Phytochemistry and antidiabetogenic property of aqueous extract of Azadirachta indica leaf in streptozotocin-induced diabetic rats

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ABSTRACT

Aim: This study was carried out to determine the phytochemicals and the antidiabetogenic property of Azadirachta indica leaf in streptozotocin-induced diabetic male rats. Methodology: The quantitative phytochemical analysis and the glucose levels were checked using standard methods. Different group of rats were pre-treated for seven, fourteen, twenty-one and twenty-eight days before the induction of diabetes to know the extent of protection the extract will have on the rats by preventing the onset of diabetes. At the end of each treatment period diabetes was induced intraperitoneally using 50mg/kg bodyweight of Streptozotocin. Results: The percentage composition of the phytochemicals in the leaves of Azadirachta indica was as follows: Alkaloids (11.36%), cardiac glycosides (6.19%), flavonoid (5.34%), hemaglutinin (6.331%), oxalate (0.0204%), phenol (4.312%), phytate (4.600%), saponin (12.55%), steroid (0.2566) and tannin (6.17%). The result obtained revealed that there was a better protection for the group of rats pre-treated for a period of three weeks and four weeks. The percentage decrease in weight after the induction of diabetes for the groups pre-treated for a period of twenty-one days at varying doses of 100, 200 and 400 mg/kg bodyweight was 6.67%, 6.05% and 4.69% respectively which was significantly (p<0.05) different from the normal untreated group (9.90%). The percentage decrease in weight after the induction of diabetes for the groups pre-treated for a period of twenty-one days at varying doses of 100, 200 and 400 mg/kg bw is 7.04%, 5.97% and 2.50% respectively which was significantly (p<0.05) different from the normal untreated group (10.8%). The percentage increase in the fasting blood glucose level for the groups pre-treated for a period of twenty-one days at varying doses of 100, 200 and 400 mg/kg bw was 65.4%, 76.3% and 72.4% respectively which is significantly (p<0.05) different from that of the normal untreated group (82.7%) and the group pre-treated with metformin at a dose of 100 mg/kg bw (84.5%). The percentage increase in the fasting blood glucose level for the groups pre-treated for a period of twenty-eight days at varying doses of 100, 200 and 400 mg/kg bw was 73.5%, 68.5% and 64.4% respectively which was significantly (p<0.05) different from that of the normal untreated group (83.3%) and the group pre-treated with metformin at a dose of 100 mg/kg bw (85.5%). The longer the period of pre-treatment, the better the reduction in the blood glucose levels recorded after the induction of diabetes. Conclusion: These results suggest that the aqueous extract of A. indica leaf has antidiabetogenic effect and could be used in the prevention as well as delaying the onset of diabetes mellitus.

Keywords: Phytochemistry, Antidiabetogenic, Azadirachta indica, Streptozotocin, Diabetic rats.

1. INTRODUCTION

Diabetes mellitus (DM) is a rapid metabolic disorder which can result from a defect in insulin secretion, insulin action, or both. Insulin deficiency in turn leads to chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism [1]. It is one of the chronic non-communicable diseases which have emerged as a leading global health challenge. It is a risk factor for vascular brain diseases, renal failure, blindness and diabetic wound [2]. During the progression of the disease the body cannot breakdown glucose properly as a result of insufficient insulin or when the insulin produced is not effectively used by the body leading to high blood glucose level. The two main type of diabetes are type 1 diabetes and type 2 diabetes. Type 1 also known as insulin-dependent diabetes is characterized by insufficient of the production of insulin as a result of damage of the beta cell of the pancreas [3]. Type 1 diabetic patients are likely to develop ketoacidosis. Type 2 diabetes known as non-insulin dependent diabetes is characterized by inefficient use of the insulin produced by the beta cells of the pancreas. 90% cases of diabetes is type 2 diabetes [4].

In 2017, it was estimated that 425 million people between the ages of 20–79 years suffered from DM. This figure is expected to rise to 629 million by 2045 [5]. In the year 2019 international diabetes federation...
reported that about 463 million individuals are affected with diabetes worldwide and the number was expected to increase by the year 2030 to 578 million and by the year 2045 it was estimated to increase to 700 million [8]. In Africa, 19 million adults (20-79) are living with diabetes and it is estimated to increase to 47 million by 2045, 45 million adults (20-76) are at a high risk of developing type 2 diabetes because of impaired Glucose Tolerance and it is estimated to reach 110 million by the year 2045, 60% of adult living with diabetes are undiagnosed, and about 9.5 billion dollars has been spent on healthcare for people with diabetes [9]. Base on a random-effects meta-analysis carried out from the year 1990 to 2017, an increasing trend of about 440% in type 2 diabetes mellitus cases was observed in Nigeria in the year 2015 over the value gotten in 1990 which was 2.2% [7]. In Nigeria, the estimated values of prevalence of diabetes in the six geopolitical zones were 3.0%, 5.9%, 3.8%, 5.5%, 4.6%, and 9.8% in the north-west, north east, north central, south west, south east, and south respectively [8].

According to International Diabetes Federation, about 2 million cases of diabetes have not been diagnosed in Nigeria leading to over 40 thousand deaths as a result of the disease and its complications [9]. Nigerians have been known to use various parts of herbs to treat diseases [10]. These plants hold active compounds that are medicinal which are ingredient in drugs [11]. Azadirachta indica (neem) is a tropical tree belonging to meliaceae family, native to India [12], Azadirachta indica is an important plant. The effect of the aqueous extract of A. indica leaf on some essential biochemical parameters has earlier been reported [13]. It is one of the most effective blood-purifiers, detoxifiers and immune system boosters. So many diseases have shown to respond to its therapeutic activity. A. indica leaf can be taken as tea. The oil can be applied externally or a few drops can be put in an empty capsule and taken internally. It enhances soil fertility [14]. About 72 countries grows A. indicatrees, Africa included.

A. indica contains active compounds that are therapeutic. The main chemical compounds are terpene, lavonoids, carotenoid, nimbin, melicain (the bitter taste) gedunin, salanin, valassin [15]. The limnoids compound in A. indicapossess insecticidal properties [16]. In Thailand the leave extract is used as an antioxidant for food and industries (pharmaceutical industry) [17, 18]. It is used in India to cure skin disease such as ringworm, scabies [19]. The mixture of neem and turmeric paste was applied on 184 individuals with scabies, 97% of the individuals got cured within 3 to 15 day of administration with no side effect [20]. The leaf extracts in diabetic induced rat improves the size, shape and number of beta cells thereby regenerating the beta cell [21]. The antidiabetogenic property of the leaves of A. indica in diabetic rats for a period of two weeks has earlier been studied. The result of the previous studies revealed that the longer the period of pre-treatment, the better the antidiabetogenic property [22]. The present study was carried out to determine the quantitative phytochemicals and antidiabetogenic property of aqueous extract of A. indica leaf in streptozotocin-induced diabetic rats at different periods (seven, fourteen, twenty-one and twenty-eight days) of pre-treatment.

2. MATERIALS AND METHODS

2.1 Sample Collection and Identification

The leaves of A. indica were collected at Nnamdi Azikiwe University Awka, Anambra State, Nigeria. The sample was identified and authenticated by a taxonomist in the Department of Botany, Nnamdi Azikiwe University, Awka. The voucher number as deposited in the herbarium of the Department of Botany, Nnamdi Azikiwe University, Awka, Nigeria is NAU/H/14.

2.3 Preparation of the Aqueous Extracts of Neem Leaf

The leaves were hand-picked, thoroughly washed and air dried at room temperature. The dried leaves were pulverized into powder using Corona manual grinding machine. One kilogramme of the pulverized leaves powder of A. indica was soaked in 3 litres of distilled water for 24 hrs for complete extraction. The aqueous extraction was sieved using muslin cloth and filtered using Whatman filter paper. The filtrate was lyophilized (freeze dried) to powder. The aqueous extract was stopped in universal bottles and preserved in the refrigerator for use. The extract was thereafter prepared by solubilizing it with distilled water before administration.

2.4 Preparation of Extract for Quantitative Phytochemical Analysis

The fresh leaves of A. indica were collected, hand-picked, washed and dried for two weeks at room temperature. It was pulverized to powder using corona manual grinding machine. The dried powdered sample was then used for the quantitative analysis as required by the standard methods used.

2.4.1 Determination of Alkaloids [23]

Five grammes (5g) of the powdered A. indica leaf sample was weighed into a 250ml beaker and 200ml of 20% acetic acid in ethanol was added and covered and allowed to stand for 4 hours at 25°C. This was filtered using filter paper no. 42 and the filtrate was concentrated using a water bath (Memmert) to one quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitate was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute NH-OH (1% ammonium solution). Then, filter with pre-weighed filter paper. The residue on the filter paper is the alkaloid, which was dried in the oven (precision electrothermal model BNP 9052 England) at 80°C. The alkaloid content was calculated and expressed as a percentage of the weight of the sample analyzed [23].

Calculation

\[ \% \text{ weight of alkaloid} = \frac{\text{weight of filter paper with residue} - \text{weight of filter paper}}{100} \times \text{Weight of sample analyzed} \]

2.4.2 Determination of Saponins [24]

Five grams (5g) of the A. indica leaf sample was put into 20% acetic acid in ethanol and allowed to stand in a water bath at 50°C for 24 hours. This was filtered and the extract was concentrated using a water bath to one-quarter of the original volume. Concentrated NH-OH was added drop-wise to the extract until the precipitate was collected by filtration and weighed. The saponin content was weighted and calculated in percentage using the formula proposed by Obadon and Ochuko [24].

Calculation

\[ \% \text{saponin content} = \left( \frac{\text{weight of filter paper + residue} - \text{weight of filter paper}}{100} \right) \times \text{Weight of sample analysed} \]

2.4.3 Determination of Tannin by Titration [25]

The Follins Dennis titrating method as described by Pearson (1976) was used. To 20g of the crushed A. indica leaf sample in a conical flask was added 100 ml of petroleum ether and covered for 24 hours. The sample was then filtered and allowed to stand for 15 minutes allowing petroleum ether to evaporate. It was then re-extracted by soaking in 100 ml of 10% acetic acid in ethanol for 24 hours. The sample was then filtered and the filtrate collected. Twenty-five ml (25ml) of NH2OH were added to the filtrate to precipitate the alkaloids. The alkaloids were heated with electric hot plate to remove some of the NH2OH still in solution. The remaining volume was measured to be 33ml. 5ml of this was taken and 20ml of ethanol was added to it. It was titrated with 0.1M NaOH using phenolphthalein as indicator until a pink end point is reached. Tannin content was then calculated in \( \% (C V_1 = C V_2) \) molarity.
The Journal of Phytopharmacology

Calculation

\[ C_1 = \frac{C_2V_2}{V_1} \]

% of tannic acid content = \( \frac{C_1 \times 100}{\text{Weight of sample analyzed}} \)

\[ C_1 = \text{Conc. of Tannic acid} \]
\[ C_2 = \text{Conc. of base} \]
\[ V_1 = \text{Volume of Tannic acid} \]
\[ V_2 = \text{Volume of Base} \]

2.4.4 Determination of Oxalate by Titration [23]

This determination involves three major steps: digestion, oxalate precipitation, and permanganate titration.

Digestion

Two (2g) of powdered A. indica leaf sample is suspended in 190ml of distilled water in a 250ml volumetric flask. Ten (10) ml of 6M HCl is added and the suspension digested at 100°C for 1 hour. Cool, and then made up to 250ml mark before filtration.

Oxalate Precipitation

Duplicate portions of 125ml of the filtrate are measured into beakers and four drop of methyl red indicator added. This was followed by the addition of NH4OH solution (dropwise) until the test solution changes from salmon pink colour to a faint yellow colour (pH 4 – 4.5). Each portion was then heated to 90°C, and 10ml of 5% CaCl2 solution is added while being stirred constantly. After heating, it was cooled and left overnight at 25°C. The solution is then centrifuged at 2500rpm for 5 minutes. The suspension is decanted and the precipitate completely dissolved in 10ml of 20% (v/v) H2SO4 solution.

Permanganate titration

At this point, the total filtration resulting from digestion of 2g of the A. indica leaf sample is made up to 300ml. Aliquots of 125ml of the filtrate is heated until near boiling and then titrated against 0.00195g iron per 1ml.

Determination of Flavonoids [26]

Ten grammes (10g) of the powdered A. indica leaf sample was extracted repeatedly with 100ml of 80% methanol at room temperature. The whole solution was filtered through Whatman filter paper No. 42 (125 mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight [26].

Calculation

\[ \% \text{Flavonoids} = \frac{(\text{weight of crucible + residue}) - (\text{weight of crucible}) \times 100}{\text{Weight of sample analyzed}} \]

2.4.5 Determination of Flavonoids [26]

To 1ml of A. indica leaf extract was added 1ml of 2% solution of 3,5-DNS (Dinitro Salicylic acid) in methanol and 1ml of 5% aqueous NaOH. It was boiled for 2 minutes (until brick-red precipitate was observed) and the boiled sample was filtered. The weight of the filter paper was weighed before filtration. The filter paper with the absorbed residue was dried in an oven at 50°C till dryness and weight of the filter paper with residue was noted. The cardiac glycoside was calculated in %.

Calculation

\[ \% \text{Cardiac glycoside} = \]
\[ \frac{(\text{weight of filter paper + residue}) - (\text{weight of filter paper}) \times 100}{\text{Weight of sample analyzed}} \]

2.4.6 Determination of Cardiac Glycosides [27]

Phytate content was determined using the method of Young and Greaves [28] as adopted by Lucas Markakes [29]. Two (2g) of each of the differently prepared powdered neem leaf sample was weighed into different 250ml conical flasks. Each sample was soaked in 100ml of 2% concentrated HCl for 3 hrs. The sample was then filtered. 50ml of each filtrate was laced in 250ml beaker and 100ml distilled water added to each sample. 10ml of 0.3% ammonium thiocyanate solution was added as indicator and titrated with standard iron (III) chloride solution which contained 0.00195g iron per 1ml.

Calculation

\[ \text{Phytic acid} = \frac{\text{Titre value} \times 0.00195 \times 1.19 \times 100}{\text{Weight of sample}} \]

2.4.8 Determination of Phenol [30]

The quantity of phenols was determined using the spectrophotometer method. The A. indica leaf sample was boiled with 50ml of (CH3CH2)2: for 15 minutes. Exactly 5ml of the boiled sample was then pipetted into 50ml flask, and 10ml of distilled water was added. After the addition of distilled water, 2ml of NH4OH solution and 5ml of concentrated CH3(C2H5)2CH2OH was added to the mixture. The sample was made up to the mark and left for 30 mins to react for colour development and measured at 505 nm wavelength using spectrophotometer.

2.4.9 Determination of Steroid Content [30]

One (1.0g) of the powdered A. indica leaf sample was weighed and mixed in 100ml of distilled water in a conical flask. The mixture was filtered and the filtrate eluted with 0.1N ammonium hydroxide solution. 2ml of the eluent was put in a test tube and mixed with 2ml of chloroform. Three (3) ml of ice-cold acetic anhydride was added to the mixture in the flask, 2 drops of (200 mg/dl) standard sterol solution was prepared and treated as described for test as blank. The absorbance of standard and test was measured, zeroing the spectrophotometer with blank at 420 nm.

Calculation

\[ \text{Mg/100ml) Absorbance of test} \times \frac{\text{Conc of Standard}}{\text{Absorbance of standard}} \]

2.4.10 Determination of Hemagglutinin [31]

To two grammes (2g) of each of the powdered A. indica leaf sample were added 20ml of 0.9% NaCl and the suspension shaken vigorously for 1 min. The supernatant was left to stand for 1 hr. The samples were then centrifuged at 2000rpm using centrifuge (PEC MEDICAL USA) for 10 min and the suspension filtered. The supernatants in each were collected and used as crude agglutination extract. The absorbance was read at 420nm.
2.5 Investigation of Antidiabetogenic (Protective) Properties

A total of one hundred (100) rats were randomized into four (4) groups (n=25) and used for the study. Each group of 25 rats was sub-divided into five (5) groups (n=5) as shown in table 1. Exactly 100mg/kg, 200mg/kg and 400mg/kg body weight of the aqueous leaf extract of A. indica leaves were consecutively administered for 7 days, 14 days, 21 days and 28 days before the induction of diabetes. Administration was by oral gavage using intubation cannular. Body weights and blood glucose levels of rats were determined before initiating the daily administration of the extracts. Body weights and blood glucose levels of rats were monitored every two days consistently for the duration of repeated dose before induction of diabetes. Diabetes mellitus was induced intraperitoneally with 50mg/kg b.w. of streptozotocin. The onset of diabetes was established by increase in the normal fasting blood sugar level (between 60mg/dl to 120mg/dl) to above 200mg/dl [32]. After induction with streptozotocin the weights of rats, the blood glucose level as well as other symptoms of diabetes were determined and monitored.

Table 1: Grouping of animals for antidiabetogenic study.

| Grouping                  | Dose per Kg | No. of Rats |
|---------------------------|-------------|-------------|
| Group A (Normal Control)  | No pretreatment | 5           |
| Group B (Metformin)       | 100mg       | 5           |
| Group C (Aqueous extract) | 100mg       | 5           |
| Group D (Aqueous extract) | 200mg       | 5           |
| Group E (Aqueous extract) | 400mg       | 5           |

2.6 Data Analysis

Data obtained 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 ± SD. The results obtained were subjected to Analysis of Variance (ANOVA) to determine if significant difference exists between the mean of the test and control groups at p<0.05.

3. RESULTS

3.1 Result of Quantitative Phytochemical Analysis

The result of the quantitative phytochemical analysis of the powdered leaves of A. indica revealed the quantities of saponin, alkaloids, cardiac glycosides, flavonoids, heamaglutinin, oxalate, phenol, phytate, steroids and tannin in the sample (Table 2). The percentage composition of the phytochemicals in the leaves of neem is as follows: Alkaloids (11.36%), cardiac glycosides (6.19%), flavonoids (5.34%), heamaglutinin (6.33%), oxalate (0.0204%), phenol (4.312%), phytate (4.600%), saponin (12.55%), steroid (0.2566) and tannin (6.17%).

Table 2: The mean of the triplicate values of the percentage composition of the phytochemical constituents of A. indica leaf.

| Phytochemicals         | Proximate Composition (%) |
|------------------------|---------------------------|
| Alkaloids              | 11.36                     |
| Cardiac Glycosides     | 6.150                     |
| Flavonoids             | 5.347                     |
| Heamaglutinin          | 6.331                     |
| Oxalate                | 0.02040                   |
| Phenol                 | 4.312                     |
| Phytate                | 4.600                     |
| Saponin                | 12.55                     |
| Steroids               | 0.2598                    |
| Tannin                 | 6.153                     |

3.2 Weight following pre-treatment with aqueous A. indica leaf extract

The groups that were pre-treated for seven days with 100, 200 and 400mg/kg bw of aqueous extract of A. indica before the induction of diabetes showed 8.12%, 6.35% and 4.64% reduction respectively in their weight 48 hours after the induction of diabetes compared to the group pre-treated with metformin (7.25%) and the untreated group (9.89%) (table 3). The result showed that a better protection against weight loss was observed in the group pre-treated with 400mg/kg bw. The groups that were pre-treated for fourteen days with 100, 200 and 400mg/kg bw of aqueous extract of A. indica before the induction of diabetes showed 5.68%, 5.56% and 4.18% reduction respectively in their weight 48 hours after the induction of diabetes compared with the group pre-treated with metformin (8.85%) and the untreated group (9.67%) (table 4). The result revealed that at two weeks all the doses tested showed better protection against weight loss.

The groups that were pre-treated for twenty-one days with 100, 200 and 400mg/kg bw of aqueous extract of A. indica before the induction of diabetes showed 6.67%, 6.05% and 4.69% reduction respectively in their weight 48 hours after the induction of diabetes compared with the group pre-treated with metformin (8.04%) and the untreated group (9.90%) (table 5). The result showed that a better protection against weight loss was observed in the group pre-treated with 400mg/kg bw. The groups that were pre-treated for twenty-eight days with 100, 200 and 400mg/kg bw of aqueous extract of A. indica before the induction of diabetes showed 7.04%, 5.97% and 2.50% reduction respectively in their weight 48 hours after the induction of diabetes compared with the untreated group (10.8) and the group pre-treated with metformin (6.02%) (table 6). The result showed that a better protection against weight loss was observed in the group pre-treated with 400mg/kg bw followed by the group pre-treated with 200mg/kg bw.

Table 3: Weight (g) profile of rats during one-week pre-treatment with 100mg/kg, 200mg/kg and 400mg/kg b.w. of aqueous extract of A. indica expressed as mean ± SD.

| Time (Days) | Untreated Rats | 100mg/kg Metformin | 100 mg/kg Aqueous Extract | 200 mg/kg Aqueous Extract | 400 mg/kg Aqueous Extract |
|-------------|----------------|---------------------|---------------------------|---------------------------|---------------------------|
| Day 0       | 124.9±2.646    | 126.6±4.393        | 126.8±4.919               | 122.6±3.131               | 128.4±4.930               |
| Day 2       | 125.4±2.302    | 128.0±4.796        | 128.4±4.827               | 125.2±2.864               | 130.2±5.630               |
| Day 4       | 126.8±2.775    | 129.6±5.899        | 129.6±52.53               | 126.0±2.915               | 132.4±6.699               |
| Day 6       | 127.0±2.345    | 131.8±5.933        | 132.2±5.069               | 128.8±2.387               | 134.2±8.686               |
| Day 7       | 129.4±2.510    | 135.2±6.261        | 135.4±4.615               | 132.2±2.775               | 137.8±3.701               |

% decrease in weight | 9.89 | 7.25 | 8.12 | 6.35 | 4.64bd

*significant reductions with respect to untreated control; †significant increase with respect to untreated control; ‡significant reduction with respect to positive control (metformin); §significant increase with respect to positive (metformin) control.
**Table 4:** Weight (g) profile of rats during two weeks pre-treatment with 100mg/kg, 200mg/kg and 400mg/kg b.w. of aqueous extract of *A. indica* expressed as mean ± SD.

| Time (Days) | Normal (Untreated) Rats | 100mg/kg Metformin | 100 mg/kg Aqueous Extract | 200 mg/kg Aqueous Extract | 400 mg/kg Aqueous Extract |
|-------------|-------------------------|---------------------|---------------------------|---------------------------|---------------------------|
| Day 0       | 124.0±2.646             | 136.2±3.962         | 128.6±5.595               | 124.6±3.362               | 122.0±3.937               |
| Day 2       | 125.4±2.302             | 136.4±4.336         | 128.8±5.450               | 124.4±2.793               | 122.4±3.564               |
| Day 4       | 126.8±2.775             | 138.8±7.362         | 132.2±5.933               | 126.6±2.881               | 122.6±4.159               |
| Day 6       | 127.0±2.345             | 139.6±3.975         | 131.2±5.586               | 127.2±1.483               | 125.0±4.637               |
| Day 8       | 131.4±2.702             | 141.6±6.986         | 131.0±4.637               | 131.3±2.872               | 127.2±3.564               |
| Day 10      | 132.8±5.450             | 143.6±5.550         | 135.5±4.435               | 131.5±4.655               | 130.0±3.317               |
| Day 12      | 133.6±2.608             | 146.0±7.348         | 137.0±4.690               | 134.0±4.690               | 134.2±4.324               |
| Day 14      | 134.4±4.669             | 149.2±6.301         | 140.8±4.193               | 138.5±2.517               | 134.0±4.062               |

**Induction of diabetes with streptozotocin after 16 hours fasting**

| Day 17      | 121.4±3.362             | 136.0±8.775         | 132.8±2.500               | 130.8±3.594               | 128.4±3.362               |

% decrease in weight after induction of Diabetes mellitus

9.67  8.85  5.68bd  5.56bd  4.18bd

*Significant reduction with respect to untreated control; **significant increase with respect to untreated control; ***significant reduction with respect to positive control (metformin); ****significant increase with respect to positive (metformin) control.

**Table 5:** Weight (g) profile of rats during three weeks pre-treatment with 100mg/kg, 200mg/kg and 400mg/kg b.w. of aqueous extract of *A. indica* expressed as mean ± SD.

| Time (Days) | Normal (Untreated) Rats | 100mg/kg Metformin | 100 mg/kg Aqueous Extract | 200 mg/kg Aqueous Extract | 400 mg/kg Aqueous Extract |
|-------------|-------------------------|---------------------|---------------------------|---------------------------|---------------------------|
| Day 0       | 124.0±2.646             | 124.4±2.608         | 123.0±3.082               | 121.0±2.739               | 122.4±2.302               |
| Day 2       | 125.4±2.302             | 126.0±1.732         | 122.6±2.302               | 124.6±2.509               | 124.4±1.817               |
| Day 4       | 126.8±2.775             | 127.2±2.049         | 124.8±2.280               | 126.4±2.408               | 126.2±1.095               |
| Day 6       | 127.0±2.345             | 128.2±2.168         | 126.6±1.517               | 119.2±3.194               | 128.4±1.342               |
| Day 8       | 131.2±2.775             | 130.8±1.304         | 127.6±1.342               | 125.8±3.421               | 130.0±1.225               |
| Day 10      | 131.4±2.702             | 133.2±2.280         | 129.0±2.345               | 128.0±3.317               | 131.6±2.074               |
| Day 12      | 133.6±2.608             | 134.4±0.894         | 131.8±0.837               | 130.2±3.633               | 134.0±2.000               |
| Day 14      | 132.8±5.450             | 137.0±1.414         | 133.6±1.517               | 132.0±3.742               | 133.8±3.633               |
| Day 16      | 134.4±4.669             | 138.6±1.140         | 134.6±2.966               | 134.2±3.633               | 137.4±2.191               |
| Day 18      | 136.0±3.464             | 140.0±2.345         | 135.5±3.416               | 136.0±3.317               | 140.2±1.924               |
| Day 20      | 137.0±3.082             | 141.2±1.923         | 139.0±2.944               | 138.0±3.317               | 142.0±2.121               |
| Day 21      | 139.4±3.361             | 144.2±1.095         | 142.5±1.915               | 142.2±3.701               | 145.0±2.000               |

**Induction of diabetes with streptozotocin after 16 hours fasting**

| Day 24      | 125.6±4.159             | 132.6±2.702         | 133.0±1.414               | 133.6±3.647               | 138.2±3.114               |

% decrease in weight after induction of DM

9.90  8.04  6.67b  6.05b  4.69bd

*Significant reduction with respect to untreated control; **significant increase with respect to untreated control; ***significant reduction with respect to positive control (metformin); ****significant increase with respect to positive (metformin) control.

**Table 6:** Weight (g) profile of rats during four weeks pre-treatment with 100mg/kg, 200mg/kg and 400mg/kg b.w. of aqueous extract of *A. indica* expressed as mean ± SD.

| Time (Days) | Normal (Untreated) Rats | 100mg/kg Metformin | 100 mg/kg Aqueous Extract | 200 mg/kg Aqueous Extract | 400 mg/kg Aqueous Extract |
|-------------|-------------------------|---------------------|---------------------------|---------------------------|---------------------------|
| Day 0       | 124.0±2.646             | 124.2±4.087         | 121.6±2.302               | 126.6±4.669               | 125.6±4.219               |
| Day 2       | 126.8±2.775             | 127.0±3.391         | 125.2±1.304               | 130.2±4.207               | 129.4±3.847               |
| Day 4       | 127.0±2.345             | 128.8±5.675         | 125.0±1.581               | 131.8±4.658               | 131.4±3.847               |
| Day 6       | 129.4±2.510             | 129.4±4.722         | 126.8±1.789               | 133.4±4.506               | 133.4±3.847               |
| Day 8       | 131.4±2.702             | 131.6±3.209         | 127.8±2.387               | 135.0±4.301               | 135.0±3.162               |
| Day 10      | 133.6±2.608             | 134.2±4.087         | 129.6±1.949               | 136.8±4.147               | 136.6±2.881               |
| Day 12      | 132.8±5.450             | 136.4±3.578         | 131.8±2.500               | 139.0±4.183               | 138.4±2.966               |
Day 14  136.0±3.464  137.8±3.564  133.0±0.817  140.0±4.183  140.0±3.391
Day 16  137.0±3.082  139.4±4.278  135.0±0.000  141.8±3.834  141.8±3.421
Day 18  139.4±4.670  142.4±4.278  137.0±0.817  143.6±3.975  143.4±3.626
Day 20  139.4±3.362  144.6±4.506  139.5±0.577  145.6±3.975  144.8±2.382
Day 22  141.6±3.578  145.8±5.167  141.3±1.258  147.8±3.633  147.5±2.380
Day 24  142.8±2.683  150.0±6.000  143.0±1.000  149.8±3.633  149.3±2.217
Day 26  145.0±4.301  148.6±3.782  144.7±0.577  151.8±3.564  151.0±1.826
Day 28  147.6±2.966  152.8±3.493  147.7±1.155  154.0±3.317  152.3±2.217

Induction of diabetes with streptozotocin after 16 hours fasting

| Day   | % decrease in weight after induction of DM |
|-------|------------------------------------------|
| Day 31| 10.8                                     |
|       | 6.02                                     |
|       | 7.04b                                    |
|       | 5.97b                                    |
|       | 2.50b                                    |

3.3 Glucose Levels following pre-treatment with A. indica leaf extract

The result of the daily fasting blood glucose levels of the different group of rats administered 100mg/kg, 200mg/kg and 400mg/kg bw. of A. indica leaf extract for 7, 14, 21 and 28 days before the induction of diabetes were shown in tables 7, 8, 9 and 10 respectively. Also, the fasting blood glucose levels of the rats were recorded 48 hrs after the induction of diabetes. The difference in percentage increase in blood glucose levels between the normal untreated rats and those administered extracts at all dose levels remain about the same after 7 days and shows marked difference from 14 days and increases(p<0.05) significantly as the duration of pre-treatment increases.

There was no significant (p<0.05) difference in the fasting blood glucose levels for the group of animals pre-treated for seven days with the aqueous leaf extracts of A. indica compared with the untreated group after the induction of diabetes (table 7). The result of the daily fasting blood glucose levels of the different group of rats administered graded doses of A. indica extract for fourteen (14) days before the induction of diabetes was shown in table 8. The groups that were pre-treated with the aqueous extract of A. indica leaf extracts for fourteen days showed a significant (p<0.05) reduction in the fasting blood glucose level after the induction of diabetes compared with the normal rats that were not pre-treated (table 8). There was a significant (p<0.05) decrease in the percentage increase in fasting blood glucose level of the groups pre-treated with the graded doses of the extract compared with the normal untreated group and the group pre-treated with a standard antidiabetic drug (metformin).

The result of the daily fasting blood glucose levels of the different group of rats administered 100mg/kg, 200mg/kg and 400mg/kg bw. of aqueous extract of A. indica for twenty-one (21) days before the induction of diabetes was shown in table 9. The groups that were pre-treated for twenty-one days with aqueous extract of A. indica leaf before the induction of diabetes showed a significant (p<0.05) decrease in their fasting blood glucose levels after the induction of diabetes compared with the normal untreated rats and the rats pre-treated with a standard antidiabetic drug (table 9). The result of the daily fasting blood glucose levels of the different group of rats administered graded doses of A. indica leaf extract for twenty-eight (28) days before the induction of diabetes was shown in table 10. The groups that were pre-treated for twenty-eight days with aqueous extract of A. indica leaf before the induction of diabetes showed a significant (p<0.05) decrease in their fasting blood glucose levels after the induction of diabetes compared with the normal untreated rats (table 10).

Table 7: Profile of fasting blood glucose (mg/dl) levels during one-week pre-treatment with 100mg/kg, 200mg/kg and 400mg/kg bw. of aqueous extract of A. indica leaf expressed as mean ± SD.

| Time (Days) | Normal (Untreated) | 100mg/kg Metformin | 100 mg/kg Aqueous Extract | 200 mg/kg Aqueous Extract | 400 mg/kg Aqueous Extract |
|-------------|--------------------|--------------------|--------------------------|--------------------------|--------------------------|
| Day 0       | 96.00±12.75        | 97.60±19.07        | 100.6±15.34              | 79.60±11.91              | 93.20±19.93              |
| Day 2       | 97.80±10.33        | 95.80±14.45        | 99.20±22.38              | 83.20±7.530              | 96.60±15.85              |
| Day 4       | 98.20±9.731        | 94.60±15.93        | 103.8±12.70              | 85.00±3.162              | 92.00±17.76              |
| Day 6       | 95.00±10.56        | 98.60±18.58        | 95.4±19.97              | 90.00±9.747              | 90.4±15.27               |
| Day 7       | 99.00±7.382        | 96.00±18.72        | 104.8±10.76              | 84.20±12.00              | 91.40±11.82              |

| Induction of diabetes with streptozotocin after 16 hours fasting |
|-----------------------------------------------|
| Day 10 | 538.8±4.592 | 533.4±94.76 | 499.0±82.91 | 554.2±46.99 | 480.8±96.42 |

| % increase in glucose level after induction of DM | 81.6 | 82 | 78.9 | 84.4 | 81.0 |

*a*significant reduction with respect to untreated control; *a*significant increase with respect to untreated control; *a*significant reduction with respect to positive control (metformin); *a*significant increase with respect to positive (metformin) control.
Table 8: Profile of glucose (mg/dl) levels during two weeks pre-treatment with 100mg/kg, 200mg/kg and 400mg/kg b.w. of aqueous extract of A. indica leaf expressed as mean ± SD.

| Time (Days) | Normal (Untreated) | 100mg/kg Metformin | 100 mg/kg Aqueous Extract | 200 mg/kg Aqueous Extract | 400 mg/kg Aqueous Extract |
|------------|--------------------|---------------------|--------------------------|--------------------------|--------------------------|
| Day 0      | 96.00±12.75        | 91.80±13.93         | 95.20±8.289              | 101.4±11.61              | 91.20±9.576              |
| Day 2      | 97.80±10.33        | 88.40±14.21         | 95.80±9.628              | 97.00±11.40              | 93.40±8.820              |
| Day 4      | 98.20±9.731        | 85.00±7.969         | 94.20±8.526              | 94.80±11.58              | 92.20±12.87              |
| Day 6      | 95.00±10.56        | 88.20±9.418         | 99.00±9.301              | 90.20±7.040              | 95.40±12.50              |
| Day 8      | 99.00±7.382        | 92.20±9.338         | 94.00±5.523              | 92.20±5.263              | 101.6±11.13              |
| Day 10     | 96.20±8.786        | 86.80±3.701         | 91.25±4.787              | 88.25±2.986              | 93.20±16.33              |
| Day 12     | 100.0±2.382        | 89.20±4.712         | 95.50±2.560              | 91.25±4.646              | 96.80±17.70              |
| Day 14     | 94.40±10.92        | 91.00±10.07         | 96.00±4.967              | 91.00±5.598              | 98.20±10.52              |

Induction of diabetes with streptozotocin after 16 hours fasting

| % increase after induction | 83.6 | 83.3 | 79.7 | 81.3 | 78.4 |

*significant reduction with respect to untreated control; †significant increase with respect to untreated control; ‡significant reduction with respect to positive control (metformin); §significant increase with respect to positive (metformin) control.

Table 9: Profile of glucose (mg/dl) levels during three weeks pre-treatment with 100mg/kg, 200mg/kg and 400mg/kg b.w. of aqueous extract of A. indica leaf expressed as mean ± SD.

| Time (Days) | Normal (Untreated) | 100mg/kg Metformin | 100 mg/kg Aqueous Extract | 200 mg/kg Aqueous Extract | 400 mg/kg Aqueous Extract |
|------------|--------------------|---------------------|--------------------------|--------------------------|--------------------------|
| Day 0      | 96.00±12.75        | 91.60±9.339         | 108.4±9.476              | 98.80±9.257              | 97.00±9.192              |
| Day 2      | 97.80±10.33        | 92.00±9.618         | 109.0±5.788              | 96.00±7.616              | 95.20±6.017              |
| Day 4      | 98.20±9.731        | 92.00±7.810         | 103.2±8.228              | 96.00±9.000              | 95.40±9.711              |
| Day 6      | 95.00±10.56        | 92.40±6.348         | 103.0±11.29              | 92.20±5.070              | 92.40±10.95              |
| Day 8      | 99.00±7.382        | 95.80±3.768         | 95.00±3.873              | 96.80±8.136              | 95.60±9.889              |
| Day 10     | 100.0±2.345        | 93.00±5.612         | 93.20±5.310              | 99.00±11.31              | 95.80±14.46              |
| Day 12     | 96.20±8.786        | 88.80±4.817         | 89.60±12.60              | 98.20±22.56              | 87.80±8.927              |
| Day 14     | 94.40±10.92        | 91.60±5.857         | 95.40±10.29              | 93.00±6.205              | 86.00±6.782              |
| Day 16     | 94.80±7.155        | 91.20±6.496         | 96.00±12.41              | 98.60±12.12              | 83.80±7.530              |
| Day 18     | 90.40±9.236        | 92.80±7.791         | 90.25±11.95              | 91.20±7.662              | 85.20±10.11              |
| Day 20     | 98.60±5.683        | 87.20±8.526         | 94.50±10.54              | 87.80±6.221              | 84.40±3.715              |
| Day 21     | 94.40±11.06        | 89.88±8.871         | 107.3±8.421              | 93.40±8.142              | 87.80±6.140              |

Induction of diabetes with streptozotocin after 16 hours fasting

| % increase in glucose level after induction of DM | 82.7 | 84.5 | 65.4bd | 76.3bd | 72.4bd |

*significant reduction with respect to untreated control; †significant increase with respect to untreated control; ‡significant reduction with respect to positive control (metformin); §significant increase with respect to positive (metformin) control.

Table 10: Profile of glucose (mg/dl) levels during four weeks pre-treatment with 100mg/kg, 200mg/kg and 400mg/kg b.w. of aqueous extract of A. indica leaf expressed as mean ± SD.

| Time (Days) | Normal (Untreated) | 100mg/kg Metformin | 100 mg/kg Aqueous Extract | 200 mg/kg Aqueous Extract | 400 mg/kg Aqueous Extract |
|------------|--------------------|---------------------|--------------------------|--------------------------|--------------------------|
| Day 0      | 96.00±12.75        | 82.40±12.14         | 87.40±9.017              | 98.40±10.83              | 108.4±8.204              |
| Day 2      | 98.20±9.731        | 83.60±5.595         | 87.40±6.148              | 94.20±11.73              | 106.0±7.382              |
| Day 4      | 95.00±10.560       | 85.60±7.668         | 86.60±6.841              | 95.60±13.85              | 86.80±7.918              |
| Day 6      | 100.0±2.345        | 82.20±7.014         | 90.40±6.229              | 86.80±10.55              | 87.60±7.537              |
| Day 8      | 99.00±7.382        | 81.00±5.874         | 88.00±5.701              | 86.40±9.633              | 86.00±7.416              |
| Day 10     | 96.20±8.786        | 82.00±11.60         | 90.20±11.37              | 89.00±11.68              | 90.00±5.568              |
| Day 12     | 94.80±7.155        | 84.80±9.757         | 83.75±9.570              | 85.60±4.980              | 89.00±7.246              |
| Day 14     | 94.40±10.92        | 86.20±8.758         | 88.75±6.238              | 95.20±10.12              | 95.40±11.46              |
The Journal of Phytopharmacology

Day 16
90.40±9.236
81.40±9.711
86.25±5.252
91.20±11.21
89.00±7.246
Day 18
98.60±5.683
85.60±8.989
75.75±4.272
94.40±12.18
89.80±11.14
Day 20
94.40±11.06
83.80±5.975
82.50±4.796
100.6±6.731
88.60±4.561
Day 22
95.80±10.73
84.40±8.532
82.00±3.606
95.60±9.555
94.40±11.35
Day 24
89.00±13.80
82.60±5.413
87.34±2.631
97.60±9.072
73.60±29.13
Day 26
93.60±10.19
85.60±4.393
85.67±6.429
97.60±10.99
80.20±35.27
Day 28
95.20±12.46
83.60±4.099
86.67±4.726
93.40±11.46
98.00±15.38

| Day 31 | 569.6±28.76 | 576.6±37.57 | 327.0±42.04 | 296.8±75.67 | 275.3±60.84 |
|--------|-------------|-------------|-------------|-------------|-------------|
| % increase | 83.3 | 85.5 | 73.5bd | 68.5bd | 64.4bd |

*significant reduction with respect to untreated control; *significant increase with respect to untreated control; *significant reduction with respect to positive control (metformin); *significant increase with respect to positive (metformin) control.

4. DISCUSSION

The percentage composition of the phytochemicals in the leaves of A. indica was as follows: Alkaloids (11.36%), cardenolides (6.19%), flavonoid (5.34%), hecamaglutinin (6.331%), oxalate (0.0204%), phenol (4.312%), phytate (4.600%), saponin (12.55%), steroid (0.257%) and tannin (6.17%). A qualitative phytochemical analysis conducted on the Aqueous, Ethanol, and Chloroform extracts of the neem leaf by Ramadas and Subramanian [33], revealed that the ethanol extract of A. indica has alkaloids, glycosides, flavonoids, saponins, tannins, phenolic compounds and reducing sugar. A Study conducted by Koom and Buddh [34], revealed that triterpenoids, phenolic compounds, carotenoids, steroids, valaminoids, ketones and tetratriterpenoids, azadirachtninoserved in Azadirachta indica might be responsible for the antibacterial activity of the leaf which is responsible for their uses as herbs in primary health care. These phytochemicals are beneficial to the plants as they serve as important metabolites that help the plant to exhibit its pharmacological role in treatment and management of diseases. The phytochemicals analyzed in the leaves of A. indica may be responsible for its antidiabetogenic property.

The percentage change in weight of the rats after the induction of diabetes compared with the normal untreated rats and the rats pre-treated with a standard antidiabetic drug decrease significantly (p<0.05) after twenty-eight days with respect to the graded doses of the A. indica extract (100mg/kg, 200mg/kg and 400mg/kg b. w. of A. indica leaf extract). A. indica extracts Pretreatment with extract of A. indica leaf hindered weight loss and this is confirmed by the findings of Chattopadhay and Bandyopadhyay [35]. Gupta et al. [36] revealed that controlled blood glucose level lowers the breakdown of structural protein leading to the decrease in weight loss.

The groups that were pretreated for twenty-one days with aqueous extract of A. indica leaf before the induction of diabetes showed a significant (p<0.05) decrease in their fasting blood glucose levels after the induction of diabetes compared with the normal untreated rats and the rats pre-treated with a standard antidiabetic drug (table 9). This result is in agreement with the findings of Ezeigwe et al. [22] who reported the controlling effect of A. indica leaf extract on blood glucose levels thereby delaying the onset of diabetes.

The groups that were pretreated for twenty-eight days with graded doses of aqueous extracts of A. indica leaf before the induction of diabetes showed a significant (p<0.05) decrease in their fasting blood glucose levels after the induction of diabetes compared with the normal untreated rats (table 10). These results suggest that the longer the administration of the extract the more its protective effects against the onset of diabetes, which confirms the result reported by Okpet et al. [37], Jelodaret et al. [21] suggested that the antidiabetic properties of neem extract is as a result of its ability to quicken the sufficient production of insulin by the pancreas which utilize glucose in the cell or possibly, the competence of the extract to regenerate the beta cell to carry out its functions. In a research work by Gupta et al. [38], it was stated that the extract shows significant protection against the oxidative damage induced by STZ in heart and erythrocytes of rats.

5. CONCLUSION

The longer the period of pre-treatment with the aqueous extract of A. indica leaves the better the protection against the onset of diabetes. This entails that administration of the extract can delay the onset of diabetes in diabetes prone subjects. This could be by quickening the sufficient production of insulin by the pancreas which activates the glucose transporters to transport glucose to the cells for effective utilization or possibly, the capability of the extract to regenerate the beta cells to produce enough insulin needed to signal the glucose transporters to carry glucose to the cells.

Ethical Approval

The authors hereby declare that “Principles of Laboratory Animal Care” were followed. The experiments were 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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Authors Contribution

Authors OCE and FCE designed the study, performed the statistical analysis, wrote the protocol and the first draft of the manuscript. Authors OCE and NNN managed the literature searches and the analysis of the study. All authors read and approved the final manuscript.
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