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Ezeigwe Obiajulu Christian
Department of Applied
Biochemistry, Faculty of
Biosciences, Nnamdi Azikiwe
University Awka, Anambra
State, Nigeria

Ezennaya Chidinma Felicia
Department of Chemistry
Biochemistry and Molecular
Biology, Faculty of Science, Alex
Ekwueme Federal University,
Ndufu-Alike Ikwo, Ebonyi,
Nigeria

Ifedilichukwu Nma Helen
Immunovirology and Vaccine
Development Unit, Department
of Medical Biotechnology,
National Biotechnology
Development Agency (NABDA),
Abuja, Nigeria

Soronnadi Vivian Nneka
Department of Science
Laboratory Technology, Imo
State Polytechnic, Umuagwo,
Ohaji, P.M.B. 1472 Owerri, Imo,
Nigeria

Chukwuemeka Ugochi Vivian
Department of Science
Laboratory Technology, Imo
State Polytechnic, Umuagwo,
Ohaji, P.M.B. 1472 Owerri, Imo,
Nigeria

Alaabo Prince Ogochukwu
Department of Biochemistry,
Michael Okpara University of
Agriculture, Umudike, Umuahia,
Abia, Nigeria

Corresponding Author:
Ezeigwe Obiajulu Christian
Department of Applied
Biochemistry, Faculty of
Biosciences, Nnamdi Azikiwe
University Awka, Anambra
State, Nigeria

Antidiabetic property and antioxidant potentials of aqueous extract of *Azadirachta indica* leaves in streptozotocin-induced diabetic rats

Ezeigwe Obiajulu Christian, Ezennaya Chidinma Felicia, Ifedilichukwu Nma Helen, Soronnadi Vivian Nneka, Chukwuemeka Ugochi Vivian and Alaabo Prince Ogochukwu

Abstract

Objective: The study was conducted to investigate the effect of the aqueous extract of *Azadirachta indica* leaves on blood glucose levels, antioxidant enzymes and inhibition of lipid peroxidation in streptozotocin (STZ)-induced diabetic Wistar albino rats.

Materials and Methods: The phytochemical analysis was done using standard methods. Diabetes was induced by a single intraperitoneal administration of 50 mg/kg body weight of STZ. Fasting blood sugar levels of the experimental rats were measured at two days interval during the 28 days of treatment. The antioxidant enzymes were assayed using standard methods. Lipid peroxidation was determined by the thiobarbituric acid-reacting substances (TBARS) assay method.

Results: Phytochemical screening reveals the presence of saponin, flavonoids, phenols, cardiac glycosides, alkaloids and tannins. The result of the antidiabetic studies showed a significant ($p < 0.05$) reduction in the fasting blood glucose level of all the extract treated groups compared with diabetic untreated rats. The reduction in fasting blood glucose levels of the extract treated groups was consistently observable from day 6 to day 28 of the treatment. The serum catalase and glutathione peroxidase activities of rats treated with the extract showed a significant increase ($p < 0.05$) compared with the diabetic untreated control. There was a significant ($p < 0.05$) reduction in malondialdehyde level of all the extract treated groups compared with diabetic untreated group.

Conclusion: These results suggest that the aqueous extract of *A. indica* at varied doses has the potential to lower fasting blood glucose levels in streptozotocin-induced diabetic rats which can be as a result of the presence of certain phytochemicals responsible for the increase in the antioxidant enzyme activity of the experimental animals.

Keywords: Diabetes, *Azadirachta indica*, streptozotocin, antioxidant enzymes

Introduction

High levels of glucose in blood leads to a chronic condition called diabetes mellitus. It is a disorder in glucose metabolism which can be caused by deficiency in insulin secretion or a combination of insulin resistance and inadequate insulin thus leading to hyperglycemia [1]. This can lead to kidney damage, eye damage, nerve damage and an increased risk of cardiovascular diseases. The rate of death from diabetes mellitus has been estimated by the International Diabetes Federation (IDF) in 2013 to be 383 million by 2035 [2]. Nigeria has been reported to have a significant increase in the incidence of diabetes mellitus with the south-south recorded to have the highest incidence [3].

There is need for a lasting solution to this epidemic worldwide problem as most of the antidiabetic oral drugs used have failed to give a lasting solution to glycemic control while exhibiting side effects [4]. These side effects include; hypoglycemia, lactic acidosis, gastrointestinal symptoms, weight gain and edema [5]. These have led to increased death rate caused by diabetes mellitus and its complications. The conventional drugs are either costly to afford by the masses with its concomitant side effects and contraindications. Recently, the use of plants in treating disease conditions has become an important area of research. These plants are otherwise called medicinal plants due to their pharmacological action to maintain health. They have being used in both traditional and modern medicine as therapeutic agents in treatment of various diseases like diabetes mellitus, typhoid, malaria, ulcer with minimal or no side effects [6].

Medicinal plants have an important role in the treatment of diabetes particularly in underdeveloped countries with insufficient resources instead of relying on expensive drugs imported from other countries. An example of such plant is *Azadirachta indica*.

Azadirachta indica commonly known as Neem plant is a medicinal plant which is used in folklore medicine in the treatment of diabetes mellitus, malaria, typhoid, viral and bacterial infections [7]. All parts of *A. indica* leaf, flower, fruits, seed, bark, and root have been reported in use as cure in different disease conditions [8]. Although studies have reported the antidiabetic property of the aqueous extracts of *A. indica* [9], more research is needed to ascertain the mechanism by which the leaves exhibit the antidiabetic potentials.

This work aims at investigating the antidiabetic properties of the aqueous extract of *A. indica* leaf and further determines the antioxidant potentials by analyzing the phytochemicals present in the leaves and the antioxidant enzymes. In this study, we have evaluated the effect of the aqueous extract of *A. indica* at graded doses of 100, 200 and 400mg/kg body weight orally administered to the Wistar albino rats on daily basis for 28 days. The parameters evaluated are blood glucose levels, antioxidant enzymes (catalase, superoxide dismutase and glutathione peroxidase) and lipid peroxidation levels.

Materials and Methods

Collection and identification of plant materials

The leaves of neem were collected from Nnamdi Azikiwe University, Awka. Anambra State. The sample was validated by a botanist in the Department of Botany, Nnamdi Azikiwe University, Awka. The voucher number as deposited in the herbarium of Nnamdi Azikiwe University, Awka is 14.

Preparation of extract

The leaves were washed and air dried at room temperature. The dried leaves were ground into powder using Corona manual grinding machine. Exactly 1kg of the ground leaves powder of *A. indica* leaves was soaked in 5 litres of distilled water for 24 hrs for aqueous extraction. The aqueous extraction was sieved and filtered using Whatman no. 1(125mm) filter paper. The filtrate was lyophilized (freeze dried) to powder. The extract was stoppered in universal bottles and preserved in the refrigerator for use. The extract was solubilized with distilled water on daily basis and administered to the experimental animals (extract treated groups) for a period of 28 days.

Chemicals

Streptozotocin, manufactured by sigma, Germany. All other chemicals used in this study were of analytical grade.

Phytochemical analysis

Phytochemical tests were carried out on the ethanol extracts using standard phytochemical methods as described by [10, 11, 12]. The phytochemicals that were assayed include anthracene glycosides, saponins, tannins, flavonoids, cyanogenic glycosides, alkaloids, cardiac glycosides, phenolic group.

Experimental animals

A total of thirty (30) male albino rats of Wistar strains were bred within the animal house of Chris Farms, Awka. They were maintained and housed in aluminium cages in the Department of Applied Biochemistry Laboratory, Nnamdi Azikiwe University, Awka with optimum condition. They

were allowed to acclimatize with the environment freely for one week before use. The animals were allowed free access to guinea growers mash pellets (Vital feed, Agro products) and water *ad libitum*. The floors of the cage were filled with saw dusts and cleaned daily.

Animal grouping and extract administration

Thirty Albino rats of Wistar strains were randomly grouped into six (Groups A-F). Group A was left uninduced. Group B to F were induced with diabetes intraperitoneally using 50mg/kg body weight of streptozotocin. Group B was diabetic untreated, Group C was treated with 100mg/kg body weight metformin (a standard antidiabetic drug used for the treatment of diabetes), group D to F were respectively treated with 100, 200 and 400 mg/kg body weight of the extract respectively. The treatment was carried out daily for a period of 28 days.

Determination of antidiabetic effects of aqueous extract of *A. Indica* Leaves

The fasting blood glucose levels of the rats were checked before the administration of Streptozotocin 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 streptozotocin 50mg/kg body weight [13]. The rats were orally given 5ml each of 5% glucose solution 2 hours after administering streptozotocin 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 [14]. Diabetes was confirmed to have been induced when the fasting blood glucose level was observed to be far much higher than normal (200mg/dl and above). Treatment was done for 28 days. While the treatment lasted, blood glucose levels were determined every two days (48 hrs.) using One Touch Glucometer and test strips.

Determination of antioxidant enzyme levels

Superoxide Dismutase (SOD) activity

Superoxide Dismutase activity was determined by its ability to inhibit the auto-oxidation of epinephrine determined by the increase in absorbance at 480nm as described by [15]. The reaction mixture (3ml) containing 2.95ml 0.05M sodium carbonate buffer pH 10.2; 0.02ml of serum and 0.03 ml of epinephrine in 0.005N HCl was used to initiate the reaction. The reference cuvette contain 2.95ml buffer, 0.03ml of substrate (epinephrine) and 0.02ml of water. Enzyme activity was calculated by measuring the change in absorbance at 480nm for 5 mins.

Catalase activity determination

Serum catalase activity was determined according to the method of Beers and Sizer as described by [16] by measuring the decrease in absorbance at 240nm due to the decomposition of H₂O₂ in a UV recording spectrophotometer. The reaction mixture (3ml) contains 0.1ml of serum in phosphate buffer (50mM, pH 7.0) and 2.9ml of 30mM H₂O₂ in phosphate buffer pH 7.0. An extinction coefficient at 240nm H₂O₂ of 40.0M⁻¹cm⁻¹ [17] was used for the calculation. The specific activity of catalase was expressed as moles of H₂O₂ reduced per minute per mg protein.

Calculation

$$\text{SOD/CAT} = \Delta A/\text{min} \times V_T/\sum X V_S$$

ΔA = change in absorbance

V_T = Total volume

V_S = Sample volume

Σ = Molar extinction coefficient

Glutathione Peroxidase Activity (GPx)

This was determined by the method of Beutter and Kelly as adapted by [18]. Hydrogen peroxide (H_2O_2) is reduced by oxidizing reduced glutathione (GSH) to form GSSG. The reaction mixture contained 1ml of 0.3M phosphate buffer (pH 7.4), 0.3ml of 10mM glutathione, 0.3ml of 15mM H_2O_2 and 1.37ml distilled water. Exactly 0.1ml serum was added to the mixture in the cuvette, shaken and absorbance was read at 340nm. Extinction co-efficient of $1.622 \times 10^{-3} M^{-1} CM^{-1}$ was used to calculate enzyme activity which was expressed in unit mg protein.

Enzyme activity was calculated using formula

$$\frac{OD/min \times V}{\Sigma xv}$$

OD = Optical Density

V = Total volume of reaction mixture

v = volume of the sample

Σ = Molar extinction coefficient

Determination of lipid peroxidation

Lipid peroxidation was determined by the thiobarbituric acid-reacting substances (TBARS) assay method of [19]. The reaction depends on the formation of complex between malondialdehyde and thiobarbituric acid (TBA). 0.4ml of serum is collected into the test tubes; 1.6ml of 0.25N HCl was added together with 0.5ml of 15% trichloroacetic acid and 0.5ml of 0.375% of thiobarbituric acid and then mixed thoroughly.

The reaction mixture was then placed in 100°C boiling water for 15 minutes, allowed to cool and centrifuged at 3000 rpm for 10 minutes. The supernatant was collected and the optical density recorded at 532nm against reagent blank containing distilled water.

The lipid peroxidation activity was calculated using the formula

$$\frac{\text{Optical density}}{\text{Time}} \times \frac{\text{extinction co-efficient}}{\text{amount of sample}}$$

Table 2: Fasting blood glucose levels of the rats used for antidiabetic studies measured after twenty-eight days of the study.

Time (Days)	Normal (Non-diabetic)	Untreated Diabetic	100mg/kg Metformin	100mg/kg Aqueous Extract	200mg/kg Aqueous Extract	400mg/kg Aqueous Extract
Initial b/f ind	90.40±10.36	98.40±22.58	89.80±9.311	89.20±11.17	91.00±5.477	98.00±21.32
Day 0	92.40±14.42	573.0±47.3b	533.4±110b	546.0±119b	488.8±141b	534.8±91.9b
Day 2	86.00±8.775	594.8±7.66b	554.8±67.8b	534.2±46.4b	517.8±91.1b	499.4±114bc
Day 4	76.20±7.050	600.0±0.00b	504.0±137b	484.3±99.bc	453.3±10bc	503.6±62.5b
Day 6	96.40±10.60	590.0±17.3b	491.4±94bc	314.8±217bc	264.0±138bc	375.0±133bc
Day 8	74.20±8.890	554.7±34.3b	375.6±158bc	454.8±138bc	254.3±147bc	185.8±124bc
Day 10	81.20±7.791	557.0±53.7b	308.8±134bc	378.3±149bc	247.0±99bc	237.3±139bc
Day 12	99.60±12.22	586.5±19.0b	339.6±165bc	489.3±90bc	296.7±211bc	243.0±77bc
Day 14	80.40±13.89	592.5±6.36b	335.2±163bc	263.7±196bc	191.3±176bc	168.0±96bc
Day 16	97.00±22.37	558.0±59.0b	339.4±58bc	303.3±174bc	286.3±79bc	241.8±185bc
Day 18	91.00±11.77	534.5±51.6b	296.8±60bc	222.0±138bc	208.0±136bc	264.5±83bc
Day 20	93.60±12.21	539.0±74.9b	287.8±143bc	209.3±219bc	185.3±53bc	282.0±75bc
Day 22	86.00±12.32	521.0±36.7b	307.4±176bc	301.7±224bc	200.3±83bc	213.3±155bc

Where the extinction coefficient value is $1.56 \times 10^{-5} M^{-1} CM^{-1}$
The unit is expressed as $\mu\text{mol}/\text{MDA}/\text{mg}$ of protein.

Statistical Analysis

Data were analysed using one-way analysis of variance (ANOVA) and POST-HOC test for multiple comparison to determine significant differences between means. The differences between the means were regarded as significant at $p < 0.05$ and the differences of the mean were expressed using SPSS version 23.

Results

Phytochemical Analysis

Phytochemical screening detected the presence of saponin, flavonoids, phenols, cardiac glycosides, alkaloids and tannins (table 1). Saponin was detected in substantial amount. Flavonoids and phenols were detected in moderate amount while cardiac glycosides, alkaloids and tannins were detected in minute quantities.

Table 1: The phytoconstituents and aqueous

S/NO	Phytoconstituents	Aqueous Extract
1	Anthracene glycosides	-
2	Alkaloids	+
3	Cardiac glycosides	+
4	Cyanogenic glycosides	-
5	Flavonoids	++
6	Phenols	++
7	Saponin	+++
8	Tannins	+

+ = Present ++ = moderately present +++ = highly present

Fasting blood glucose levels

The result of the fasting blood glucose levels before and after induction of diabetes were shown in table 2. The fasting blood glucose levels significantly ($p < 0.05$) increased after induction of diabetes with Streptozotocin when compared with the fasting blood glucose levels of the normal control. There was a gradual reduction of glucose levels in the treated groups, which was significant ($p < 0.05$) in contrast to the untreated group from the 6th day of treatment to the 28th Day. The group treated with 100mg/kg of the extract showed the highest reduction in glucose level followed by 200mg/kg and 400mg/kg body weight on the 28th day of treatment. These reduction were found to be significant ($p < 0.05$) when compared with the group treated with 100mg/kg body weight of metformin. The fasting blood glucose levels of the diabetic untreated group remained high without any significant reduction throughout the period of the experiment.

Day 24	83.20±13.65	580.5±3.53b	321.2±159bc	299.0±165bc	295.3±181bc	123.3±58bc
Day 26	86.80±5.450	587.0±12.7b	262.6±255bc	250.0±178bc	255.7±211bc	174.0±112bc
Day 28	80.60±6.269	563.5±26.1b	253.8±119bc	152.7±198bc	208.0±114bc	220.0±103bc

A. Significant reduction with respect to normal control; B. Significant increase with respect to normal control; C. Significant reduction with respect to diabetic untreated control; D. Significant increase with respect to diabetic untreated control

Antioxidant enzymes

The result of the antioxidant enzyme activities were as shown in table 3. The catalase activity of the group treated with the extracts at varied doses significantly ($p < 0.05$) increased when compared with the diabetic untreated. The extract at a dose of 100mg/kg body weight also showed a significant ($p < 0.05$) increase in the catalase activity compared with the normal control.

There was no significant ($p < 0.05$) difference in the mean values of the superoxide dismutase activity (SOD) of the

extract treated groups compared with the diabetic untreated and normal control groups. Glutathione Peroxidase activity increased significantly ($p < 0.05$) in all groups administered graded doses of the extract compared with the diabetic untreated and normal control groups. The increase was highest in the group that was treated with 200mg/kg body weight of the extract followed by 400mg/kg body weight and was least in the group treated with 100mg/kg body weight of the extract.

Table 3: The effect of treatment with different doses of aqueous extract of neem leaf respectively for a period of twenty-eight days on the antioxidant enzymes.

Anti-oxidant Enzymes	Normal (Non-diabetic rats)	Diabetic Untreated	100mg/kg Metformin	100mg/kg Aqueous Extract	200mg/kg Aqueous Extract	400mg/kg Aqueous Extract
CAT (IU/L)	0.2620±0.356	0.040±0.014	0.5700±0.462b	0.3200±0.276bd	0.1600±0.173a	0.130±0.086ad
SOD (UI/L)	0.9842±0.010	0.969±0.005	0.9858±0.0078	0.9960±0.0227	0.9890±0.0053	0.985±0.0158
Glut Px(IU/l)	0.8780±0.276	0.790±0.226	1.0620±0.5162	1.5330±1.0101	2.6533±0.8411	2.557±1.468bd

A. Significant reduction with respect to normal control; B. Significant increase with respect to normal control; C. Significant reduction with respect to diabetic untreated control; D. Significant increase with respect to diabetic untreated control

Lipid Peroxidation

The aqueous extract of *A. indica* leaves inhibited lipid peroxidation at all the doses administered (table 4). There was a significant ($p < 0.05$) reduction in the malondialdehyde level of the groups treated with the graded doses of the extract

compared with the diabetic untreated group. The group that was treated with a standard antidiabetic drug (metformin) also reduced significantly ($p < 0.05$) compared to the diabetic untreated group.

Table 4: The effect of treatment with different doses of aqueous extract of neem respectively for a period of twenty-eight days on the lipid peroxidation level.

Lipid Peroxidation inhibition	Normal (Non-diabetic)	Diabetic Untreated rats	100mg/kg Metformin	100mg/kg Aqueous Extract	200mg/kg Aqueous Extract	400mg/kg Aqueous Extract
MDA Level (umol/L)	0.228±0.1329	0.590±0.349	0.2380±0.445c	0.360±0.192c	0.290±0.1770c	0.3150±0.103c

A. Significant reduction with respect to normal control; B. Significant increase with respect to normal control; C. Significant reduction with respect to diabetic untreated control; D. Significant increase with respect to diabetic untreated control

Discussion

Herbal therapy remains the most safe, accessible and affordable remedy to people for the alleviation of various diseases. Such plants include *Azadirachta indica*. *Azadirachta indica* leaves extract is used in the treatment of various diseases, one which is diabetes mellitus in folklore medicinal practice. Phytochemical analysis carried out on the aqueous extract of *A. indica* leaves revealed the presence of high content of saponin while phenols and flavonoids were moderately present. These phytochemicals could be responsible for the antidiabetic property of *A. indica* leaf extract. Results of phytochemical analysis seems to be in line with the findings of [20]. Reports have demonstrated that the presence of these phytochemicals can protect human against certain ailments including diabetes mellitus [21, 22].

It has been reported that streptozotocin causes oxidative stress in experimental animals thereby increasing the formation of reactive oxygen species. Streptozotocin can cause organ specific toxicities and metabolic alterations [23]. Streptozotocin induces diabetes in rats by damaging the pancreatic β cells thereby resulting to hypoinsulinemia and hyperglycemia [24]. The aqueous extract of *A. indica* leaves lowered fasting blood glucose levels significantly ($p < 0.05$) towards recovery in streptozotocin-induced diabetic rats (table 2). The graded doses administered to the experimental subjects for a period

of 28 days revealed that aqueous extract of *A. indica* is more effective than the conventional drug used for the treatment of diabetes. These results indicate that the aqueous extract of *Azadirachta indica* can be used as a hyperglycemic remedy even at a dose of 100 mg/kg weight. This report agrees with the results of [25, 26]. The reduction of blood glucose levels in the treated rats can be due to the presence of the phytochemicals; flavonoids, polyphenols, tannins and saponins. The presences of phytochemicals in the aqueous extract was reported by [8].

Saponins is highly present in the aqueous extract and have been reported to have the ability of reducing blood glucose levels. Its mechanism of action is reported to be by enhancing the liver glycogen storage, increasing glucose utilization by the liver and decreasing gluconeogenesis through the inhibition of two major enzymes glucose -6- phosphatase and fructose -6-bisphosphatase [27]. Saponin can also cause hypoglycemia by the rejuvenation of insulin and its release from the beta cells of the pancreas [28] and an increase in the expression of GLUT4 [29].

The phytochemical screening also showed the present of flavonoids which have also being reported to have a hypoglycemic effect by increasing insulin secretion, enhancing insulin mediated glucose uptake in cells, increasing calcium uptake and inhibiting the enzyme activity of aldose

reductase^[30]. Phenols is also reported of exhibiting its antidiabetic effects. Its mechanism of action includes; increasing glucose uptake and the synthesis of glycogen^[31]. Hyperglycemia causes an increase in oxidative stress causing a decrease in the antioxidant enzyme activities with increase in the production of reactive oxygen species^[32]. It has been reported that substances that can reduce the oxidative stress can be therapeutic for diabetes mellitus^[33]. Research is performed to seek natural antioxidant substances which are safe with little or no side effects. Recently, the focus is on medicinal plants with phytochemical compounds due to the potentials of promoting health^[34]. This study also investigated the antioxidant effects of the aqueous extract of *A. indica*. The result showed an increase in the level of antioxidant enzymes activities; catalase, superoxide dismutase and glutathione peroxidase in the groups treated with the aqueous extract of *A. indica* leaves. Studies have shown that the presence of phytochemicals such as phenols, flavonoids and tannins in the plant indicates its ability to act as an antioxidant. The antioxidants of contents of medicinal plants contribute to their remedy of disease conditions^[35]. Plants extracts with high amount of phenols and flavonoids has also shown antioxidation activities^[36]. So the increase in antioxidant enzyme activities may be due to the presence of the phytochemicals.

Lipid peroxidation proceeds through a free radical chain reaction which has been associated with biomembrane damage; this damage has been shown in different disease conditions such as diabetes, cancer and cardiovascular diseases^[36]. The extract significantly inhibited lipid peroxidation by lowering the malondialdehyde level in the extract treated groups compared to the diabetic untreated groups. Evidence had shown that the extracts of *A. indica* reduces *in vivo* lipid peroxidation activities and cellular damage associated with oxidative stress^[38] by its antioxidant effects^[39, 40].

Conclusion

The oral administration of different doses of the extract showed a decrease in fasting blood glucose levels, increase antioxidant enzymes activities and inhibited lipid peroxidation. The presence of important phytochemicals detected in the extract protects cells as powerful antioxidants which repair damages caused to cells by highly reactive oxygen species. These results show that the aqueous extract of *A. indica* leaves can be used as an alternative antidiabetic remedy for the treatment and management of diabetes.

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