Azadirachtolide: An anti-diabetic and hypolipidemic effects from Azadirachta indica leaves

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ABSTRACT: Introduction: Azadirachta indica (Meliaceae) leaves are used traditionally in the Indian Ayurvedic medicinal system to treat diabetes. The aim of the present study is to investigate the effect of azadirachtolide (tetranortriterpenoid from Azadirachta indica leaves) on blood glucose and serum lipid profiles on streptozotocin-induced diabetic rats. Methods: Streptozotocin-induced diabetic rats were used for the study. Azadirachtolide (at a dose 50 and 100 mg/kg) was administrated intra-peritoneally in diabetic rats once a week for 30 days. Biochemical parameters notably fasting blood sugar, total cholesterol, triglycerides, low-density lipoprotein, very low-density lipoprotein and high-density lipoprotein were determined. The in vitro alpha amylase and alpha glucosidase inhibitory effects of azadirachtolide were measured and IC₅₀ values were determined. Results: Azadirachtolide exhibited significant (P < 0.05) anti-diabetic as well as hypolipidemic effects by lowering FBS, TC, TG, LDL, and VLDL levels; but also with elevation of HDL level in diabetic rats. Azadirachtolide showed appreciable alpha amylase (IC₅₀ value of 55.80 ± 1.7 µg/ml) and alpha glucosidase inhibitory effects (IC₅₀ value of 47.85 ± 1.4 µg/ml) compared with acarbose (IC₅₀ value of 83.33 ± 1.8 µg/ml). Conclusion: The present study indicated that azadirachtolide possesses anti-hyperglycemic and anti-lipidemic effects. Thus, results suggested azadirachtolide has a beneficial effect in the management of diabetes associated with abnormal lipid profile and related cardiovascular complications.

KEYWORDS: Azadirachta indica, Azadirachtolide, Anti-diabetic, Hypolipidemic

INTRODUCTION

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion or insulin action, or both.[1] Broad research on diabetes has resulted in the development of a number of oral hypoglycemic agents including biguanides, sulphonylureas and thiozolidinediones which are available commercially for the management of diabetes. However, these drugs also produce undesirable side effects.[2] Hence, there is a need to develop alternative anti-diabetes medicines. The herbal medicines are widely used for the treatment of disease because of their effectiveness, safety, affordability and acceptability.[3] Medicinal plants including their phyto-compounds have been used in the Indian traditional systems of medicine for treatment of diabetic populace all around the world with less known scientific basis of their functioning.[4-7] Hence, phyto-products from medicinal plants need to be investigated by scientific methods for their anti-diabetic activity. Various medicinal effects have been reported for anti-inflammatory, anti-arthritis, antipyretic, antifungal, anti-bacterial, diuretic, immunomodulatory and anti-tumor properties. Phyto-compounds such as azadirachtins, nimocinol, isomeldenin, 2, 3′-dehydrosalanol gedunin, nimbin, nimolicinol from Azadirachta indica have been reported in the leaves.[8]

Tetranortriterpenoids has been reported for anticancer, antiviral, anti-allergic and anti-inflammatory activities.[9-12] There is no report on azadirachtolide (tetranortriterpenoid from Azadirachta indica leaves) for antidiabetic and hypolipidemic activities. Therefore, the effect of azadirachtolide (tetranortriterpenoid from Azadirachta indica leaves) on blood glucose and serum lipid profiles on streptozotocin-induced diabetic rats was investigated. Further, in vitro alpha amylase and alpha glucosidase an inhibitory effect of azadirachtolide was evaluated.

MATERIALS AND METHODS

Chemicals and reagents
Streptozotocin, starch azure, porcine pancreatic amylase, alpha glucosidase from yeast Saccharomyces cerevisiae, para-nitrophenyl gluco-pyanoside and Tris-HCl buffer were procured from Sigma Chemicals, USA. Dimethyl sulfoxide, acetic acid, calcium chloride, ethanol, chloroform, petroleum ether, potassium bromide,
deuterated chloroform, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, Whatmann filter paper and sodium carbonate were purchased from Merck, India. Thin layer chromatography plates were obtained from Merck (silica gel 60 F_{254} grade, Germany). Diagnostic kits and reagents for fasting blood sugar, total cholesterol, triglyceride, high density lipoprotein, low density lipoprotein and very low density lipoprotein were obtained from Merck, India. Acarbose was gifted by Zota Pharmaceutical Pvt. Ltd., Chennai. Glibenclamide (Aventis) was procured from local medical market.

Plant materials
Azadirachta indica leaves (Rutaceae) were collected from the locality of IIT Kharagpur campus, West Bengal, India in the month of September and October 2007. The leaves were inspected to be healthy and botanically identified and authenticated by Dr. M. Senthilkumar, Plant Biotechnologist, Prathyusha Institute of Technology and Management, Chennai. The herbarium Azadirachta indica leaves was deposited in the Prathyusha Institute of Technology and Management (PITAM) against voucher no. PITAM/ CH/00015/ 2007. Azadirachta indica leaves after collection were dried at room temperature (27-30°C) for 25-30 days. After complete drying (inspection), the dried materials were ground into fine powder using a domestic electric grinder (Product: GX 21, Bajaj appliances, Mumbai, India) and used for extraction.

Extraction and isolation
Dried plant powder of Azadirachta indica leaves (500 g) was extracted with ethanol (1 L) at room temperature. Then extract was filtered (Whatmann filter paper, 110mm, Cat. no 1001 110). The filtrate was evaporated by rotary evaporation (Buchi Rotavapor R-210) to get a dark greenish solid residue. These greenish solid residues (15 g) was successively extracted with petroleum ether (3.5 g) and chloroform (5.2 g) and subjected for in vitro alpha amylase inhibitory activity. The chloroform fraction showed appreciable alpha amylase inhibitory compared to petroleum ether fraction. The chloroform fraction was subsequently subjected to column chromatography using gradient elution using acetone and chloroform as solvents (10% acetone in chloroform for 15 mins, 20% acetone in chloroform for 15 mins and 30% acetone in chloroform for 15 mins). The fractions obtained with 20% acetone in chloroform afforded compound-I (10 mg). These fractions were subjected to preparative TLC with mobile phase hexane: ethyl acetate (8.5:1.5) for isolation of compound-I. Compound-I was identified as azadirachtolide by comparing its FTIR, ESI-MS and NMR with previously published literature (Ragasa et al., 1997).

General experimental procedure
HPTLC (CAMAG, Switzerland) analyzes was performed using silica gel 60 F_{254} TLC plate. All collected fractions were spotted (10 µl) on a silica gel 60 F_{254} (Merck, Darmstadt, Germany) TLC plate. The plate was air dried and then developed using the solvent system hexane: ethyl acetate (8.5:1.5) in a CAMAG-twin-trough glass chamber previously saturated with mobile phase vapor for 20 min. After developing the plate, it was dried at 105°C for 15 min and then it was scanned using Scanner 3 (CAMAG, Switzerland) at 254nm using WinCATS 4 software. IR spectrum was recorded using a Thermo Nicolet Nexus 870 FT-IR Spectrophotometer using potassium bromide pellets. Mass spectrum was recorded on Electro-Spray Ionization Mass Spectroscopy (Waters, UK). NMR spectra were recorded in CDCl3 in a Bruker 400 MHZ spectrometer using Topspin software.

In vitro alpha amylase inhibitory assay
The assay was carried out following the standard protocol with slight modifications.\textsuperscript{13} Starch azure (2 mg) was suspended in a tube containing 0.2ml of 0.5 M Tris-Hel buffer (pH 6.9) containing 0.01 M calcium chloride (substrate). The tube was boiled for 5 min and then pre-incubated at 37°C for 5 min. Azadirachtolide was dissolved in 0.1% of dimethyl sulfoxide in order to obtain concentrations of 10, 20, 40, 60, 80 and 100 µg/ml. Then 0.2 ml of azadirachtolide of a particular concentration was put in the tube containing the substrate solution. 0.1 ml of porcine pancreatic amylase in Tris-Hel buffer (2units/ml) was added to the tube containing the azadirachtolide and substrate solution. The reaction was carried out at 37 °C for 10 min. The reaction was stopped by adding 0.5 ml of 50% acetic acid in each tube. The reaction mixture was then centrifuged (Eppendorf -5804 R) at 3000 rpm for 5 min at 4°C. The absorbance of resulting supernatant was measured at 595 nm (Perkin Elmer Lambda 25 UV-VIS). The concentration of the azadirachtolide required to inhibit 50% of alpha amylase activity under the conditions was defined as the IC_{50} value. The experiments were repeated thrice with the same protocol.

The alpha amylase inhibitory activity was calculated as follows:

\[
\text{Alpha amylase inhibitory activity} = \frac{(\text{Ac}+) - (\text{Ac}–) - (As–Ab)}{(\text{Ac}+) - (\text{Ac}–)} \times 100
\]

Where, Ac+, Ac–, As, Ab are defined as the absorbance of 100% enzyme activity (solvent with enzyme alone), 0% enzyme activity (solvent without enzyme), a test sample (with enzyme) and a blank (a test sample without enzyme) respectively.

In vitro alpha glucosidase inhibitory assay
The assay was performed using a standard protocol.\textsuperscript{14} Alpha glucosidase (2U/ml) was premixed with 20 µl of azadirachtolide at various concentrations (10, 20, 40, 60, 80 and 100 µg/ml) and incubated for 5 min at 37°C. 1mM para-nitrophenyl glucopyranoside (20 µl) in 50mM of phosphate buffer (pH 6.8) was added to initiate the reaction. The mixture was further incubated at 37°C for 20 min. The reaction was terminated by addition of 50 µl of 1 M sodium carbonate and the final volume was made up to 150 µl. Alpha glucosidase activity was determined spectrophotometrically at 405nm on a Biorad microplate reader.
by measuring the quantity of para-nitrophenol released from pNPG. The assay was performed in triplicate. The concentration of azadirachtolide required to inhibit 50% of alpha glucosidase activity under the conditions was defined as the IC_{50} value. The experiments were repeated thrice with same protocol.

Animal studies
Adult male Wistar Rats (weighing 150-200 g) were used for this investigation. The animals were acclimatized to the laboratory conditions for a period of 2 weeks prior to the experiment. They were maintained at an ambient temperature (25 ± 2 °C) and relative humidity (40-60%), with 12/12 h of light/dark cycle. The animals were maintained on balance diet and water ad libitum. Institutional Animal Ethical Committee (IAEC) approved the study and all the experiments were carried out following the guidelines of CPCSEA, India.

Induction of diabetes and blood sample collection
A freshly prepared solution of streptozotocin (45mg/kg) in 0.1M citrate buffer pH 4.5 was injected intra-peritoneally in overnight fasted rats. After 3 days, blood was collected from the tail vein of overnight fasting rats under the supervision of a veterinary surgeon using aseptic conditions. The FBS level of blood was checked regularly up to the stable hyperglycemia stage, usually one week after streptozotocin injection. Animals with marked hyperglycemia (FBS 250 mg/dl) were selected for the study.[15]

Experimental design
Group I - Normal control
Group II - Diabetic control
Group III - Diabetic +50 mg/kg (i.p.) azadirachtolide
Group IV – Diabetic +100 mg/kg (i.p.) azadirachtolide
Group V - Diabetic + 0.5 mg/kg (i.p.) glibenclamide

The experiment was carried on five groups (I, II, III, IV and V) of six rats each. Group-I served as normal control. Group-II served as diabetic control. Group-III diabetic +50 mg/kg (i.p.) of azadirachtolide. Group IV-diabetic +100 mg/kg (i.p.) of azadirachtolide. Group V-diabetic + 0.5 mg/kg (i.p.) glibenclamide and served as positive control. The azadirachtolide was suspended in 0.3% w/v sodium carboxy methyl cellulose (Sodium CMC) as a vehicle and injected intra-peritoneally into rats once a week for a month with a dose of 50 mg/kg and 100 mg/kg body weight. The blood samples were collected from each rat by retro-orbital vein-puncture. Biochemical parameters were estimated at the beginning and after 30 days of experiment.

Biochemical parameters
Biochemical parameters notably fasting blood sugar (FBS), total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) levels and high-density lipoprotein (HDL) level in blood serum were measured spectrophotometrically (Semi-Autoanalyser, Microlab 300, Merck) as per the manufacturers instructions using diagnostic kits and reagents obtained from Merck, India.

Statistical analysis
All values were expressed mean ± standard deviation. Statistical analysis of in vivo results were performed by one-way analysis of variance (ANOVA) followed by Student's t-test. P < 0.05 was considered statistically significant. In vitro inhibitory assay statistical difference and linear regression analysis were performed using Graphpad prism 5 statistical software.

RESULTS
Azadirachtolide (10 mg) was isolated from 500 g of dried leaves of Azadirachta indica (Figure 1). HPTLC analyzes indicated that F_2 contained azadirachtolide and the retention factor (R_f) values of azadirachtolide was found to be 0.31 (Figure 2). The F_2 fractions were subjected to preparative TLC with the solvent system hexane: ethyl acetate (8:5:1.5) to get the compound-1 (azadirachtolide).

FTIR (KBr disc) is shown in Figure 3: peak at 3444 cm⁻¹ indicated presence of OH group, peak at 2925 cm⁻¹, 2854 cm⁻¹ was due to presence of C-H, peak at 1666 cm⁻¹ showed presence of C=O group and peak at 1736 cm⁻¹ indicated presence of ester carbonyl group, peak at 1666 cm⁻¹ showed presence of C=O group and peak at 1458 cm⁻¹ indicated presence of CH-CH bending (Figure 3).

ESI-mass spectroscopy showed the presence of a molecular weight peak of azadirachtolide at 593. ESI-MS (m/z, % intensity): m/z 593 [M-H]. Proton NMR (CDCl₃ solvent) showed senecioyloxy substituent δ 1.88 (3H), δ 2.20 (3H), δ 5.70 (1H). an acetate δ 1.97 (3H). Four additional methyl singlet δ 0.8, δ 1.25, δ 1.28, δ 1.30, two olefinic hydrogen δ 5.57, δ 5.71, methylene hydrogen bonded to oxygenated carbons δ 4.15 (1H), δ 3.81 (1H), δ 3.68 (1H), δ 3.59 (1H) and methine hydrogen bonded to oxygenated carbons δ 4.12 (1H), δ 4.15 (1H), δ 5.30 (1H), δ 5.47 (1H).

Azadirachtolide showed appreciable alpha amylase (IC_{50} value of 55.80 ± 1.7µg/ml) and alpha glucosidase inhibitory effects (IC_{50} value of 47.85 ± 1.4µg/ml) as compared with acarbose (IC_{50} value of 83.33 ± 1.8µg/ml) (Figure 4). The body weight was slightly increased in normal control rats compared to initial body weight whereas streptozotocin-induced diabetic rats showed loss of body weight (172.6 ± 2.05 g) after 30 days as compared with initially weight of diabetic rats (186.6 ± 1.24 g). However, body weight of diabetic rats was restored by treating with
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Streptozotocin treatment resulted in elevation of fasting blood glucose, triglycerides, total cholesterol, low density lipoproteins, very low density lipoproteins and a reduction in high density-lipoprotein levels as compared to the normal control rats (Table 2).

Intra-peritoneal administration of azadirachtolide (at a dose of 50 mg/kg and 100 mg/kg, once a week for 30 days) exhibited significant ($P < 0.05$) reduction in fasting blood sugar levels (204.0 ± 2.94 and 198.3 ± 2.86 mg/dl in diabetic rats. Diabetic rats treated with azadirachtolide (at a dose of 50 mg/kg and 100 mg/kg, i.p.) once a week for 30 days on being compared with diabetic rats exhibited significant ($P < 0.05$) reduction in fasting blood sugar levels (204.0 ± 2.94 and 198.3 ± 2.86 mg/dl respectively). The standard glibenclamide (0.5 mg/kg, i.p.) also showed anti-diabetic activity with reduction of fasting blood sugar level (215.0 ± 2.18 mg/dl) on 30 days as compared to the diabetic control. There was a significant ($P < 0.05$) reduction in triglycerides, total cholesterol, low density lipoprotein and very low density lipoprotein levels of diabetic rats treated with azadirachtolide (50 and 100 mg/kg, i.p.) on being compared with diabetic control. Also, there was a significant ($P < 0.05$) elevation of HDL level in azadirachtolide (50 and 100 mg/kg, i.p.) treated diabetic rats.

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**Figure 2:** HPTLC peaks of collected column fractions CE-Crude extract (Pink peak), F$_1$-10% acetone in chloroform (Violet peak), F$_2$-20% acetone in chloroform (Green peak), F$_3$-30% acetone in chloroform (Orange peak).

**Figure 3:** FTIR Spectrum of azadirachtolide.
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In the present study, an increase in blood sugar levels in diabetic rats was observed after the induction of diabetes by streptozotocin. This was prevented by treating diabetic rats with azadirachtolide (at a dose 50 and 100 mg/kg, i.p.) once a week for 30 days. The standard drug glibenclamide has been used to treat diabetes, which stimulate insulin secretion from pancreatic beta cells, it may be suggested that the mechanism of action of azadirachtolide is similar to glibenclamide. The azadirachtolide (at a dose 50 and 100 mg/kg, i.p.) treated diabetic rats showed a significant reduction in both fasting blood sugar levels and some lipid parameters (TC, TG, LDL, and VLDL).

Some biological active compounds such as mimbidin, sodium nimbidate, nimbin, nimbolide, gedunin, azadirachtin, mahmoodin, gallic acid, catechin, margoone, isomargolone, cyclic trisulphide, cyclic tetrasulphide and polysacharides were isolated from leaves and seeds of Azadirachta indica.[21] Aqueous extract of neem leaf significantly reduced the blood glucose level of male albino rats of Wistar strains.[22] The combined ethanolic extracts of Azadirachta indica and Vernonia amygdalina leaf extracts showed anti-hyperglycemic effect on alloxan induced albino wistar rats.[23] The weight loss in diabetic rats may be associated with lipid lowering activity of azadirachtolide or due to its influence on various lipid regulation systems. Treatment with azadirachtolide (at a dose 50 mg/kg and 100 mg/kg body weight) in diabetic rats may have potential role to prevent formation of atherosclerosis and coronary heart disease. The present in vivo study showed that intra-peritoneal

**Table 1: Body weights of streptozotocin-induced diabetic rats after treatment with azadirachtolide.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial body weight</th>
<th>Final body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>191.0 ± 0.81</td>
<td>200.0 ± 0.63</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>186.6 ± 1.24</td>
<td>172.6 ± 2.05</td>
</tr>
<tr>
<td>50 mg/kg of azadirachtolide</td>
<td>183.0 ± 1.60</td>
<td>178.3 ± 1.69*</td>
</tr>
<tr>
<td>100 mg/kg of azadirachtolide</td>
<td>182.0 ± 1.63</td>
<td>177.6 ± 1.69*</td>
</tr>
<tr>
<td>0.5 mg/kg of glibenclamide</td>
<td>180.3 ± 0.47</td>
<td>176.3 ± 1.24*</td>
</tr>
</tbody>
</table>

*P<0.05 compared with treated diabetic groups Vs Diabetic control. n = 6/group.
Values are expressed as mean ± S.D

**DISCUSSION**

The present study was designed to explore the effect of azadirachtolide (tetranortriterpenoid from Azadirachta indica leaves) on blood glucose and serum lipid profiles on streptozotocin-induced diabetic rats. A comparison of the FTIR, ESI-MS, NMR spectra of isolated fraction showed significant similarity with previously reported azadirachtolide data.[16] Intra-peritoneal administration of 50mg/kg and 100 mg/kg of azadirachtolide once a week for 30 days showed anti-diabetic and hypolipidemic effects in diabetic rats. Lipid abnormalities associated with atherosclerosis is the major cause of cardiovascular disease in diabetes. High level of TC and LDL are major coronary risk factors.[17] Further, several studies suggested that TG itself is interdentally related to coronary heart disease.[18,19] The abnormalities in lipid metabolism lead to elevation in the levels of serum lipid and lipoprotein that in turn play an important role in occurrence of premature and severe atherosclerosis, which affects patients with diabetes.[20]

In the present study, an increase in blood sugar levels in diabetic rats was observed after the induction of diabetes by streptozotocin. This was prevented by treating diabetic rats with azadirachtolide (at a dose 50 and 100 mg/kg, i.p.) once a week for 30 days. The standard drug glibenclamide has been used to treat diabetes, which stimulate insulin secretion from pancreatic beta cells, it may be suggested that the mechanism of action of azadirachtolide is similar to glibenclamide. The azadirachtolide (at a dose 50 and 100 mg/kg, i.p.) treated diabetic rats showed a significant reduction in both fasting blood sugar levels and some lipid parameters (TC, TG, LDL, and VLDL). Some biological active compounds such as mimbidin, sodium nimbidate, nimbin, nimbolide, gedunin, azadirachtin, mahmoodin, gallic acid, catechin, margoone, isomargolone, cyclic trisulphide, cyclic tetrasulphide and polysacharides were isolated from leaves and seeds of Azadirachta indica.[21] Aqueous extract of neem leaf significantly reduced the blood glucose level of male albino rats of Wistar strains.[22] The combined ethanolic extracts of Azadirachta indica and Vernonia amygdalina leaf extracts showed anti-hyperglycemic effect on alloxan induced albino wistar rats.[23] The weight loss in diabetic rats may be associated with lipid lowering activity of azadirachtolide or due to its influence on various lipid regulation systems. Treatment with azadirachtolide (at a dose 50 mg/kg and 100 mg/kg body weight) in diabetic rats may have potential role to prevent formation of atherosclerosis and coronary heart disease. The present in vivo study showed that intra-peritoneal
administration of azadirachtolide (at a dose 50 and 100 mg/kg) exhibited anti-diabetic and hypolipidemic effects in streptozotocin-induced diabetic rats.

One of the therapeutic approaches for type 2 diabetes is to reduce the post-prandial hyperglycemia. Alpha amylase and alpha glucosidase are the enzymes involved in the metabolism of carbohydrates. Alpha amylase degrades complex dietary carbohydrates to oligosaccharides and disaccharides, which are ultimately converted into monosaccharide. Liberated glucose is then absorbed by the gut and results in postprandial hyperglycemia. Inhibition of alpha amylase and alpha glucosidase limits postprandial glucose levels by delaying the process of carbohydrate hydrolysis and absorption.[24] The plant based alpha amylase and alpha glucosidase inhibitor offers a prospective therapeutic approach for the management of post-prandial hyperglycemia.[25] In this study, azadirachtolide showed appreciable alpha amylase and alpha glucosidase inhibitory effects compared with acarbose.

CONCLUSION

The present study indicated that azadirachtolide (at a dose 50mg/kg and 100 mg/kg body weight) exhibited anti-diabetic and hypolipidemic effects in streptozotocin-induced diabetic rats. Therefore, azadirachtolide could be used as an anti-diabetic agent in the management of diabetes associated with abnormalities of lipid profiles.

ACKNOWLEDGMENTS

Authors would like to acknowledge Prof. P.K. Dutta, Head, School of Medical Science and Technology, IIT Kharagpur and for his valuable support in the research work. The authors would like to acknowledge the Central Research Facility (CRF) of Indian Institute of Technology, Kharagpur for providing the facility of FTIR, ESI-MS and NMR.