION

Diabetes is known to manifest due to free radical generation leading to oxidative stress. The increasing widespread of diabetes menacing the quality of human life requires extensive and qualitative research into development of efficient hypoglycemic agent free of adverse effects. Thus, medicinal plants are continually being investigated using animal model of the disease with the anticipation of developing a comparatively safe anti-diabetic plant-based products^{19,20}. In the present study, the effect of different fractions of crude ethanol extract of *A. indica* leaf on bodyweight and fasting blood glucose level of streptozotocin-induced diabetic rats was investigated. Intraperitoneal administration of streptozotocin to rats significantly ($p<0.05$) increased the blood glucose levels 48 hours after injection as well as decreased bodyweight.

Phytochemicals are naturally occurring chemical compounds with antioxidant activity found in plants which provide health benefits to humans. The antioxidant activity of the plant extracts can be as a result of the important phytochemicals detected in it and may be responsible for the hypoglycemic potential of the fractions of the crude ethanol extract of *A. indica* leaf. Phytochemical analysis of the ethanol extract of *A. indica* revealed the presence of important phytochemicals such as alkaloids, cardiac glycosides, cyanogenic glycosides,

flavonoids, phenols, saponin and tannins²¹. Flavonoids have the capacity to act as powerful antioxidants which can protect the human system from reactive oxygen species and other free radicals thereby lowering blood glucose concentration²². It has been reported that saponin can cause hypoglycemia²³.

In this study, sequential extraction techniques using different solvents in order of increasing polarity (n-hexane, chloroform, ethyl-acetate, n-butanol and water) were used to obtain AILF from the crude ethanol extract (since the nature, polarity and solubility of the antidiabetic bioactive constituents in the leaf of AI were not known). In general n-hexane is used to extract compounds of low polarity such as fatty acids, waxes, some alkaloids and terpenoids; chloroform and ethyl-acetate extracts both medium polarities and some polar compounds such as flavonoids, tannins and some terpenoids; whereas, n-butanol extracts highly polar compounds like carbohydrates, amino acids and their derivatives²⁴.

The data from this study revealed that ethyl acetate fractions of AI leaf produced significant ($p<0.05$) reduction in fasting blood glucose level in diabetic rats (Table 3). The ethyl acetate fraction may exert its effect due to increased insulin secretion or improvement in glucose uptake in tissue as it contains bioactive compounds like flavonoids, phenols and saponins that are also present in other plants which produce similar effects²⁵. The implication is that AILF stimulates

increased glucose utilization and glucose tolerance through body tissues of the diabetic rats. Among all the AILF administered, ethyl acetate fraction was observed to have the most significant hypoglycemic potency.

V. CONCLUSION

The results of this research suggest that ethyl acetate fraction of *A. indica* leaf exhibits better hypoglycemic property compared with the n-hexane, chloroform and n-butanol fractions. The hypoglycemic property of the ethyl acetate fraction makes it a useful alternative to the standard antidiabetic drugs and can serve as an adjuvant in the development of antidiabetic drugs.

ETHICAL APPROVAL

All authors hereby declare that “Principles of Laboratory Animal Care” were followed. All experiments have been examined and approved by the ethics committee of Nnamdi Azikiwe University, Awka, Nigeria in accordance with the Institutional Animal Care and Use policy in Research, Education and Testing.

COMPETING INTERESTS

The authors state no conflict of interest in this research.

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