Attenuation of nonenzymatic glycation, hyperglycemia, and hyperlipidemia in streptozotocin-induced diabetic rats by chloroform leaf extract of *Azadirachta indica*

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**INTRODUCTION**

*Azadirachta indica* (AI), also known as neem, belongs to the family Meliaceae, which is one of the most useful medicinal plants.¹⁴ AI has been used traditionally in the control of diabetes mellitus in many countries. The blood sugar level-lowering activity of AI oil and leaf extracts has been reported in various models of diabetic animals.¹² The aqueous extract of neem leaves significantly decreases the blood sugar level and prevents glucose-induced hyperglycemia in normal rats, streptozotocin diabetic rats and alloxan-induced diabetic rabbits.¹³ The ethanol extract of neem leaves has also been shown to demonstrate antilipid peroxidative, antihyperglycemic and anti-hypercholesterolemic activities as well as to reduce the triglyceride serum levels in a diabetic rat model.¹⁶ Treatment of AI combined with *Vernonia amygdalina* was assessed to determine the markers of hepatotoxicity in serum-included glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) activities, total protein, albumin and urea, which indicated that neem provides the best protection against hepatic dysfunction.¹⁵ More recent studies on 90 noninsulin-dependent male diabetic rats show that the capsules prepared with neem leaf are potentially helpful in the reduction of the diabetic symptoms and blood pressure of the subjects.¹⁶ In another study, it was observed that chronic treatment with the ethanolic extract of neem in diabetic rats has an effect on blood glucose, pancreatic islet histopathology, and oxidative status of the pancreas. The result showed improved islet morphology and oxidative status in neem-treated diabetic rats.¹⁷ The current investigation is an attempt to study the hypoglycemic effect of the chloroform leaf extract on streptozocin-induced diabetes in rats.

**ABSTRACT**

Background: The hypoglycemic effects of hexane, chloroform and methanol extracts of leaves of *Azadirachta indica* (AI) were evaluated by oral administration in streptozotocin-induced severe diabetic rats (SD). **Materials and Methods:** The effect of chronic oral administration of the extract for 28 days was evaluated in streptozotocin diabetic rats. Lipid peroxidation, glycogen content of liver and skeletal muscles, insulin, superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), oxidized glutathione (GSSG) levels were determined. In addition, advanced glycation end product formation (AGEs) was evaluated. **Results:** The most active extracts were obtained with chloroform. Chloroform extract from AI shows increased levels of SOD, GSH, GSSG and CAT, hepatic glycogen content, glucose-6-phosphatase and insulin plasma levels, which also decreased the glucokinase (GK), lipid peroxidation and insulin resistance. The chloroform extract exhibited significant inhibitory activity against advanced glycation end product formation with an IC₅₀ average range of 79.1 mg/ml. **Conclusion:** *Azadirachta indica* can improve hyperlipidemia and hyperinsulinaemia in streptozocin-induced diabetic rats and, therefore, AI can be potentially considered to be an antidiabetic-safe agent.

**Key words:** *Azadirachta indica*, antihyperglycaemic, antihyperlipidaemic, antiglycation

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diabetic models in rats that are known as insulin-deficiency models.

**MATERIALS AND METHODS**

Fresh leaves of *AI* were collected at Colima State Mexico and a sample specimen (no. 6532) was deposited in the Herbarium of the Escuela Nacional de Ciencias Biologicas, IPN, for further reference.

**Animals**

The study was conducted in the Wistar male albino rats strain, weighing about 180–225 g. Before and during the experiment, the animals were fed with the laboratory normal diet and water *ad libitum*. The procedures involving animals and their care conformed to the international guidelines Principles of Laboratory Animals Care.

**Preparation of plant extracts**

About 300 g of leaves was dried and powdered in a mechanical grinder. This powder material was extracted with 1 L of hexane, chloroform and methanol consecutively using a soxhlet apparatus. These extracts were filtered and concentrated by rotary vacuum evaporator for complete removal of solvent. An aqueous extract of the suspension was prepared using 2% (v/v) Tween-80 and used for oral administration.

**Study on glucose-supplied animals (Oral glucose tolerance test)**

After overnight fasting, a 0-min blood sample was taken from the diabetic control rats group and another sample was taken from the group of rats treated with the plant extract. The rats of both groups were supplied with glucose orally (2 g/kg, p.o.) 30 min after the administration of the drug or the vehicle (for control). Blood glucose levels were measured at 30, 60, 120, and 180 min after the glucose was supplied to assess the effect of different doses of chloroform extract of the leaves of *AI* on blood glucose levels of the animals.

**Induction of diabetes mellitus**

Rats were not supplied with meals for 24 h before the induction of diabetes by STZ injection. Experimentally induced SD conditions were developed in a group of normoglycemic male Wistar albino rats by a single intravenous injection of STZ at a dose of 65 mg/kg body weight/rat in 0.5 ml of physiological saline. This single dose of STZ produced SD or type-1 diabetes, having fasting blood glucose level more than 250 mg/dl after 24 h of STZ injection, and this diabetic state was maintained throughout the experimental schedule:

Severe diabetic: it was treated with AI extracts, 300 mg/kg body weight, and the animals of control group received only 2% Tween-80 solution.

All groups were treated with the extract of chloroform or vehicle for 28 days. The fasting blood glucose was monitored on day 0 at 2, 4, 6, 8, and 12 h after the administration of the extracts. The drug solutions or vehicle or standards such as tolbutamide (40 mg/kg body weight) or glibenclamide (0.5 mg/kg body weight) were administered orally by gastric intubations once daily at 9:00 am. On the 28th day, diabetic animals were sacrificed by an ether overdose and the insulin level was determined.[8,9] Plasma blood glucose levels were determined by using the glucose oxidase–peroxidase (GOD-POD) method.

**Measurements of reduced superoxide dismutase, catalase, glutathione, and oxidized glutathione**

Livers were homogenized with a homogenizer (Ultra Turrax T25, Rose Scientific Ltd., Edmonton, Canada) in 10 volumes of a 50 mM sodium phosphate buffer (pH 7.4) at 4°C and the supernatant obtained was used for the following antioxidant enzyme measurements using commercial kits: reduced superoxide dismutase (SOD) (Oxis International), catalase (CAT) (Cayman Chemical company, Michigan USA), glutathione peroxidase (Oxis International), and glutathione reductase (Oxis International, California USA).

**Serum insulin**

Diabetic rats were orally administered with 300 mg/kg extracts daily for 28 days. Afterwards, blood samples were withdrawn in order to examine the insulin levels. Serum insulin was measured using a GLAZYME INSULIN-EIA TEST. The level of insulin in serum was expressed in µIU/ml.

**Insulin tolerance test**

Insulin tolerance test was performed at the end of repeated administration of the plant extract. After overnight (18 h) fasting, the insulin (0.5 U/kg body weight) solution was administered subcutaneously (s.c.). Blood sample were collected before administration of insulin and at 30, 60, and 120 min after the administration of insulin.

**Assay of Glycogen content, Glucose-6-Phosphatase, and glucokinase activities in the liver**

Rats of each group were orally administered with 300 mg/kg of extracts for 28 days; afterwards, they were sacrificed and their livers and skeletal muscle were removed. Glycogen content of liver and skeletal muscles was determined by using anthrone reagent and the results were expressed in mg/g of tissue. The amount of phosphate liberated per unit time, determined as blue phosphor–molybdous complex, was measured using a spectrophotometer at 700 nm, which showed the Glucose-6-Phosphatase...
(G6Pase) activity. Protein content of the liver extract was quantified with Bradford reagent. G6Pase activity (mU) was expressed as mmol of phosphate released/min/mg of protein.

GK activity was measured using a spectrophotometric method as previously described by Briefly. β-nicotinamide-adenine dinucleotide phosphate (NADPH) generated by GK was measured using a spectrophotometer at 340 nm. GK activity was estimated by the standard method, i.e. by subtracting the rate of NADPH formation in the presence of 0.5 mM glucose from that obtained in the presence of 100 mM glucose. Protein concentration was quantified with Bradford reagent, and one unit of enzyme activity (mU) was defined as mmol of substrate molecules converted by 1 mg protein per minute.

**Inhibition test on protein glycation in vitro**
Evaluation for glycation inhibitory activity of extracts was performed as previously described. Bovine serum albumin (10 mg/mL) was incubated with 250 mM D-fructose in the presence or absence of test materials for 21 days in 0.2 M potassium phosphate buffer (pH 7.4) at 37°C. After incubation, the fluorescence was measured at excitation 370 nm and emission 440 nm. Both incubations and measurements were carried out in triplicate. Aminoguanidine hydrochloride (Sigma-Aldrich Corporation, St. Louis, USA) (AG) dissolved in distilled water was used as a positive control. The concentration of each test sample showed 50% inhibition of the activities (IC<sub>50</sub>), which was estimated from the least-squares regression line of the logarithmic concentration plotted against the remaining activity.

**Statistical analysis**
Data are shown as mean ± SEM and statistical analyses were performed by means of the Student’s t-test or by one-way analysis of variance (ANOVA), followed by Dunnett’s multiple-comparison test (DMRT) using SPSS software when appropriate. The statistical significance was assumed at P-levels <0.05. Concentrations to produce 50% of the maximal responses (EC<sub>50</sub>) were calculated by a nonlinear curve fitting procedure using Sigma Plot version 8.0.

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**RESULTS**

**Effect on oral glucose tolerance test**
Table 1 shows the changes in the levels of blood glucose in the diabetic groups after oral administration of glucose (2 g/kg). The diabetic rats exhibit a significant increase in the blood glucose at 60 and 90 min. The extract of Al significantly (P < 0.05) decreased the blood glucose levels at 60, 90 and 120 min after glucose loading when compared with the control, which decreased after 60 min. Al-treated animals tend to give values to near-normal.

**Thiobarbituric acid assay**
Table 2 shows thiobarbituric acid reactive substances (TBARS) as index of lipid peroxidation in plasma, which increased in diabetic rats during the 28-day duration of the experiment. Chloroform extracts are significantly lower compared with those in the diabetic control.

**Glycogen level and Glucose-6-Phosphatase**
As shown in Table 3, the glycogen content was increased by AI as compared with the diabetic group. The result in our studies shows that there is an increase in the glycogen content of the liver and skeletal muscle of diabetic rats treated with extracts. G6Pase activity was assessed in all groups [Table 2]. Compared with the diabetic control, the G6Pase effect was decreased by the AI chloroform extract. GK activity was increased by the AI chloroform extract. AI extracts decreased the G6Pase activity and increased the glycogen and GK activity in the liver, which indicates increased hepatic glucose uptake and decreased hepatic glucose release. Thus, the chloroform extract enables hypoglycemic activity probably by reducing HGO via decreasing G6Pase activity and increasing GK activity.

**Effect on glutathione, superoxide dismutase, catalase, and oxidized glutathione**
Table 4 shows the results of the antioxidant defense system in the liver. In STZ-diabetic rat livers, the SOD, GSH, GSSG, and CAT levels were significantly lower in the diabetic control rats compared with the nondiabetic rats. The supplementation of chloroform extract applied to rats results in an increase in these parameters, restoring them toward the control levels.

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**Table 1: Effect of the chloroform extract of Azadirachta indica on oral glucose tolerance**

| Groups (300 mg/kg) | Blood glucose levels (mg/dl) |
|-------------------|-----------------------------|
|                   | 0 min | 30 min | 60 min | 90 min | 120 min |
| Normal            | 93.30 ± 2.11 | 184.61 ± 2.03 | 159.28 ± 2.64 | 130.49 ± 1.98 | 101.67 ± 1.24 |
| Diabetic control  | 275.32 ± 5.29 | 350.72 ± 7.12 | 406.07 ± 7.69 | 378.23 ± 5.01 | 340.11 ± 5.13 |
| Diabetic + Al     | 253.41 ± 4.25 | 335.28 ± 6.49* | 299.26 ± 6.05* | 160.12 ± 3.17* | 143.72 ± 2.26* |
| Diabetic + Tolbutamide (40 mg/kg) | 272.16 ± 5.19 | 347.19 ± 6.73* | 378.23 ± 5.01 | 160.12 ± 3.17* | 127.76 ± 2.58* |

Values are mean ± SEM for six animals in each group. *P < 0.05 when compared with diabetic controls, Al: Azadirachta indica.
Effect of *Azadirachta indica* on plasma insulin

The serum insulin level was decreased in diabetic rats with streptozotocin-induction as compared with the control group. There was also a significant difference of this parameter between the two groups [Table 3]. After 28 days of extract supplementation to SD rats, there was a significant increase in the serum insulin level with respect to the SD group.

**Insulin tolerance test**

Three hundred milligrams per kilogram of AI tended to decrease the blood glucose levels after 30 min when compared with the controls [Table 5].

Effect of AI on advanced glycation end products formation

The BSA-fructose model adopted in this study provides a useful tool for assessing the effect of extracts on nonenzymatic glycation process. Chloroform extracts of the leaves of AI displayed comparable activity to aminoguanidine, an advanced glycation end products (AGEs) inhibitor tested in clinical trials for the treatment of diabetic complications [Table 6].

**DISCUSSION**

Streptozotocin, a monofunctional nitrosourea derivative,
derivatives of leaves of *Azadirachta indica* on the formation of advanced glycation end products

| Treatment               | Inhibitory effect IC₅₀ (µg/ml) |
|-------------------------|-------------------------------|
| AI–chloroform           | 79.1                          |
| Aminoguanidina          | 27.2                          |

In conclusion, leaves of *AI* have beneficial effects on the regulation of blood glucose levels in diabetic rats, as indicated by a decrease in insulin resistance, maintenance of serum lipid profiles, and liver glycogen levels. These effects can be attributed to the antioxidant properties of the chloroform extract, which prevent oxidative stress and damage in β-cells. Furthermore, the extract may improve glucose tolerance, suggesting the effectiveness of the extract, which may involve increasing the peripheral utilization of glucose. The possible mechanism of antihyperglycemic action of this extract appears to be both pancreatic and extrapancreatic, which have been supported here by the significant increase of G6Pase activity in the liver in SD rats. The extrapancreatic effect may be due to the sensitization of insulin receptor in target organs or by inhibiting insulinase activity in both the liver and the kidney. The glucose tolerance test has been indicated that this extract did not execute the antihyperglycemic effect by modulating the absorption of glucose in the intestine.

Excessive hepatic glycogenolysis and gluconeogenesis associated with decreased utilization of glucose in the tissue is the fundamental mechanism underlying hyperglycemia in the diabetic state. Aberration of liver glycogen synthesis or glycogenolysis in diabetes may be due to a lack of or resistance to insulin, which is essential to activate the glycogen synthase system. The significant increase of liver glycogen level in the extract-treated diabetic groups may be due to reactivation of the glycogen synthase system. The experimental results indicate that leaves of *AI* possess significant antidiabetic activity and are capable of maintaining serum lipid profile and liver glycogen level.

Insulin resistance is a condition where normal or elevated insulin level produces an attenuated biological response. In diabetes, insulin resistance is the central pathophysiological event.

Hyperglycemia-induced oxidative stress may also cause liver cell damage. The administration of extracts improves impairments of SOD, GSH, GSSG, and CAT activities in SD. These results suggest that the extract prevents oxidative stress, acts as a suppressor against liver cell damage, and inhibits the progression of liver dysfunction induced by chronic hyperglycemia. In other studies, polyphenols from *AI* were found in higher concentrations, and had a high antioxidant capacity. Although antioxidant activity and total phenolics were not evaluated in the current study, these are correlated with antiglycation activity.

The possible mechanism of antihyperglycemic action of this extract appears to be both pancreatic and extrapancreatic, which have been supported here by the serum insulin assay in SD rats. The extrapancreatic effect of this extract has been focused here by the significant recovery of G6Pase activity in the liver in SD rats. The extrapancreatic effect may be due to the sensitization of insulin receptor in target organ or by inhibiting insulinase activity in both the liver and the kidney. From the glucose tolerance test, it has been indicated that this extract did not execute the antihyperglycemic effect by modulating the absorption of glucose in the intestine.

**CONCLUSION**

In conclusion, leaves of *AI* have beneficial effects on
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