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Full Length Research Paper

ANTIDIABETIC PROPERTY AND ANTIOXIDANT POTENTIALS OF ETHANOL EXTRACT OF *AZADIRACHTA INDICA* LEAF IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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ABSTRACT

The purpose of this study was to investigate the antidiabetic property and antioxidant potentials of ethanol extract of *Azadirachta indica* leaves in streptozotocin-induced diabetic rats. The phytochemical analysis was done using standard phytochemical methods. Diabetes was induced in the rats by a single intraperitoneal dose of 50mg/kg body weight of streptozotocin. Blood glucose levels of diabetic rats were measured at two days interval during the 28 days of treatment. Superoxide Dismutase (SOD) activity, Catalase (CAT) activity and Glutathione Peroxidase (GPx) activity were determined using standard diagnostic methods. Lipid peroxidation was determined by the thiobarbituric acid-reacting substances (TBARS) assay method. Qualitative Phytochemical screening revealed that the ethanol extract of *A. indica* leaf contains saponin in substantial quantity. Alkaloids and Tannins were moderately present while cardiac glycosides, cyanogenic glycosides, flavonoids and phenols were detected in a very minute quantity. The ethanol extract of *A. indica* leaf significantly (p<0.05) lowered the fasting blood glucose level of streptozotocin-induced diabetic rats. There was a significant (p<0.05) increase in the SOD activity (1.023±0.051) of the group of rats treated with the ethanol extract of neem leaf at a dose of 400mg/kg b.w compared with the diabetic untreated group (0.969±0.005). There was a significant (p<0.05) increase in the CAT activity (0.3050±0.4101) of the group of rats treated with the ethanol extract of neem leaf at a dose of 200mg/kg b.w compared with the diabetic untreated group (0.040±0.014). Also, there was a significant (p<0.05) increase in the GPx activity (2.623±0.516) of the group of rats treated with the ethanol extract of *A. indica* leaf at a dose of 400mg/kg b.w compared with the diabetic untreated group (0.790±0.226). The malondialdehyde (MDA) level (0.363±0.174, 0.313±0.0896 and 0.2875±0.0922) significantly (p<0.05) decreased in the groups administered graded doses (100, 200 and 400mg/kg b.w) of

ethanol extract of *A. indica* leaf respectively compared with the diabetic untreated group (0.590±0.349). The ethanol extract contains important phytochemicals which may be responsible for its antidiabetic property and antioxidant potentials in streptozotocin-induced diabetic rats. It lowers blood glucose level in STZ-induced diabetic rats favourably towards recovery and improved health.

Keywords: Neem leaf, Hypoglycaemia, Streptozotocin, Antioxidant enzymes, Malondialdehyde.

INTRODUCTION

Diabetes is a metabolic disorder characterized by high blood glucose level resulting from defective insulin action, or glucose transporters which can be linked to insulin resistance. Diabetes is a serious, chronic disease that occurs either when the pancreas does not produce enough insulin (a hormone that regulates blood sugar, or glucose), or when the body cannot effectively use the insulin it produces (WHO, 2016). Type 2 diabetes mellitus was first described as a part of metabolic syndrome in 1988 (Patlak, 2002). The worldwide prevalence of diabetes has continued to increase noticeably. Globally, as of 2011, an estimate of 366 million people had diabetes mellitus, with type 2 making up about 90% of the cases (Jumpup, 2007). The number of people with type 2 diabetes mellitus is increasing in every country with about 80% of people with diabetes mellitus living in low-income and middle-income countries. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs involved in the metabolism of glucose. The major cause of the abnormalities observable in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues (Baynes, 2015).

There are different causes of diabetes but by far the majority of cases are classified as either type 1 or type 2 diabetes. Some of the symptoms of type 1 diabetes already known include weight loss, polyphagia, polydipsia, constipation, fatigue, cramps, blurred vision, polyphagia and candidiasis (Bearse *et al*., 2004). Long lasting type 1 diabetes mellitus patients are most likely to be susceptible to some microvascular complications (Hove *et al*., 2004) and macrovascular disease (Pittas, 2009). Most cases of Type 2 diabetes are easily diagnosed because of the complications associated with it. Complications of type 2 diabetes include cardiovascular diseases leading to myocardiac infarction (Alberti *et al*., 2004).

The major challenge for health care providers today is finding a lasting medicine for the management of diabetes mellitus. The high cost of conventional drugs used in the treatment of diabetes as well as the attendant undesirable side effects and contraindications has continued to impede the successful treatment of diabetes. This has triggered the recourse to complementary alternative medicine in the treatment of diabetes without following up their effect on essential biochemical parameters. Many people depend solely on medicinal plants for the treatment of diabetes due to its

affordability and availability even when the efficacy of the herbal remedy has not been established. Neem plant is one of the most versatile medicinal plants with a wide spectrum of biological activity. Traditional healers make use of the leaves of neem to treat diabetes. Scientific report support the hypoglycemic activity of neem leaves (Khosla, *et al*., 2000). Maragathavali *et al.* (2012) even reported that chewing of neem leaf in the morning for twenty-four days protected the body from diabetes. However, there is still need to investigate the antidiabetic property and antioxidant potential of the leaves of neem.

MATERIALS AND METHODS

Sample Collection and Identification

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.

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 neem were soaked in 1 litre of 80% ethanol for 24 hrs. It was sieved and filtered using Whatman no 1(125mm) filter paper. The filtrate was evaporated to dryness using rotary evaporator at 50^0 C and the paste put in a stoppered universal bottle and stored in the refrigerator until needed. The paste was dissolved in distilled water before use.

Chemicals

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

Experimental Animals

A total of thirty (30) male wistar albino rats were purchased from the animal house of Chris Farms, Awka and used for the study. They were maintained and housed in aluminium cages in the Department of Applied Biochemistry Laboratory, Nnamdi Azikiwe 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 Vital feed distributors, Awka.

Phytochemical Analysis

Phytochemical tests were carried out on the ethanol extracts using standard phytochemical methods as described by Harbone (1993), Sofowora (1993), Trease and Evans (1996). The phytochemicals that were assayed include anthracine glycosides, saponins, tannins, flavonoids, cyanogenic glycosides, alkaloids, cardiac glycosides, phenolic group.

Determination of Anti-Diabetic effects of ethanol extract of neem leaf

A total of thirty (30) male wistar albino rats were used for the study. Twenty-five (25) of the rats were made diabetic and subsequently divided into five groups of five rats each. The remaining 5 non-diabetic rats were used as control subjects. Groups A, B and C were orally administered 100mg,

200mg and 400mg/kg body weight of ethanol extract of neem leaf respectively. Group D received 100mg/kg body weight of metformin (a standard drug used in the treatment of diabetes), group E did not receive any treatment and group F was a control group of 5 non-diabetic rats that received 1ml of distilled water in place of treatment regimen. The initial blood glucose levels of the rats were checked and recorded using One Touch Glucometer and test strips before the induction of diabetes. 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 (Rakieten, *et al*., 1963). The rats were orally given 5ml each of 5% glucose solution 2 hours after injection of 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 Trinder (1972). Diabetes was confirmed to have been induced when the fasting glucose level was observed to be far much higher than normal (between 60mg/dl to 120mg/dl) to above 200mg/dl. 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 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 Sun and Zigma (1978). The reaction mixture (3ml) containing 2.95ml of 0.05M sodium carbonate buffer at pH 10.2; 0.02ml of liver homogenate and 0.03 ml of epinephrine in 0.005N HCl was used to initiate the reaction. The reference cuvette contains 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 5min.

Catalase activity determination

Serum catalase activity was determined according to the method of Beers and Sizer as described by Uso *et al*., (2005) by measuring the decrease in absorbance at 240nm due to the decomposition of H_2O_2 in a UV recording spectrophotometer. The reaction mixture (3ml) contain 0.1ml of serum in phosphate buffer (50mM, pH 7.0) and 2.9ml of 30mMH_2O_2 in phosphate buffer pH 7.0. An extinction coefficient at 240nm H_2O_2 of $40.0M^{-1}cm^{-1}$ (Aebi 1984) was used for the calculation. The specific activity of catalase was expressed as moles of H_2O_2 reduced per minute per mg protein.

Calculation

$CAT = \Delta A/min \times V_T/\sum \times Vs$

ΔA = change in absorbance

V_T = Total volume

Vs = Sample volume

$\sum$ = Molar extinction coefficient

Determination of Glutathione Peroxidase Activity (GPx)

This was determined by the method of Beutter and Kelly as adapted by Anthony

et al., (2003). 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 15mMH_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 x $10^{-3}M^{-1}CM^{-1}$ was used to calculate enzyme activity which was expressed in unit mg protein.

Enzyme activity was calculated using formula

$$\frac{OD/min \times V}{\sum \times v}$$

OD = Optical Density
V = Total volume of reaction mixture
v = volume of the sample
$\sum$ = Molar extinction coefficient

Lipid Peroxidation

Lipid peroxidation was determined by the thiobarbituric acid-reacting substances (TBARS) assay method of Buege and Aust (1978). 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 0.25NHCl 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^oC 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:

$$\frac{\text{optical density}}{\text{time}} \times \frac{\text{extinction co-efficient}}{\text{amount of sample}}$$

where the extinction coefficient value is 1.56 x $10^{-5}M^{-1}CM^{-1}$

The unit is expressed as umol/MDA/mg of protein.

RESULTS AND DISCUSSION

Phytochemical Analysis

The result of the qualitative phytochemical screening showed that the ethanol extracts of neem contain saponin in substantial quantity. Alkaloids and Tannins were moderately present in the ethanol extract while cardiac glycosides, cyanogenic glycosides, flavonoids and phenols were detected in minute quantities (Table 1).

Table 1: Result of the qualitative phytochemical analysis

S/NO	Phytoconstituents	Ethanol extract
1	Anthracine glycosides	-
2	Alkaloids	++
3	Cardiac glycosides	+
4	Cyanogenic glycosides	+
5	Flavonoids	+
6	Phenols	+
7	Saponin	+++
8	Tannins	++

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

Fasting Blood Glucose Profiles of Diabetic Rats treated with Ethanol neem leaf extract

The result of twenty-eight (28) days treatment with ethanol leaf extracts of *A. indica* following induction of diabetes with streptozotocin is shown in table 2. The group of rats administered different doses of the ethanol leaf extracts of neem showed significant (p<0.05) reduction in the fasting blood sugar level from the 4th day of treatment compared with the diabetic untreated group. The groups treated with the ethanol extract of neem at different doses showed significant (p<0.05) reduction in fasting blood glucose profiles compared with the group treated with metformin with a better reduction observed in the group that was treated with 400mg/kg b.w ethanol extract of *A. indica* leaf. The fasting blood glucose level of the diabetic untreated rats remained consistently high without any significant reduction. The reduction in the fasting blood glucose level of rats administered 400mg/kg b.w ethanol extract of *A. indica* leaf were more pronounced compared with the groups administered 100mg/kg b.w and 200mg/kg b.w.

Table 2: Fasting blood sugar 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 Ethanol Extract	200mg/kg Ethanol Extract	400mg/kg Ethanol Extract
Initial b/find	90.40±10.36	98.40±22.58	89.80±9.311	76.60±11.01	91.80±11.14	79.20±5.020
Day 0	92.40±14.42	573.0±47.36	533.4±110.7	533.6±50.78	555.4±88.94	569.4±46.50
Day 2	86.00±8.775	594.8±7.662	554.8±67.86	492.2±101.2	595.0±7.550	535.0±94.48
Day 4	76.20±7.050	600.0±0.00	504.0±137.0	436.0±91.51	465.4±129.7	343.4±86.09
Day 6	96.40±10.60	590.0±17.32	491.4±94.13	345.0±77.64	246.8±122.7	182.2±94.04
Day 8	74.20±8.890	554.7±34.31	375.6±158.9	350.8±104.0	293.8±197.0	192.2±87.21
Day 10	81.20±7.791	557.0±53.74	308.8±134.0	190.5±137.6	368.6±200.1	286.8±89.21
Day 12	99.60±12.22	586.5±19.01	339.6±165.8	157.8±110.3	157.8±128.8	223.8±86.11
Day 14	80.40±13.89	592.5±6.364	335.2±163.5	217.8±109.4	179.8±67.34	211.2±68.13
Day 16	97.00±22.37	558.0±59.09	339.4±58.05	183.3±126.6	156.5±71.55	99.00±39.75
Day 18	91.00±11.77	534.5±51.62	296.8±60.29	320.8±155.2	221.3±133.6	119.0±71.82
Day 20	93.60±12.21	539.0±74.95	287.8±143.2	286.5±75.52	255.0±96.95	197.8±112.6
Day 22	86.00±12.32	521.0±36.77	307.4±176.6	258.0±211.8	211.8±138.6	166.2±112.5
Day 24	83.20±13.65	580.5±3.536	321.2±159.4	282.4±198.8	198.8±87.58	181.0±109.7
Day 26	86.80±5.450	587.0±12.73	262.6±255.6	207.8±144.7	184.0±99.68	145.8±97.10

Day 28	80.60±6.269	563.5±26.16	253.8±119.8	206.3±167.4	177.0±156.7	143.8±79.17

Result of Antioxidant Enzyme Activity by Neem Leaf Extract

Results show that while diabetes did not have any effect on the superoxide dismutase activity, it caused the perturbation of catalase and glutathione peroxidase, both of which are involved in antioxidant activity (See table 3). The *A. indica* leaf extract had some ameliorative effect on the catalase enzyme activity (p<0.05) with the 200mg/kg bw dose showing the best restorative effect even when compared with metformin.

There was a significant (p<0.05) increase in the glutathione peroxidase (GPx) activity of the group of rats treated with the graded doses of ethanol extracts of neem leaf compared with the diabetic untreated group. The increase was dose-dependent as can be seen from table 3. The GPx level in the diabetic untreated group reduced when compared with the normal control group.

Table 3: The effect of treatment with different doses of ethanol extract of *A. indica* leaf 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 Ethanol Extract	200mg/kg Ethanol Extract	400mg/kg Ethanol Extract
CAT (IU/L)	0.2620±0.356	0.040±0.014	0.5700±0.4628*a	0.1275±0.1159*a	0.3050±0.4101*a	0.070±0.018
SOD (UI/L)	0.9842±0.010	0.969±0.005	0.9858±0.0078	0.9885±0.0066	0.9898±0.0103	1.023±0.051*
GluthPx(IU/l)	0.8780±0.276	0.790±0.226	1.0620±0.5162	0.1275±0.1159	1.6875±2.0680*a	2.623±0.516*a

*** significant increase (p<0.05) with respect to diabetic untreated rats. [a] significant increase with respect to normal non-diabetic rats.**

Result of Lipid Peroxidation Inhibition by Neem Leaf Extract

Induction of diabetes apparently increased malondialdehyde (MDA) level in rats. The MDA level decreased (p<0.05) significantly in the groups administered ethanol extracts of neem leaf compared with the diabetic untreated group (table 4). *A. indica* leaf extract helped to normalize MDA level in a dose-dependent fashion. The group that was administered 100mg/kgbw of metformin did not show any significant difference in the MDA level compared with the normal control. The MDA level of the diabetic untreated group increased (p<0.05) significantly when compared with the normal control group.

Table 4: The effect of treatment with different doses of ethanol extract of *A. indica* leaf on the lipid peroxidation for a period of twenty-eight days.

Lipid Peroxidation inhibition	Normal (Non-diabetic)	Diabetic Untreated rats	100mg/kg Metformin	100 mg/kg Ethanol Extract	200mg/kg Ethanol Extract	400mg/kg Ethanol Extract
MDA Level(umol/L)	0.228±0.1329	0.590±0.349	0.2380±0.445*	0.363±0.174*	0.313±0.0896*	0.2875±0.0922*

***significant decrease ($p<0.05$) with respect to diabetic untreated rats.**

DISCUSSION

Qualitative Phytochemical analysis of ethanol extract of *A. indica* leaf reveal that it contains substantial amount of saponin, moderate amounts of alkaloids and tannins and minute amounts of phenol, flavonoids, cardiac and cyanogenic glycosides. It is well-known that plants produce these chemicals to protect themselves, but it has similarly been demonstrated that many phytochemicals can protect human against diseases (Kimura *et al*., 2005). Saponins have been reported to cause hypoglycaemia (Lacaille-Dubois *et al*., 2000; Traore *et al*., 2000). Diabetes is known to manifest due to oxidative stress and free radicals' generation. Flavonoids have the capacity to act as powerful antioxidants which can protect the human system from free radicals and reactive oxygen species (Atmani *et al*., 2009).

Antioxidants exert their activity by scavenging the free radicals thereby giving rise to fairly stable radical. Radical-scavenging activities of the tannins and phenolics suggest that they may be responsible for the antidiabetic activity of neem leaf extract. Phytochemicals are biologically active and naturally occurring chemical compounds found in plants, which provide health benefits for humans. It is well-known that plants produce phytochemicals to protect themselves. However, it has also been demonstrated that many phytochemicals can protect human against diseases (Nostro *et al*., 2000).

Oxidative stress has been shown to have a marked effect in the cause of diabetes mellitus which results to a reduction in the antioxidant status of affected subjects. The antioxidant enzymes (CAT and GPx) activities were significantly ($p<0.05$) increased in the rats treated with the different doses of the neem leaf extract compared with the diabetic untreated rats which showed a decrease in the antioxidant activities (table 3). The results obtained from this research shows that decrease in the activities of antioxidant enzymes in STZ-induced animals and attainment of near normalcy in neem treated rats indicates that oxidative stress caused by STZ could be nullified due to the effect of the ethanol extracts of neem leaf. This observation is in line with the reports of Hossain *et al*. (2013) who reported the free radical scavenging properties of neem leaf due to rich source of important antioxidants.

Diabetes induces lipid peroxidation as seen in increased concentration of malondialdehyde (MDA) level in diabetic untreated rats. However, the administration of ethanol extracts at specific doses caused a significant (p<0.05) decrease in MDA concentration of the diabetic rats (table 4). This difference was observed to be significant (p<0.05) compared with the diabetic untreated rats. However, the decrease in MDA levels of those administered the different doses of the extracts were dose-dependent. Constituents of *A. indica* such as flavonoids, phenols and tannins have antioxidant activity. These phytochemicals might be responsible for the lipid peroxidation inhibition by scavenging free radicals (Doaa *et al*., 2011).

CONCLUSION

The ethanol extract of *A. indica* leaf contains important phytochemicals which may be responsible for its antidiabetic property and antioxidant potentials in streptozotocin-induced diabetic rats. It lowers blood glucose concentration in streptozotocin-induced diabetic rats and has antioxidant property against streptozotocin-induced diabetic rats. The ethanol extract of *A. indica* leaf also prevented lipid peroxidation in streptozotocin-induced diabetic rats.

CONFLICT OF INTEREST

Authors hereby declare no conflict of interest.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" were followed. All experiments have been examined and approved by the ethics committee of Nnamdi Azikiwe University, Awka, Nigeria.

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