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Antidiabetic and antidiabetogenic properties of the aqueous extracts of *Azadirachta indica* leaves on alloxan induced diabetic wistar rats

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Key words: *Azadirachtaindica*, Aqueous extract, Hyperglicaemia, Antidiabetic, Antidiabetogenic.

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Abstract

There is a surging interest in the use of plant derived products in the treatment of a lot of diseases in recent decades. This present study was designed to evaluate the anti-diabetic and anti-diabetogenic activity of aqueous extract of *Azadirachta indica* leaf in normal as well as alloxan induced diabetic rats. The phytochemical analyses as well as the 24-hour acute toxicity test of the orally administered aqueous extract were carried out using standard methods. Diabetes was induced in the rats by a single intraperitoneal dose of 200mg/kg$_{bw}$ of Alloxan. The aqueous extract of *Azadirachta indica* was administered orally in three (3) doses of 50mg/kg$_{bw}$, 100mg/kg$_{bw}$ and 150mg/kg$_{bw}$ for six days. Antidiabetogenic potential of the extract was investigated by pretreatment of rats with 100mg/kg bw of the aqueous extract before diabetic induction. The phytoconstituents identified in the aqueous extract were saponin, condensed tannins, flavonoids, alkaloids and phenolic group. The result of the 24-hour acute toxicity test of the orally administered aqueous extract of *A. indica* leaves gave an LD_{50} of 4.47g/kg$_{bw}$. All the doses of the aqueous extract showed a progressive daily percentage reduction in blood sugar level and were significantly ($P<0.05$) effective in reducing blood sugar level in alloxan induced hyperglycaemic rats on the sixth day of treatment when compared to the control diabetic rats that were treated with glibenclamide (a standard antidiabetic drug) at a dose of 0.214mg/kg$_{bw}$ on that same day. The 100mg/kg$_{bw}$ had the highest reduction in blood glucose level. Pre-treatment with the aqueous extract at a dose of 100mg/kg$_{bw}$ for fourteen days showed a significant protection ($P<0.05$) from alloxan induced diabetogenic effect resulting in a 39.5% reduction in blood glucose level when diabetes was induced. The efficacy of the extract in protecting diabetic induction with alloxan was better at two weeks than after 3 days. These results suggest that the leaf extract of *A. indica* has both anti-diabetic and antidiabetogenic effects and could be of great use in the treatment and management of diabetes mellitus, controlling blood sugar level as well as in preventing or delaying the onset of diabetes mellitus.

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Introduction

From time immemorial, medicinal plants have been of great importance to the health of man, and the whole world. Man has continually explored plants in order to assess the importance of developing natural, sustainable, and affordable drugs and cosmetics since the dawn of civilization (Owolabi *et al.*,2013).

Diabetes mellitus has become a major public health problem globally. It is the seventh leading cause of death aswell as the third if all the fatal complications are taken into consideration. (Ene *et al.,* 2007; Apea and Faruk 2013). The disease is a disorder of carbohydrate, fat and protein metabolism characterized by chronic hyperglycemia, and glucosuria with secondary disturbance of protein and fat metabolism (Ene, *et al.,* 2007). It can progress undetected for a long time. Type 1 diabetes is directly linked with deficient insulin secretion from β-pancreatic cells. Type 2 diabetes on the other hand, is closely associated with obesity/overweight, sedentary lifestyle, unhealthy dietary-habit, and genetic predisposition and characterized by initial phases of progressive insulin resistance (Hazra *et al.*, 2011). Orthodox options for treatment presently includes: repaglinide, metformin, tolbutamide, phenformin, pioglitazone, rosiglitazone, chlorpropamide, glipizide, etc. these drugs are quite effective but their uses are usually affected by high cost, undesirable side effects, and accessibility, thus giving rise to the sustained interest in plant derived natural products for treatment of diabetics till date. (Hazra *et al.*, 2011).

*Azadirachta indica (*Neem) has been extensively used in ayurveda, unani and homoeopathic medicine and has become a cynosure of modern medicine in india and other parts of the world. Neem oil, bark and leaf extracts have been reported to be used in folk medicine to control leprosy, intestinal helminthiasis, respiratory disorders, and constipation and also as a general health promoter. Its uses for the treatment of rheumatism, chronic syphilitic sores and indolent ulcer have also been shown. Neem oil also found use in control of various skin infections. The bark, leaf, root, flower and fruit all show potentials to cure blood morbidity, biliary afflictions, itching, skin ulcers, burning sensations and pthysis (Biswas *et al.*, 2002). Neem amongst other components contains isoprenoids (like diterpenoids and triterpenoids containing protomeliacins, limonoids, azadirone and its derivatives, gedunin and its derivatives, vilasinin type of compounds and C-secomeliacins such as nimbin, salanin and azadirachtin) and nonisoprenoids, which are proteins or amino acids and carbohydrates (polysaccharides), sulphur compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcone, coumarin and tannins, aliphatic compounds, etc (Biswas *et al.*, 2002) these are attributable for some of its medicinal activities. Antidiabetic potentials of extracts of different parts of *Azadirachta indica* using different solvents have been reported by some trado-medical practioners and some literatures (Mamun-or-Rashid *et al.,* 2014; Khosla *et al.,* 2000; Bopana *et al.,* 1997). Majority of this work have concentrated on just antidiabetic effect of this plant with no attention on the prophylactic (antidiabetogenic) potentials. In view of the potential medicinal benefits of the neem plant and the dramatic rise in the prevalence of diabetes mellitus, this present study investigates the anti-diabetic and anti-diabetogenic (prophylactic) efficacy of aqueous extract of *Azadirachta indica* leaf in normal as well as alloxan induced diabetic rats.

Materials and methods

Sample Collection

The leaves of *A. indica* were collected from the Reserve Forest of Ministry of Agriculture, Awka. Anambra State.

Sample Identification

The sample was identified by a taxonomist in the Department of Botany, NnamdiAzikiwe University, Awka.

Sample Preparation

The leaves were properly washed and air dried at room temperature for two weeks. The dried leaves were ground into powder using corona manual grinding machine. Exactly 300g of the ground leaves

of *A. indica* were soaked in 3 litres of water for 24 hrs. it was sieved and filtered using whatman no 1(125mm) filter paper. The filtrates were separately lyophilized (freeze dried) to powder, and put in separate stoppered universal bottles.

Chemicals

Alloxan monohydrate, Bromine water, lead acetate, hydrochloric acid, tetraoxosulphate (vi) acid, sodium chloride, ferric chloride, meyer'reagent, wagner's reagent, millon's reagent manufactured by sigma, Germany. All other chemicals used in our study were of analytical grade.

Phytochemical Screening

Phytochemical tests were carried out on the aqueousextract using standard phytochemical tests as described by Harbone (1973), Sofowora (1993), Teases and Evans (1996). The following phytochemicals were assayed for: Anthracine Glycosides, Saponins, Tannins, Flavonoids, Cyanogenic Glycosides, Alkaloids, Cardiac Glycosides and Phenolic Group.

Experimental Animals

Exactly fifty (50) wistar albino rats and 48 mice were purchased from the animal house at the Faculty of Pharmaceutical Sciences, NnamdiAzikiwe University, Agulu, Anambra State. They were maintained and housed in cages in the Department of Applied Biochemistry Laboratory, NnamdiAzikiwe University, Awka. They were allowed to acclimatize with the environment for one week before use. The animals were kept on guinea growers mash pellets that were obtained from Eke market, Awka. The animals were weighed and their glucose levels were measured accordingly using One Touch basic Glucometer and test strips (Code-12).Blood was collected using orbito-recto method (that is from the tail). Diabetes mellitus was induced intraperitoneally with alloxan (200mg/kg).

Acute toxicity test (**LD_{50}** Determination) of *A. indica*.

The Median Lethal Dose (LD_{50}) was determined using Wistar Albino Mice. Test animals were randomly divided into four (4) groups of six mice each and administered graded doses of 2g, 3.5g, 4.5g and 5.5g of aqueous extract of *A. indica* per kg body weight.

Investigation of Anti-diabetic Properties of Aqueous extracts of Azadirachtaindica Leaves on Alloxan Induced Wistar Rats

The blood glucose level of the rats was checked before the administration of alloxan using One Touch Glucometer and test strips. The rats were then fasted for 16 hours, but with free access to water after which they received an intraperitoneal injection of alloxan200mg/kg body weight (Etuk, 2010). The rats were orally given 20ml each of 5% glucose solution after 2 hours to prevent hypoglycemia. The animals were allowed free access to food and water after alloxan administration. After 48 hours of the alloxan administration, blood was collected orbito rectally and their glucose concentrations were determined using a One Touch Glucometer (Life scan, USA) and test strips based on method of Trinder (1972). Diabetes is confirmed to have been induced if the glucose level is observed to be far much higher than normal (above 140mg/dl).

Determination of Anti-Diabetic Effects of Extracts

The diabetic rats were divided by random selection into five groups of five rats each. The three test groups were administered 50mg, 100mg and 150mg doses of aqueous extract of *A. indica* leaves per kg body weight respectively. Administration in all instances was by gavage using intubation cannular. These treatments were repeated for six consecutive days. The positive control group of five rats was similarly administered 5mg of glibenclamide (a standard anti-diabetic drug) per kg body weight for six consecutive days. Another group of five rats used as negative control did not receive any treatment.

Determination of the Anti-Diabetogenic Effects of Extracts

Exactly 100mg/kg body weight of the aqueous extract was administered for three consecutive days and fourteen consecutive days to two respective groups of rats by gavage using intubation cannular. Exactly

100mg/kg body weight of the aqueous leaf extractwas administered for fourteen consecutive days to another group of rats by gavage using intubation cannular. Subsequently induction of diabetes in these groups of rats was carried out. Blood glucose levels of therats were determined before the administration of the extract and consistently for the three days and fourteen days of repeated dose before induction of diabetes. After treatment with alloxan the blood glucose level was again determined to establish to what extent the extracts prevented onset of diabetes.

Statistical Analysis of Results

Data obtained from the experiments were analyzed using the Statistical Package for Social Sciences (SPSS) software for windows version 21 (SPSS Inc., Chicago, Illinois, USA). All the data were expressed as Mean ± SEM. The limit of significance was set at $p<0.05$. Data obtained were subjected to test of significance using Student's t-test to determine if significant difference exists between the mean of the test and control.

Results

Results of the Phytochemical Analysis of A. indica Leaf Extracts

The results obtained from the qualitative phytochemical analysis of the aqueous extract of the leaves of *A. indica* are shown in table 1.

Table 1. Phytochemical analysis of the aqueous extract of *A. indica*.

S/No	Phytochemical constituents	Test	Aqueous extract
1	Anthracine Glycosides		-
2	Saponin	i. Frothing test	+++
		ii. Emulsion test	+++
3	Tannins	i. Bromine Water test	+
		ii. Acid test	-
		iii. Lead Acetate test	-
4	Flavonoids	i. Ferric Chloride test for Phenolic nucleus	-
		ii.Lead Acetate test	++
		iii. Sodium Hydroxide test	++
5	Cyanogenic Glycosides		-
6	Alkaloids	i.Wagner's reagent test	+
		ii.Meyer's reagent test	+
7	Cardiac Glycosides	i.HuppertSalkowski test	+
8	Phenolic Group (Tyrosine Millions method)		+

+ Present ++ moderately present +++ Appreciable amount.

Median Lethal Dose (LD_{50}) *of Aqueous Extracts of A. indica Leaf*

The result of a 24 hour acute toxicity test of orally administered aqueous extract of *A. indica* leaf in albino mice is shown in table 2.

It was observed that those fed with 2g/kg body weight of the extract were slightly weak but no death was recorded but at 3.5g/kg body weight, they appear weaker with 1 death recorded. At 4.5g/kg body weight, 3 mice died while the rest were extremely weak and at 5.5g/kg body weight, 5 mice died.

From the above data the LD_{50} was determined by plotting percentage mortality against log dose. From the plot shown in fig 1, the LD_{50} was derived. Log dose at 50% = 3.65.

LD_{50} was derived by determining the antilog of log dose.

Therefore $\log^{-1} 3.65 = 4.47$g/kg.

Animal Studies

Assessment of Anti-Diabetic Efficacy of Aqueous Extracts of A. indica leaves

Figures 2a, 2b, and 2c, show the effect of treatment with graded doses of aqueous extract of *A. indica* leaves on the blood glucose profile of the alloxan induced diabetic albino rats. Diabetes was induced within 48hrs as shown by the increased blood glucose level on day 2 in all instances. The subsequent profile reveals the glucose level pattern over subsequent days following treatment with extracts.

Table 2. Percentage mortality for the acute toxicity test of the Aqueous Extract.

Dose group (n=6)	Log dose	No of death	% mortality
2.0g/kg	3.30	0	0%
3.5g/kg	3.54	1	16.7%
4.5g/kg	3.65	3	50%
5.5g/kg	3.74	5	83.3%

Table 3. Percentage change in blood glucose level following the administration of 0.214mg/kg$_{bw}$ Glibenclamide.

Time (Days)	Blood Glucose Level(AVE±SEM)	%age decrease (*)or increase (#)
0	595.0 ± 3.87	-
1	566.8 ± 21.92	*4.74
2	537.5 ± 26.23	*9.66
3	583.0 ± 7.18	*2.02
4	539.3 ± 17.16	*9.36
5	508.0 ± 40.25	*14.62
6	483.7 ± 35.6	*18.71

There was a significant (P>0.05) reduction in the blood glucose level for the group that were administered 50mg/kg$_{bw}$ aqueous extract of *A. indica* leaves when compared to the group that was administered 0.214/kg$_{bw}$ glibenclamide. On the sixth day of administration, 52.9% reduction in blood glucose level were observed with the aqueous extract of *A. indica* leaves while glibenclamide showed 18.7% reduction on the same day. The reduction in blood glucose levels for the aqueous extract of *A. indica* leaves was consistent and significant.

Table 4. Percentage change in blood glucose level following the administration of 5ml Distilled Water.

Time (Days)	Blood Glucose Level (AVE±SEM)	% decrease (*)or increase (#)
0	530.0 ± 47.6	-
1	565.6 ± 21.92	6.72#
2	504.0 ± 50.37	4.91*
3	576.5 ± 3.57	8.77#
4	565.8 ± 13.1	6.75#
5	517.8 ± 56.25	2.30*
6	553.5 ± 29.87	4.43#

The aqueous extract of *A. indica*leaves at 100mg/kg$_{bw}$ showed a significant (P<0.05) reduction in blood glucose level when compared to the group treated with the standard antidiabetic drug (glibenclamide) also. The observed reduction in blood glucose profile on the sixth day of treatment for the aqueous extract at 100mg/kg$_{bw}$ was 55.7%. The extent of reduction observed following treatment with the aqueous extract at 100mg/kg$_{bw}$ was consistent and significant as can be seen in fig. 2b.

Table 5. Percentage change in blood glucose level following the administration of 50mg/kg$_{bw}$ Aqueous Extract.

Time (Days)	Blood Glucose Level (AVE±SEM)	% decrease (*) or increase (#)
0	569.2 ± 21.37	-
1	522.2 ± 33.67	8.25*
2	469.0 ± 17.42	17.60*
3	432.8 ± 32.88	23.96*
4	361 ± 111.1	36.29*
5	300.0 ± 40.3	47.29*
6	268.0 ± 32.2	52.93*

Table 6. Percentage change in blood glucose level following the administration of 100mg/kg$_{bw}$ Aqueous Extract.

Time (Days)	Blood Glucose Level	%age decrease (*) or increase (#)
0	569.2 ± 34.92	-
1	477.6 ± 33.19	11.42*
2	379.4 ± 51.22	29.64*
3	330.8 ± 49.12	38.65*
4	368.6 ± 68.66	31.46*
5	304.6 ± 48.88	43.51*
6	238.8 ± 45.80	55.71*

The aqueous extract of *A. indica* showed a significant ($p<0.05$) decrease in blood glucose level at 150mg/kg body weight when compared to the group of rats that were administered a standard antidiabetic drug (glibenclamide). The extent of reduction in blood glucose profile for the aqueous extract at 150mg/kg body weight was 46.0%. The decrease observed was not consistent on the fourth and fifth day of administration although significant on the sixth day for the aqueous as can be seen in fig. 2c

Table 7. Percentage change in blood glucose level following the administration 150mg/kg$_{bw}$ Aqueous Extract.

Time (Days)	Blood Glucose Level (AVE±SEM)	% age decrease (*) or increase (#)
0	464.4 ± 79.87	-
1	388.0 ± 68.79	16.45*
2	213.2 ± 52.5	54.09*
3	158.4 ± 42.72	65.89*
4	258.0 ± 72.01	44.44*
5	161.6 ± 40.08	65.20*
6	250.8 ± 77.65	45.99*

The ethanolic extract of *A. indica* at 100mg/kg$_{bw}$ showed a significant ($p<0.05$) reduction in blood glucose level after three days of pre-treatment before inducing diabetes with alloxan when compared to the animals that were induced without pre-treatment. The extent of reduction was 37.7%. The aqueous extract of *A. indica* at 100mg/kg$_{bw}$ did not cause any significant reduction after three days of pre-treatment before inducing diabetes with alloxan when compared to the animals that were induced without pre-treatment.

Fig 3b. reveals that the aqueous extract of *A. indica* at 100mg/kg$_{bw}$ showed a significant ($p<0.05$) lower blood glucose level after induction of diabetes following fourteen days of pre-treatment when compared to the animals that were induced without pre-treatment. The reduction in blood glucose profile occasioned by pre-treatment for fourteen days with 100mg/kg$_{bw}$ with aqueous extract of *A. indica* leaves was 39.5%. These observations suggest that extracts of *A. indica* leaves have antidiabetogenic effects and that the effects are dependent on the duration of pre-treatment.

Discussion and conclusion

Phytochemical screening of the aqueous extract of *A. indica*leafrevealed the presence of saponins, condensed tannins, flavonoids, alkaloids, cardiac glycosides and phenolic group. This is in line with the results of the phytochemical screening of *A. indica* leaf as reported by Atangwho, *et al.*, (2009). These phytoconstituents are responsible for a lot of the medicinal potential that has been reported.

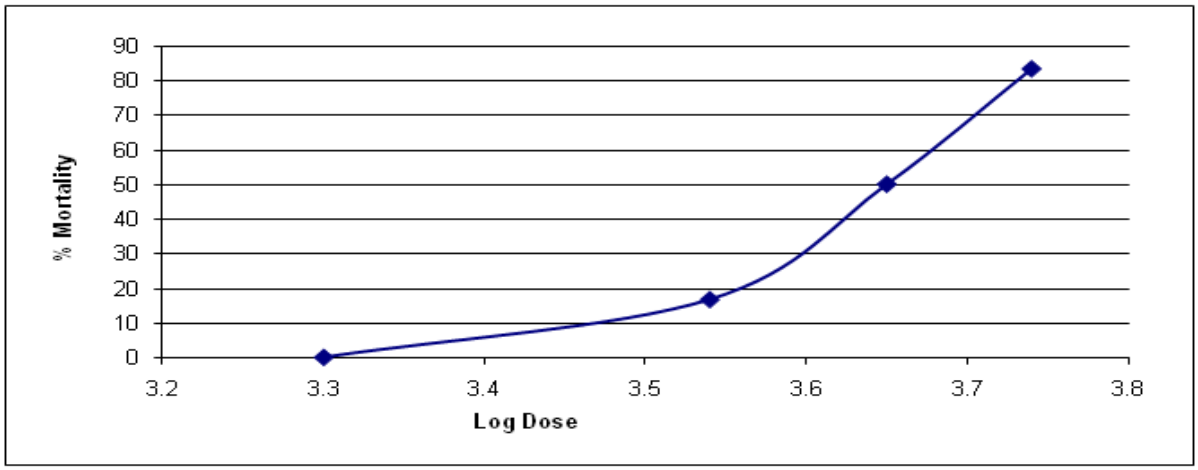

Fig. 1. LD_{50} for orally administered aqueous extract of *A. indica*.

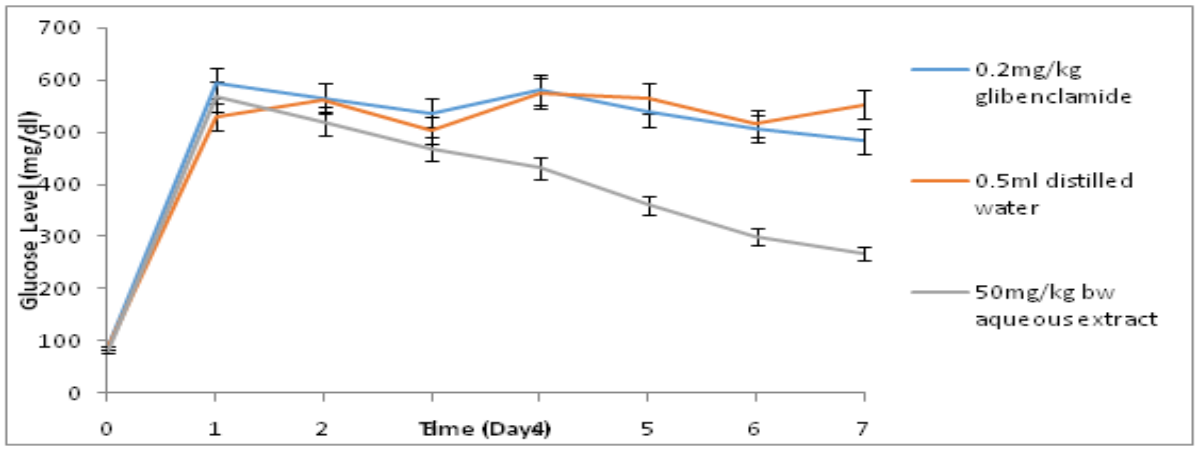

Fig. 2a. Blood glucose profile of treatment with glibenclamide and 50mg of *A. indica* extract per kg body weight following induction of diabetes.

The result of a 24–hour acute toxicity study of the orally administered aqueous extract of *A. indica* leaves showed that the median lethal dose (LD_{50}) is about 4.47g/kg_{bw}. The reported LD_{50} of the intraperitoneally administered aqueous extract of *A. indica* leaf is 4.8g/kg_{bw} (Agaie, *et al.*, 2000). This extract can be said to be "non-toxic" as close to 5000mg/$kg_{bw}$is usually assumed to be 'non-toxic'.

The blood sugar reducing potency of the extract varied considerably with different doses of the aqueous extracts of *A. indica* leaf. The rats that received 50mg/kg_{bw} aqueous extract of *A. indica* showed a significant ($p<0.05$) decrease in the blood glucose level when compared with those that received the standard drug (glibenclamide) at 0.214mg/kg_{bw}. The aqueous extract at 50mg/kg_{bw} showed 52.9% reduction on the sixth day of treatment which was higher when compared to 18.7% reduction for the group that was treated with a standard antidiabetic drug (glibenclamide) at 0.214mg/kg_{bw}.

There was a significant ($p<0.05$) decrease in blood sugar level at 100mg/kg_{bw} of aqueous extract of *A.*

indica on the sixth day of treatment when compared to the rats that received glibenclamide for the same number of days. The percentage level decline on the sixth day of treatment for the aqueous of *A.indica*at 100mg/kg$_{bw}$was 55.7%.

At a dose of 150mg/kg$_{bw,}$ the effect of the aqueous extract varied.The percentage sugar level decline of 46% was recorded on the 6th day. The result showed that the extract was more efficient at a dose of 100mg/kg. These results suggest that the aqueous extract of *A. indica* is potent in lowering the blood glucose level at the graded doses of 50mg/kg$_{bw}$, 100mg/kg$_{bw}$ and 150mg/kg$_{bw}$. This result is in line with the reports of Biswas *et al.* (2002) and Khosla *et al.* (2000). The mechanism of the antidiabetic properties of the extract is not well known. Jelodar *et al.* (2005) had suggested that the antidiabetic properties of the extract may be related to the ability of the extract to stimulate sufficient production of insulin by the pancreas that aided in the peripheral utilization of glucose in the cells or a possible ability of the extract to regenerate the β-cells to carry out its functions.The antidiabetic potential of according to Chattopadhyay, *et al.*, (1993) be due to inhibition of the action of epinephrine on glycogenolysis and peripheral utilization of glucose.

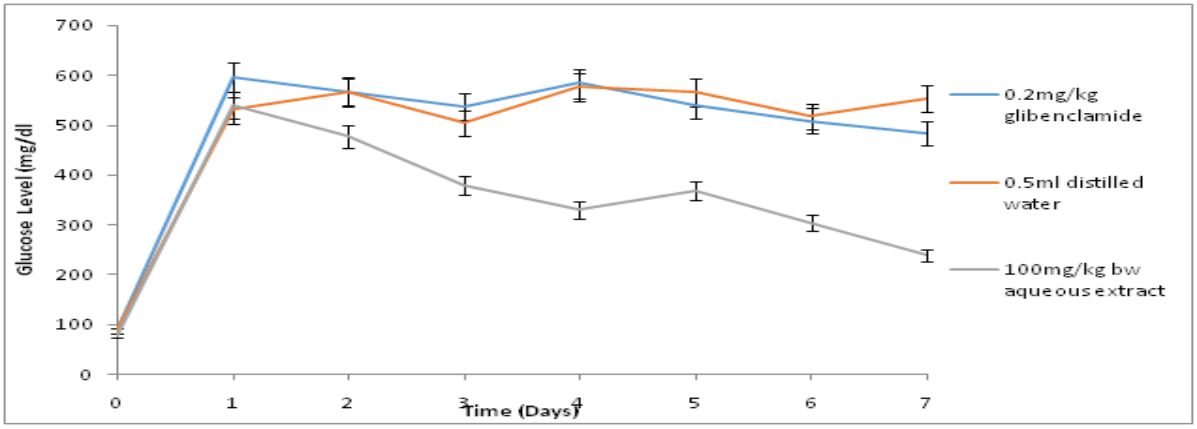

Fig 2b. Blood glucose profile of treatment with glibenclamide and 100mg of *A. indica* extract per kg body weight following induction of diabetes.

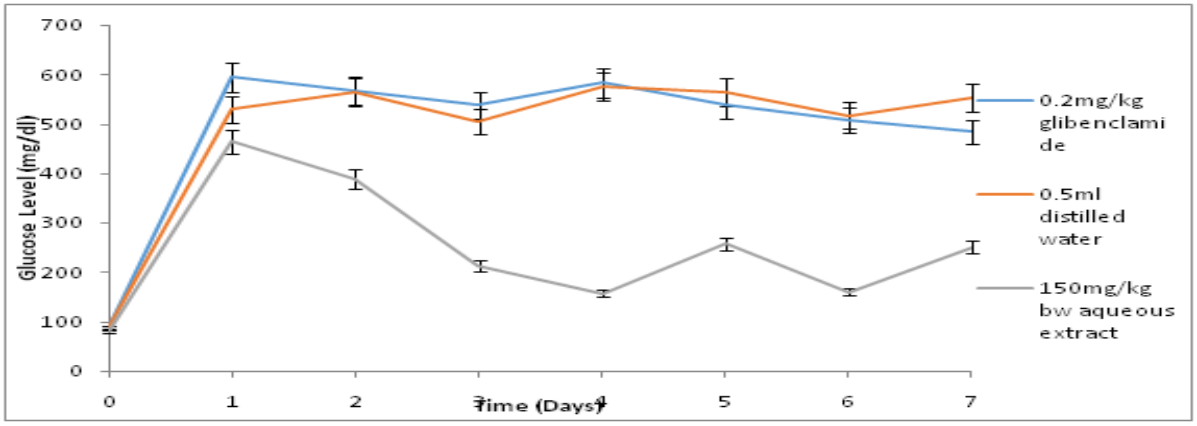

Fig 2c. Blood glucose profile of treatment with glibenclamide and 150mg of *A. indica* extract per kg body weight following induction of diabetes.

The results of the anti-diabetogenic studies suggest that the rats administered 100mg/kg$_{bw}$ethanolic extract of *A. indica*leaves for three and fourteen days respectively before inducing diabetes with alloxan showed a significantly ($P<0.05$) lower blood sugar level on the fourteenth day when compared with rats that were induced without pre-treatment. This effect was not significant early on in the 3rd day. The

percentage difference in sugar level was 39.5%. The result shows that prolonged pretreatment with the aqueous extract may confer some protective effect from diabetic onset.

These results agree with the earlier studies on some extracts of this plant on diabetic rats as reported by Chandra, *et al.*,(2008) and Henry, *et al.*, (2012).

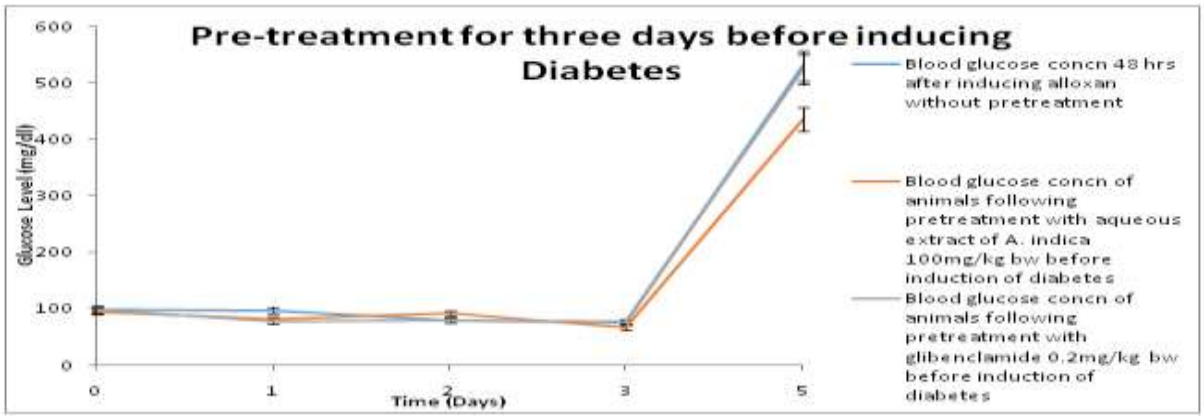

Fig. 3a. Blood glucose profile comparing the glucose level without pre-treatment before induction of diabetes and pre-treatment with 100mg/kg$_{bw}$ aqueous extract of *A. indica* leaves for three days and subsequent induction of diabetes (see appendix 1 for the table).

In conclusion, the aqueous extract of *A.indica* leafexhibited significant antihyperglycemic activities inalloxan-induced diabetic rats. Its effect was comparable to that of commonly used standard drugs, Glibenclamide at doses lower than the 1/10 of the determined LD_{50}. In addition, it showed strong potential as a prophylactic measure for diabetics. However further work is necessary to investigate the active ingredients present in this extract that confers the anti-diabetic and anti-diabetogenic properties using activity guided assays.Mechanisms of action of the extract and its constituents can be determined by devoting efforts to the inhibitory effects on biomolecules involved in sugar metabolism. Its longterm toxicological effect on some vital organs-liver, kidney- and system- reproductive, endocrine-should also be investigated critically. It is expected that this research will stimulate interest in*A. indica*leaves as a remedy for diabetes mellitus.

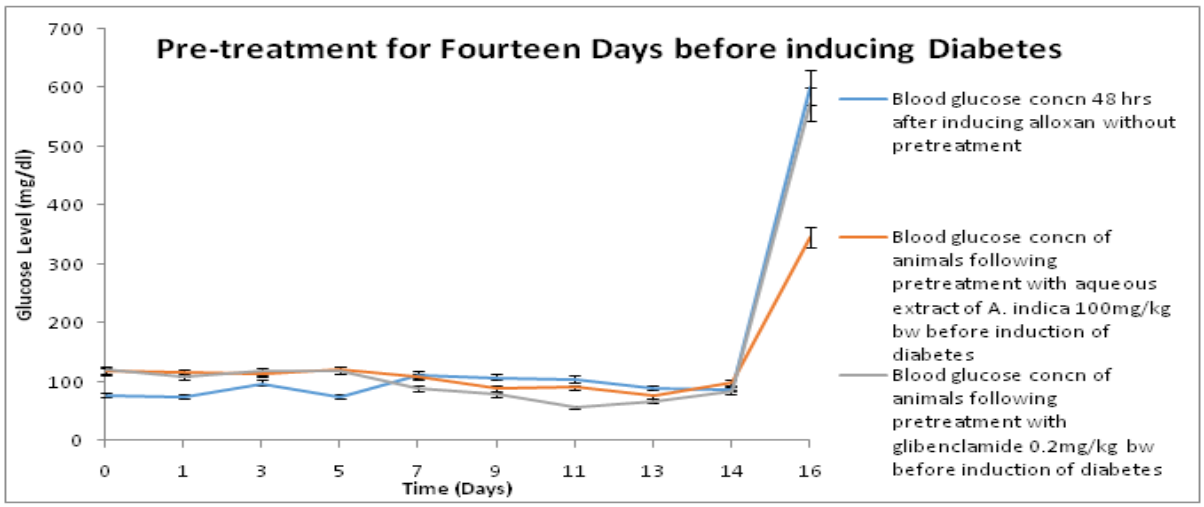

Fig. 3b. Blood glucose profile comparing the glucose level without pre-treatment before induction of diabetes and pre-treatment with 100mg/kg$_{bw}$ aqueous extract of *A. indica* leaves for fourteen days and subsequent induction of diabetes.

Conflict of interest

Authors hereby declare no conflict of interest.

Consent

This section is not applicable since none of the experiments was carried out in human subjects.

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 NnamdiAzikiwe University, Awka, Nigeria.

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