Antidiabetic and antioxidant activity of *Annona squamosa* extract in streptozotocin-induced diabetic rats

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ABSTRACT

Introduction: The aim of the study is to analyse the antioxidant effect of oral administration of aqueous extract of *Annona squamosa (A. squamosa)* leaf on blood glucose, haemoglobin, glycosylated haemoglobin, plasma insulin, antioxidant enzymes and lipid peroxidation in liver and kidney to streptozotocin (STZ)-induced diabetic rats.

Methods: Aqueous extract of *A. squamosa* on blood glucose, haemoglobin, glycosylated haemoglobin, plasma insulin, serum lipid and the levels of lipid peroxides and antioxidant enzymes, such as catalase, superoxide dismutase, glutathione peroxidase and reduced glutathione, were examined in the liver and kidney tissues of control and experimental groups.

Results: Oral administration of *A. squamosa* aqueous extract to diabetic rats for 30 days significantly reduced the levels of blood glucose, lipids and lipid peroxidation, but increased the activities of plasma insulin and antioxidant enzymes, like catalase, superoxide dismutase, reduced glutathione and glutathione peroxidase.

Conclusion: The *A. squamosa* aqueous extract supplementation is useful in controlling the blood glucose level, improves the plasma insulin, lipid metabolism and is beneficial in preventing diabetic complications from lipid peroxidation and antioxidant systems in experimental diabetic rats; therefore, it could be useful for prevention or early treatment of diabetes mellitus.

Keywords: *Annona squamosa*, antioxidant, blood glucose, diabetes mellitus, free radicals, oxidative stress

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INTRODUCTION

Diabetes mellitus is an endocrine disorder that is characterised by hyperglycaemia[1]. The pharmaceutical drugs are either too expensive or have undesirable side effects. Treatment with sulphonylureas and biguanides are also associated with side effects[2]. However, for a number of reasons, complementary medicine has grown in popularity in recent years. Dietary measures and traditional plant therapies as prescribed by Ayurvedic and other indigenous systems of medicine were used commonly in India. Many indigenous Indian medicinal plants have been found to be useful to successfully manage diabetes and some of them have been tested and their active ingredients isolated[3,4]. The World Health Organisation (WHO) has also recommended the evaluation of the plants' effectiveness and conditions where we lack safe modern drugs[5].

In recent years, much attention has been focused on the role of oxidative stress, and it has been reported that oxidative stress may constitute the key and common event in the pathogenesis of secondary diabetic complications[6]. Free radicals are continuously produced in the body as a result of normal metabolic processes and interaction with environmental stimuli. Oxidative stress results from an imbalance between radical-generating and radical-scavenging systems that has increased free radical production or reduced activity of antioxidant defences or both. Implication of oxidative stress in the pathogenesis of diabetes mellitus is suggested not only by oxygen free-radical generation but also due to non-enzymatic protein glycosylation, auto-oxidation of glucose, impaired glutathione metabolism, alteration in antioxidant enzymes and formation of lipid peroxides[7-10]. In addition to reduced glutathione (GSH), there are other defense mechanisms against free radicals, such as the enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), whose activities contribute to eliminate superoxide, hydrogen peroxide and hydroxyl radicals[11].

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Many of the complications of diabetes mellitus, including retinopathy and atherosclerotic vascular disease, the leading cause of mortality in diabetes mellitus, have been linked to oxidative stress, and antioxidants have been considered as treatments[12]. Plants often contain substantial amounts of antioxidants, flavonoids and tannins and the present study suggests that antioxidant action may be an important property of plant medicines associated with the hypoglycaemic effect on diabetes mellitus[13].

Annona squamosa (A. squamosa), commonly known as custard apple, is cultivated throughout India, mainly for its edible fruit. The plant is attributed with medicinal properties, which include antifertility and antitumour activities in rats[14]. The young leaves of *A. squamosa* along with five grains of black pepper are used extensively for its antidiabetic activity by tribal men in and around the villages of the Aligarh district, India[15]. The treatment is particularly popular in the Lodha community where the plant is considered to be a sacred fruit. The formula is being successfully used by some Unani and Allopathic physicians and is in existence to date. *Piper nigrum* seeds have been used in herbal medicine due to its antirheumatic, anti-inflammatory, diuretic and antidiabetic properties[16].

The aqueous leaf extract has been reported to ameliorate hyperthyroidism, which is often considered to a causative factor for diabetes[17,18]. The antidiabetic activity of the aqueous extract has been reported in streptozotocin (STZ)-nicotinamide type 2 diabetic rats[19]. The objective of this investigation was to ascertain the scientific basis for its use in the treatment of diabetes mellitus. Therefore, this study was designed to investigate the protective effect of *A. squamosa* on lowering the blood glucose level, tissues lipid peroxides and enzymic antioxidants in STZ-induced diabetic rats.

METHODS

STZ was purchased from Sigma Chemical Co, St. Louis, MO, USA. All the other chemicals used were of analytical grade and purchased from commercial sources.

The young leaves of the plant *A. squamosa* were collected from The Survey of Medicinal Plant Unit, Regional Research Institute of Unani Medicine, Aligarh, India. Identification of the samples was done by using standard botanical monographs. They were further confirmed with the Department of Botany, Aligarh Muslim University, Aligarh, India and a voucher Specimen (HB 546) was deposited in the department herbarium.

The aqueous extract was prepared by cold maceration of 250 g of the shade-dried leaf powder in 500 ml of distilled water allowed to stand overnight, and boiled for 5-10 minutes till the volume was reduced to half its original volume. The solution was then cooled, filtered, concentrated, dried in vacuo (yield 36 g) and the residue stored in a refrigerator at 2-8°C for subsequent experiments.

Male albino Wistar rats, weighing 150-180 g obtained from Central Animal house, J.N. Medical College, Aligarh Muslim University, Aligarh, India, were used for the present investigations. The animals were maintained on standard rat feed supplied by Hindustan Lever Ltd, India. The experiments were conducted according to the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines (IAEC).

The animals fasted overnight and diabetes was induced by a single intraperitoneal injection of freshly-prepared STZ (55 mg/kg body weight of rats) in 0.1 M citrate buffer (pH 4.5)[20]. The animals were allowed to drink 5% glucose solution overnight to overcome the drug-induced hypoglycaemia. Control rats were injected with citrate buffer alone. The animals were considered as diabetic, if their blood glucose values were above 250 mg/dL on the third day after the STZ injection. The treatment was started on the fourth day after the STZ injection and this was considered the first day of treatment. The treatment was continued for 30 days.

The rats were divided into four groups comprising eight animals in each group as follows:

Group 1: Control rats given only buffer.

Group 2: Diabetic controls (STZ 55 mg/kg body weight of rats).

Group 3: Diabetic rats treated with protamine-zinc insulin *i.p.* injection (6 units/kg body weight of rats/day)[21].

Group 4: Diabetic rats treated with *A. squamosa* (300 mg/kg body weight of rats/day) in aqueous solution orally for 30 days.

After completion of treatment, the animals were sacrificed. Blood was collected in tubes containing potassium oxalate and sodium fluoride. Plasma was used for the estimation of glucose using the O-Toluidine method reported by Sasaki el al[22]. The levels of haemoglobin and glycosylated haemoglobin were estimated using the methods of Drabkin and Austin[23], and Nayak and Pattabiraman[24], respectively. Plasma insulin level was assayed by enzyme-linked immuno sorbent assay kit (ELISA, Boerhringer Mannheim, Germany).

The liver and kidney tissues were excised and rinsed in ice-cold saline. Tissues were cut into small pieces and homogenised with a Potter-Elvehjem tight-

fitting glass-Teflon homogeniser in Tris-HCl buffer (pH 7.4). The homogenate was centrifuged and the supernatant was used for various measurements. The following analyses were carried out: serum total cholesterol (TC), high density lipoprotein (HDL-C) and triglycerides (TG) were estimated using the standard kit of Ranbaxy laboratories, New Delhi, India. Low density lipoprotein (LDL-C)[25], thiobarbituric acid reactive substances (TBRAS) (lipid peroxides) and hydroperoxides were estimated according to the methods of Ohkawa et al[26] and Jiang et al[27], respectively.

GSH was estimated using the method of Sedlak and Lindsay[28]. The activity of SOD was assayed using the method of Marklund and Marklund[29]. The activity of GPx was assayed using the method of Lawrence and Burk[30]. CAT activity was assayed using the method of Aebi[31]. Protein was estimated using the method of Lowry et al[32]. All spectrophotometric measurements were carried out in a Camspec UV-Visible (Camspec M330B, UK) spectrophotometer.

All the grouped data were statistically evaluated using the Statistical Package for Social Sciences (SPSS) version 7.5 (Chicago, IL, USA). Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant differences test. p-values of less than 0.05 were considered to indicate statistical significance. All the results were expressed as mean ± standard deviation (SD) for eight animals in each group.

RESULTS

A significant increase in the level of blood glucose and a decrease in body weight were observed in diabetic rats when compared to control rats. Administration of *A. squamosa* and insulin to diabetic rats significantly decreased the level of blood glucose and increased body weight gain to near control level. The diabetic rats showed a significant decrease in the levels of total haemoglobin and a significant increase in the level of glycosylated haemoglobin (HbA_1c) and plasma insulin level as compared to diabetic rats. Administration of *A. squamosa* or insulin to diabetic rats restored the total haemoglobin and HbA_1c to almost control levels (Table I).

The serum lipid profile is shown in Table II. The values of TC, HDL-C and LDL-C of those treated with *A. squamosa* extract returned to values nearing that of the control group. This showed that treatment with *A. squamosa* significantly improved the lipid profile in

Table I. Effect of treatment *A. squamosa* leaf extract for 30 days on blood glucose, body weight, total haemoglobin, glycosylated haemoglobin and plasma insulin of control and experimental groups of rats.

Group	Blood glucose (mg/dL)		Change in body weight (g)	Total haemoglobin (g/dL)	Glycosylated haemoglobin (Hb %)	Plasma insulin (μU/ml)
	Initial	Final				
Control	76.0 ± 5.6	87.0 ± 5.7	32.4 ± 2.3	15.21 ± 1.12	7.4 ± 1.37	16.21 ± 0.69
Diabetic	269.8 ± 8.0*	296.0 ± 8.6*	-34.1 ± 2.0*	11.56 ± 0.71*	15.3 ± 1.56*	5.42 ± 0.31*
Diabetic + insulin	260.7 ± 7.7*	90.6 ± 6.0*	20.0 ± 1.1*	14.66 ± 0.68*	8.2 ± 0.93*	11.30 ± 0.52*
Diabetic + *A. squamosa*	264.4 ± 8.5*	98.1 ± 5.9*	21.6 ± 1.3*	15.20 ± 0.80*	8.5 ± 1.12*	12.11 ± 0.65*

Values are given as mean ± SD for groups of eight animals each. Values are statistically significant at *p<0.05.

Diabetic rats were compared with control rats; *A. squamosa*-treated diabetic rats were compared with diabetic rats; insulin-treated diabetic rats were compared with diabetic rats.

Table II. Effect of treatment *A. squamosa* leaf extract for 30 days on serum lipid profile of control and experimental groups of rats.

Parameter (mg/100 ml)	Control	Diabetic	Diabetic + insulin	Diabetic + *A. squamosa*
TC	129.2 ± 10.3	279.5 ± 13.2*	155.0 ± 10.5*	168.2 ± 7.8*
LDL-C	60.2 ± 4.5	185.4 ± 8.3*	73.1 ± 6.2*	78.7 ± 9.0*
HDL-C	42.4 ± 5.1	58.6 ± 4.2*	66.4 ± 6.8*	64.1 ± 5.6*
TG	88.0 ± 7.6	187.2 ± 10.5*	103.3 ± 9.5*	109.2 ± 10.6*

Values are given as mean ± SD for groups of eight animals each. Values are statistically significant at *p<0.05.

Diabetic rats were compared with control rats; *A. squamosa* treated diabetic rats were compared with diabetic rats; insulin-treated diabetic rats were compared with diabetic rats.

Table III. Effect of treatment *A.squamosa* leaf extract for 30 days on level of TBARS and hydroperoxides in liver and kidney of control and experimental groups of rats.

Group	TBARS (mM TBARS/100 g of wet tissue)		Hydroperoxides (mM hydroperoxides/100 g of wet tissue)	
	Liver	Kidney	Liver	Kidney
Control	0.93 ± 0.07	1.30 ± 0.14	73.4 ± 3.4	56.4 ± 4.6
Diabetic control	1.71 ± 0.45*	2.28 ± 0.31*	99.2 ± 5.2*	80.3 ± 3.4*
Diabetic + insulin	1.05 ± 0.31*	1.59 ± 0.11*	76.2 ± 4.5*	69.1 ± 2.1*
Diabetic + *A. squamosa*	0.97 ± 0.05*	1.41 ± 0.10*	73.1 ± 3.9*	71.20 ± 3.0*

Values are given as mean ± SD for groups of eight animals each. Values are statistically significant at *p<0.05.

Diabetic rats were compared with control rats; *A. squamosa* treated diabetic rats were compared with diabetic rats; insulin treated diabetic rats were compared with diabetic rats.

Table IV. Effect of treatment *A. squamosa* leaf extract for 30 days on superoxide dismutase, catalase, glutathione peroxide and reduced glutathione in livers of control and experimental groups of rats.

Group	Control	Diabetic	Diabetic + insulin	Diabetic + *A. squamosa*
Liver				
SOD (U/mg protein)	22.56 ± 1.76	15.63 ± 1.38*	18.52 ± 2.00*	18.34 ± 1.45*
CAT (U/mg protein×10^3)	0.231 ± 0.025	0.117 ± 0.014*	0.179 ± 0.022*	0.201 ± 0.018*
GPx (U/mg protein)	0.195 ± 0.042	0.138 ± 0.031*	0.163 ± 0.047*	0.179 ± 0.060*
GSH (mg/100 g tissue)	55.6 ± 3.00	30.3 ± 2.34*	54.4 ± 3.20*	51.0 ± 1.64*

Values are given as mean ± SD for groups of eight animals each. Values are statistically significant at *p<0.05.

Diabetic rats were compared with control rats; *A. squamosa* treated diabetic rats were compared with diabetic rats; insulin-treated diabetic rats were compared with diabetic rats.

Table V. Effect of treatment *A.squamosa* leaf extract for 30 days on superoxide dismutase, catalase, glutathione peroxide and reduced glutathione in kidneys of control and experimental groups of rats.

Group	Control	Diabetic	Diabetic + insulin	Diabetic + *A. squamosa*
Kidney				
SOD (U/mg protein)	13.14 ± 1.61	9.24 ± 1.28*	14.15 ± 1.16*	13.16 ± 1.40*
CAT (U/mg protein×10^3)	0.121 ± 0.19	0.080 ± 0.008*	0.116 ± 0.027*	0.134 ± 0.030*
GPx (U/mg protein)	0.064 ± 0.007	0.044 ± 0.006*	0.058 ± 0.009*	0.051 ± 0.005*
GSH (mg/100 g tissue)	40.3 ± 2.25	29.0 ± 1.26*	36.2 ± 1.95*	38.7 ± 2.17*

Values are given as mean ± SD for groups of eight animals each. Values are statistically significant at *p<0.05.

Diabetic rats were compared with control rats; *A. squamosa* treated diabetic rats were compared with diabetic rats; insulin-treated diabetic rats were compared with diabetic rats.

diabetic animals. Table III shows the concentration lipid peroxidation and hydroperoxides in the liver and kidneys of both control and experimental groups of rats. There was a significant elevation in tissue lipid peroxidation and hydroperoxides in diabetic rats. Administration of *A. squamosa* or insulin to diabetic rats decreased the levels of tissue lipid peroxidation and hydroperoxides to normal levels. The concentration of tissues SOD, CAT, GSH and GPx were significantly decreased in diabetic rats when compared to the control group. Administration of *A. squamosa* extract and insulin to diabetic rats tend to bring the activities of these enzymes to near normal level (Tables IV and V).

DISCUSSION

Currently-available drug regimens for management of diabetes mellitus have certain drawbacks and therefore, there is a need for safer and more effective antidiabetic drugs[2-4]. This study was undertaken to assess the antidiabetic effect of *A. squamosa* leaves. In

the present study, the oral treatment of *A. squamosa* leaf extract decreased the blood glucose levels in diabetic rats. It has been reported that using medicinal plant extract to treat STZ-induced diabetic rats results in activation of *β*-cells and insulinogenic effects[33].

A. squamosa may also have brought about hypoglycaemic action through stimulation of surviving *β*-cells of islets of Langerhans to release more insulin. This was clearly evidenced by the increased levels of plasma insulin in diabetic rats treated with *A. squamosa*. Since the percentage fall in plasma glucose levels was different in models with varying intensity of hyperglycaemia, it implies that the antihyperglycaemic effect of that plant is dependent on the dosage of diabetogenic agent, which in turn leads to *β*-cell destruction[34]. A number of other plants have also been observed to exert hypoglycaemic activity through insulin-release stimulatory effects[35,36].

The decreased level of total haemoglobin in diabetic rats is mainly due to the increased formation of HbA_1c. HbA_1c was found to increase in patients with diabetes mellitus and the amount of increase is directly propotional to the fasting blood glucose level[37]. During diabetes mellitus, the excess glucose present in the blood reacts with haemoglobin to form HbA_1c[38]. HbA_1c is used as a marker for estimating the degree of protein glycation in diabetes mellitus. Administration of *A. squamosa* to diabetic rats reduced the glycosylation of haemoglobin by virtue of its normoglycaemic activity and thus decreases the levels of glycosylated haemoglobin in diabetic rats. This normalisation of glycosylated haemoglobin indicates decreased glycation of proteins

The concentrations of lipids, such as cholesterol, TG, LDL-C and HDL-C, were significantly higher in diabetic rats than in the control group. A variety of derangements in metabolic and regulatory mechanisms, due to insulin deficiency, are responsible for the observed accumulation of lipids[39]. The impairment of insulin secretion results in enhanced metabolism of lipids from the adipose tissue to the plasma. Further, it has been reported that diabetic rats treated with insulin shows normalised lipid levels[40]. Thus, the results indicate that *A. squamosa* shows insulin-like action by virtue of its lipid lowering levels.

Oxidative stress has been shown to play a role in the causation of diabetes mellitus. Antioxidants have been shown to have a role in the alleviation of diabetes mellitus[41]. In diabetes mellitus, oxygen free radicals (OFRs) are generated by stimulating H_2O_2 in-vitro, as well as in-vivo, in pancreatic *β*-cells[42]. OFR-scavenging enzymes can respond to conditions of oxidative stress with a compensatory mechanism that increases the enzyme activity in diabetic rats[43]. In our study, concentrations of lipid peroxides and hydroperoxides were increased in liver and kidneys of diabetic rats, indicating an increase in the generation of free radicals. Increased lipid peroxidation in diabetes mellitus can be due to increased oxidative stress in the cell as a result of depletion of antioxidant scavenger systems. The present finding indicates significantly increased lipid peroxidation of rats exposed to STZ and its attenuation by *A. squamosa* treatment. This suggests that the protective role of *A. squamosa* leaf extracts could be due to the antioxidative effect of flavonoids present in the leaf, which in turn act as strong superoxide radicals and singlet oxygen quenchers.

Numerous studies have revealed lowered antioxidant and enhanced peroxidative status in diabetes mellitus[44]. In the current study, the SOD, CAT and GPx activities were significantly reduced in the liver and kidneys of diabetic rats. These observations emphasise the critical importance of maintaining the antioxidant potential of the pancreatic *β*-cell in order to ensure both its survival and insulin secretion capacity during times of increased oxidative stress. The decreased activities of SOD and CAT in both liver and kidneys during diabetes mellitus may be due to the production of reactive oxygen free-radical that can themselves reduce the activity of these enzymes.

Reduced glutathione is a potent-free radical scavenger GSH within the islet of *β*-cell and is an important factor against the progressive destruction of the *β*-cell following partial pancreatectomy[45]. Depletion of GSH results in enhanced lipid peroxidation. This can cause increased GSH consumption and can be correlated to the increase in the level of oxidised glutathione (GSSG). Treatment of *A. squamosa* resulted in the elevation of the GSH levels, which protect the cell membrane against oxidative damage by regulating the redox status of protein in the membrane[46]. SOD, CAT and GPx are enzymes that destroy the peroxides and play a significant role in providing antioxidant defences to an organism. GPx and CAT are involved in the elimination of H_2O_2. SOD acts to dismutate superoxide radical to H_2O_2, which is then acted upon by GPx. The functions of all three enzymes are interconnected and a lowering of their activities results in the accumulation of lipid peroxides and increased oxidative stress in diabetic rats. Treatment of *A. squamosa* increased the activity of these enzymes and thus may help to avoid the free radicals generated during diabetes mellitus.

The study suggested that diabetic animals are exposed to oxidative stress and *A. squamosa* can partially reduce the imbalances between the generation of reactive oxygen species (ROS) and the scavenging enzyme activity. According to these results, *A. squamosa*

could be a supplement, as an antioxidant therapy, and may be beneficial for correcting the hyperglycaemia and preventing diabetic complications due to lipid peroxidation and free radicals. The *A. squamosa* leaf is not only similar to insulin in having a hypoglycaemic effect, it also controls the antioxidant level and could be used to improve the lipid metabolism. Longer duration studies of *A. squamosa* and its isolated compounds on chronic models are necessary to develop a potent antidiabetic drug.

It can be concluded from the data that *A. squamosa* leaf extract supplementation is beneficial in controlling the blood glucose level, improves the lipid metabolism and prevents diabetic complications from lipid peroxidation and antioxidant systems in experimental diabetic rats. This could be useful for prevention or early treatment of diabetic disorders.

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