Hypoglycaemic and antidiabetic effect of aqueous extract of leaves of *Annona squamosa* (L.) in experimental animal

Rajesh Kumar Gupta[1,2], Achyut Narayan Kesari[2], Geeta Watal[2], P. S. Murthy[1], Ramesh Chandra[1], Kapil Maithal[1] and Vibha Tandon[1,*]

[1]Dr B. R. Ambedkar Centre for Biomedical Research, University of Delhi, Delhi 110 007, India
[2]Department of Chemistry, University of Allahabad, Allahabad 211 002, India

Natural remedies from medicinal plants are considered to be effective and safe alternative treatment for diabetes mellitus. Our aim was to demonstrate the hypoglycaemic and antidiabetic activity of the water extract of *Annona squamosa* (custard apple) in diabetic animals with a view to explore its use for the treatment of diabetes mellitus in humans. Hot-water extract of the leaves of *A. squamosa* was prepared by boiling fresh and air-dried leaves (25°C for 5 days) with water (20 ml/g) for 2 h and was tried by oral route in alloxan (80 mg/kg bw)-induced diabetic rabbits and streptozotocin (STZ) (50 mg/kg bw)-induced diabetic rats. The hot-water extract of leaves of *A. squamosa* at a dose 350 mg/kg bw reduced the fasting blood glucose (FBG) level slightly by 6.5% within 1 h and the peak blood glucose at 1 h during glucose tolerance test (GTT) was reduced by 15% in normal healthy rats. The same dose of water extract showed antidiabetic activity in two species of animals, namely rabbits and rats with induced diabetes. In the case of alloxan-induced diabetes in rabbits, a significant ($P < 0.001$) reduction in FBG by 24.4% and improvement in glucose tolerance as indicated by 36.8 and 40.5% fall in blood glucose at 1 h and 2 h during GTT was observed. In STZ diabetic rats also, there was 16.5% decrease in FBG, 24 and 67% reduction in blood glucose at 1 h and 2 h during GTT. After ten days of treatment of a group of STZ diabetic rats with 350 mg/kg bw of the water extract, there was 75.5% fall in FBG level and no sugar in fasting urine was observed. Treatment of alloxan-induced severely diabetic rabbits for 15 days with a dose of 350 mg/kg bw of extract reduced FBG by 48.7% and sugar in urine was reduced by 75%. It brought about fall in the levels of total cholesterol by 41.3% with increase of 29.14% in high density lipoprotein (HDL) cholesterol and 70% fall in low density lipoprotein (LDL) cholesterol, including 25% decrease in triglyceride (TG) levels. After 15 days of treatment, glycosylated haemoglobin level (HbA1c) was reduced by 30%, with a 10.8% increase of total haemoglobin. Extract enhanced the serum insulin level by 27.6% in diabetic animals during GTT and insulin release by 38.1% from isolated pancreatic islets. Water extract increases uptake of glucose in psoas muscles by 57% in the presence of insulin and inhibits glucose absorption by 18.1% through the isolated intestinal segments. Water extract inhibits the activity of glucose-6-phosphatase in isolated rat microsomes. No mortality was found up to 15 times the effective dose. The present study reveals that *A. squamosa* has both hypoglycaemic and antidiabetic activity. It seems to act by enhancing insulin level from pancreatic islets, increased utilization of glucose in muscle and inhibited the glucose output from liver. It reverses the abnormal lipid profile seen in diabetic animals. Its margin of safety is high. Extract obtained from leaves of *A. squamosa* is useful in maintaining healthy blood sugar and cholesterol levels.

IT is well known that the incidence of diabetes mellitus is high all over the world, especially in Asia. Different types of oral hypoglycaemic agents such as biguanides and sulphonylurea are available along with insulin for the treatment of diabetes mellitus[1], but have side effects associated with their uses[2,3]. There is a growing interest in herbal remedies because of their effectiveness, minimal side effects in clinical experience and relatively low costs. Herbal drugs or their extracts are prescribed widely, even when their biological active compounds are unknown. Even the World Health Organization (WHO) approves the use of plant drugs for different diseases, including diabetes mellitus. Therefore, studies with plant extracts are useful to know their efficacy and mechanism of action and safety. Medicinal plants useful in diabetes were reviewed recently[4,5]. The plant *Annona squamosa* (Annonaceae), is commonly called custard apple in English and sharifa in Hindi[6]. This plant is reputed to possess varied medicinal properties[7]. Its use as an insecticidal agent has been investigated by several workers[8]. Various phytochemical, pharmacological, antibacterial and antiovulatory studies have already been carried out with the seed extract[9]. Ayurvedic practitioners use stem and leaf extracts as indigenous uterotonic drug[10]. Post-coital antifertility activity of *A. squamosa*[11] is reported in the seed extract, while aerial parts are inactive. From the leaves of *A. squamosa*, a tetrahydroisoquinoline alkaloid

*For correspondence. (e-mail: vtandon@acbrdu.edu)

with cardiotonic activity[12] and a bioactive acetogenin from its bark[13] have been isolated. Some workers isolated flavonoids from leaves[14]. Aporphine alkaloids[15,16], terpine derivatives[17], glycoside [18] and a novel diazepine, squamolone[19] were isolated from this plant. Ethanol extract of the leaves and stem is reported to have anti-cancer activity[20]. In the Ayurvedic system of medicine, herbal extracts but not purified compounds have been used from centuries because many constituents with more than one mechanism of action are considered to be beneficial. Here we report detailed studies on the hypoglycaemic and antihyperglycaemic activity of the hot-water extract from leaves of *A. squamosa*, with a view to provide scientific evidence on modern lines.

Material and methods

Chemicals

Alloxan, streptozotocin, collagenase, ficoll (type 400) and HEPES were purchased from Sigma-Aldrich Co, USA and bovine serum albumin (BSA) from Calbiochem USA. Glucose, total cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides (TG) were assayed using kits from Ranbaxy Diagnostics, New Delhi. One-touch glucometer (Accu-chek sensor) of Roche Diagnostics, Germany and Uristix were purchased from Bayer Diagnostics India Ltd; insulin from Abbot India was used. For *in vitro* assay of insulin, Mercodia insulin ELISA kit from Uppsala, Sweden was used.

Animals

Wistar strain of rats, weighing about 150–200 g and New Zealand albino rabbits weighing about 1–1.25 kg were obtained from National Institute of Communicable Diseases (NICD) Delhi, and used in the experiments. Animals were kept in our animal house at an ambient temperature of 25°C and 45–55% relative humidity, with 12 h each of dark and light cycles. Animals were fed pellet diet of Golden feed, Delhi and water ad-libitum. For experimental purpose the animals were kept fasting overnight but were allowed free access to water. *Principles of Laboratory Animal Care* (NH publication no. 85–23, revised 1985) was followed.

Preparation of plant extract

Leaves of *A. squamosa* were collected from the garden of Indian Agricultural Research Institute (IARI) Delhi, in March and April, and identified by C. R. Babu, University of Delhi. Leaves were washed well with water. Then 50 g of fresh/air-dried (25°C for 5 days in the absence of sunlight) leaves was extracted in 1 l of boiling water for 2 h and concentrated to half of the volume by boiling in a water bath. The resulting dark-brown extract was cooled and filtered using Whatman No. 1 filter paper. The filtrate was centrifuged at 10,000 rpm in sorvall centrifuge at room temperature (25°C) and the sediment was discarded. The supernatant extract was concentrated up to 100 ml on rotavapour under reduced pressure. The concentrated crude extract was lyophilized into powder (5 g) and used for the study. Extract prepared by using fresh leaves as well as air-dried leaves showed the same activity.

Estimation

Blood glucose was estimated daily using one-touch glucometer to check whether hyperglycemia had stabilized or not, and it was done to prevent cruelty to the animals. The kit was used for initial, weekly and final estimations given in this study. It is well known that there are differences between the glucose values obtained by the two methods. We also found a difference of ± 30 mg/dl in the venous blood using the kit method and capillary blood by one-touch glucometer method. Total cholesterol, HDL cholesterol, triglyceride levels in serum were measured spectrophotometrically by methods prescribed by the manufacturer[21,22] and very low density lipoprotein (VLDL) and low density lipoprotein (LDL) cholesterol were calculated by Friedewald's formula: VLDL cholesterol = Triglyceride/5 and LDL cholesterol = Total cholesterol – (VLDL + HDL cholesterol)[23]. Urinary sugar was detected by reagent-based uristrix from Bayer. Insulin level in serum and *in vitro* medium was estimated by insulin ELISA that is a solid phase, two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule.

Induction of experimental diabetes

A freshly prepared solution of streptozotocin (STZ) (50 mg/kg bw) in 0.1 M citrate buffer, pH 4.5 was injected intraperitoneally to overnight starved rats[24]. Similarly, intravenous injection of alloxan (80 mg/kg bw) was used for inducing diabetes in rabbits. Fasting blood glucose (FBG) level was estimated at the time of induction of diabetes and post prandial glucose (PPG) was checked regularly till stable hyperglycaemia, usually one week with STZ and two weeks with alloxan. Depending on their glucose levels, the animals were divided arbitrarily into three groups, subdiabetic animals, with nearly normal FBG of 80–120 mg/dl but showing abnormal glucose tolerance; diabetic animals with FBG of 120–250 mg/dl, and severely diabetic animals showing FBG above 250 mg/dl.

Method

Initial screening of the extract for hypoglycaemic activity was done in normal healthy rats by conducting glucose

tolerance test (GTT). The antihyperglycaemic or antidiabetic effect was tried in diabetic animals by two methods: (i) by studying the effect of different doses of the water extract on blood glucose levels of sub diabetic and diabetic rabbits during GTT, the most effective dose was also tested on STZ-induced sub diabetic rats during GTT, and (ii) by giving the effective dose of the water extract (350 mg/kg bw) once daily for 10 days to STZ diabetic rats and 15 days for severely diabetic rabbits and observing the change in FBG, PPG level, glycosylated haemoglobin (HbA1c), urine sugar and improvement in glucose tolerance. To assess the ability of the water extract to reverse abnormal lipid constituents seen in severely diabetic rabbits, total lipid profile was estimated. Attempt has been made to understand the mechanism of action of the plant extract: (a) Effect of the water extract on serum insulin level of normal and diabetic rats was studied during GTT. (b) Rat pancreatic islets were incubated with plant extract and insulin release was measured *in vitro*. (c) Isolated psoas muscle of rat was incubated with extract to study the effect of extract on insulin–receptor interaction. (d) The effect of extract on glucose absorption through small intestine was also studied *in vitro*. (e) Effect of water extract on activity of glucose-6-phosphatase (G-6-Pase) in isolated rat microsomes was studied. Toxic effect of the water extract was studied by LD_{50}.

Assessment of hypoglycaemic activity of water extract in normal healthy rats

Initial testing was carried out with different oral doses (50–500 g/kg bw) of the leaf extract in normal healthy male rats. Five groups of four rats each were used in the experiment; group-1 served as untreated control. To animals of groups 2–7, different doses of plant extracts, i.e. 50, 100, 200, 300, 350 and 400 mg/kg bw of the water extract were given correspondingly and water was given to untreated control group animals. In overnight-fasted rats, fasting blood was taken. The different doses of the water extract 200, 300, 350, 400 mg/kg bw were given orally with the help of feeding cannula and its effect on FBG was studied up to 2 h. To the same animals, 4 g/kg bw of aqueous glucose solution was given orally after collecting 2 h blood sample (which serves as 0 h sample for GTT) and glucose tolerance was studied up to another 3 h. Total period of blood collection was up to 5 h.

Assessment of antidiabetic activity of water extract by GTT in diabetic rabbits and rats

In order to study the effect of *A. squamosa* water extract in both rodent and non rodents, investigations were carried out in rats and rabbits. Different doses (200, 300, 350 and 400 mg/kg bw) of extract with 350 mg/kg bw of standard drug tolbutamide were tested initially to find out the effective dose. GTT-based activity testing was carried out on sub diabetic and diabetic rabbits[25] in which the same animals served as their own control. Five groups of four rabbits each were used in the experiment. To the animals of groups a, b, c and e, different doses, 200, 300, 350 and 400 mg/kg bw respectively, of plant extract were given and to group f, standard antidiabetic drug tolbutamide was given, which served as positive control. In overnight-fasted untreated diabetic rabbits, the initial fasting blood sample was drawn from ear vein (for FBG) and then the same volume of water as that of the extract was given orally. After 90 min, blood was again drawn, this gives the '0 h' value for GTT. GTT was performed to get the pattern of the untreated diabetic rabbits. This gives the pre-treatment GTT pattern. The same animals were again fasted overnight after a week. Fasting blood sample was drawn, and then the extract was given. After 90 min blood sample was drawn again. This served as 0 h sample of the treated diabetic rabbits. GTT was performed by giving aqueous glucose solution (2 g/kg bw). This gives the GTT pattern of treated diabetic rabbits. Similarly, out of four doses tested in the rats, the effective dose was 350 mg/kg bw (Table 1, group d).

Treatment of diabetic rats

Three groups of five rats each were used in the experiment. Group 1 served as normal healthy control group and group 2 as diabetic untreated control. Group 3 was treated with a daily single dose 350 mg/kg bw for 10 days. At the beginning and end of the experiment, FBG and urine sugar were estimated. Blood glucose level (PPG) was estimated daily during the treatment period.

Treatment of severely diabetic rabbits

Three groups of six rabbits each were used in the experiment. Group 1 served as normal healthy control group and group 2 as diabetic untreated control. Group 3 was treated with a daily single dose of 350 mg/kg bw for 15 days and the same amount of water was given to the control-group animals. At the beginning and end of the experiment, blood and urine were collected from fasted animals. FBG, PPG, HbA1c, TC, HDL cholesterol and TG were estimated and LDL and VLDL were calculated. Urine sugar was also estimated.

Studies on mechanism of action of water extract

Effect of water extract on serum insulin level of normal and diabetic rats: The effect on serum insulin level was studied in normal and diabetic rats during GTT. Two groups of four overnight-fasted diabetic rats were used in the experiment. Group 1 served as untreated diabetic control and group 2 was treated with a single dose of 350 mg/kg

Table 1. Effect of different doses of water extract of *Annona squamosa* on blood glucose levels during GTT in alloxan diabetic rabbits. Group a; Dose of 200 mg/kg bw; group b, 300 mg/kg bw; group c, 350 mg/kg bw; group d, 350 mg/kg bw STZ diabetic rat; group e, 400 mg/kg bw; group f, effect of a dose of 350 mg/kg bw of tolbutamide

Group	FBG	0 h	1 h	2 h	3 h
Group a					
200 mg/kg bw of water extract p.o.	88 ± 10.2	79 ± 8.6	151 ± 8.2	136 ± 9.4	81 ± 6.8
No extract	88 ± 10.0	86 ± 8.0	182 ± 10 .4	170 ± 8.4	109 ± 9.2
Group b					
300 mg/kg bw of water extract p.o.	133 ± 8.8	117 ± 7.4	206 ± 10.4	159 ± 8.6	132 ± 9.4
No extract	132 ± 10.0	135 ± 8.4	306 ± 9.6	240 ± 10.8	159 ± 10.2
Group c (rabbits)					
350 mg/kg bw of water extract p.o.	159 ± 8.4	115 ± 8.2*	190 ± 10.0*	103 ± 8.8*	93 ± 7.8
No extract	156 ± 9.6	152 ± 9.4	301 ± 10.8	173 ± 10.0	92 ± 8.2
Group d (rats)					
350 mg/kg bw of water extract p.o.	104 ± 8.6	96 ± 10.0	266 ± 7.8*	106 ± 8.0*	77 ± 10.6
No extract	114 ± 9.4	117 ± 9.6	350 ± 10.2	325 ± 8.6	105 ± 8.8
Group e					
400 mg/kg bw of water extract p.o.	155 ± 8.0	134 ± 9.4	191 ± 9.2	163 ± 8.6	121 ± 10.2
No extract	148 ± 10.2	155 ± 10.4	290 ± 8.4	193 ± 10.4	173 ± 9.8
Group f					
350 mg/kg bw of tolbutamide p.o.†	114 ± 10.0	93 ± 10.4	163 ± 8.8*	122 ± 8.4	102 ± 10.2
No drug	109 ± 9.6	106 ± 8.6	240 ± 9.4	138 ± 10.6	112 ± 9.8

$N = 4$ in each group; Values of BGL are given in mean ± S.D.
†Provided orally, *$P < 0.001$ when compared with control (no drug) animals.

bw of the extract. The extract was administered orally after withdrawing the fasting blood sample. Further blood samples were drawn after 1 and 2 h of extract administration. These samples will show effect of the plant extract on plasma glucose and plasma insulin levels during fasting for 2 h. To study the effect of the plant extract on serum insulin levels during GTT, to the same animals 2 g/kg bw of aqueous glucose solution was given orally after collecting 2 h blood sample (which serves as 0 h sample for GTT) and insulin released during glucose tolerance was studied up to another 3 h. Total period of blood collection was up to 5 h. The same method was followed for the normal animals. The serum was separated and frozen immediately and insulin concentration was measured.

Effect of water extract on insulin release by pancreatic islets: Pancreatic islets were isolated[26] using collagenase digestion method. Pancreas was taken out from anaesthetized rats (bw 200 g) and free insulin was removed by injecting Hank's[1] balanced salt solution (pH 7.4). Fat was removed by rapid trimming. The fat-free pancreas was minced finely and incubated for 15 min at 37°C with rapid magnetic stirring in a solution of crude collagenase (approximately 6 mg/ml of incubation medium) in Hank's buffer containing 0.3% glucose and 1% bovine serum albumin (BSA), adjusted to pH 7.4 by gassing with 95% O_2 and 5% CO_2 atmosphere[27]. Further separation from acinar tissue was carried out with ficoll (Type-400) gradient (25, 23, 20 and 11%) centrifugation[28]. After centrifugation for 10 min at 800 *g*, the acinar tissue was left in 25% ficoll. The islets which settle at 20–11% interface, were picked up with Pasteur pipette. Purity of the islets was identified using Gomoris chromium hematoxylin phloxin stain[29]. The islets were divided into 10–20 numbers per batch and preincubated with glucose–Krebs ringer bicarbonate buffer (KRB) containing $NaHCO_3$ (0.2%) HEPES (0.38%), insulin-free BSA (0.1%) and 11.1 mM glucose for 5 min at 37°C in CO_2 incubator[30]. Then various concentrations of the plant extract (50, 100, 150 µg) or buffer for controls were added and incubation continued for 2 h in CO_2 incubator under 95% O_2 and 5% CO_2 atmosphere. Aliquots of 50 µl were removed from the incubation mixture at 0, 30, 60 and 120 min, and were frozen immediately until insulin assay was performed. The experiments were carried out in normal healthy and streptozotocin-induced diabetic rats.

Effect of water extract on uptake of glucose in rat psoas muscle tissue: Psoas muscle was isolated from two anaesthetized adult rats (kindly identified by M. Fahim, University of Delhi) and placed immediately in KRB containing 11.1 mM glucose. Muscle tissue was cut into pieces of equal mass, about 0.250 g, and preincubated for 5 min in CO_2 incubator as mentioned above. Four sets (in triplets) including muscle tissue alone, muscle tissue with insulin (25, 50 mU/l), muscle tissue with both insulin and water extract (50, 100 and 150 µg) and muscle tissue with water extract (50, 100 and 150 µg) alone were incubated for 2.5 h in CO_2 incubator under 95% O_2 and 5% CO_2 atmosphere. Aliquots of 25 µl were removed from incubation mixture at 0, 30, 60, 90, 120 and 150 min, and changes in glucose concentration were measured.

Effect of water extract on glucose absorption through a rat gut preparation[31]: An overnight-fasted rat was sacri-

ficed under anaesthesia and the abdomen removed. Small segments (each about 5 cm) close to the duodenum were rinsed with KRB by pushing the solution gently from the syringe. The segments were placed in well-oxygenated KRB-containing 11.1 mM glucose along with various concentrations of the plant extract (50, 100 and 150 µg) and buffer as control, incubated at 37°C for 2 h in CO_2 incubator under 95% O_2 and 5% CO_2 atmosphere. Aliquots of 25 µl were removed from the incubation mixture at 0, 30, 60, 90 and 120 min, and change in glucose concentration in the medium was studied.

Effect of water extract on activity of G-6-Pase: Microsomes were isolated from STZ-induced diabetic rats. Overnight-fasted rats were euthanized by decapitation and livers were removed, placed in an ice-cold buffer at pH 7.4 containing 250 mmol/l sucrose, 25 mmol/l HEPES–KOH, 2.5 mmol/l EDTA and 0.1 mmol/l phenylmethylsulphonyl fluoride and homogenized using a homogenizer. The homogenate was centrifuged at 12,000 *g* for 10 min, and the resulting supernatant was centrifuged for 1 h at 100,000 *g*. The pellet was resuspended at a protein concentration of 40 mg/ml in the homogenization buffer and G-6-Pase activity was measured by monitoring the release of phosphate from G-6-P. Microsomes (1.0 µl of the preparation described above) were incubated at room temperature in 200 µl of a buffer at pH 7.2, containing 50 mmol/l HEPES, 100 mmol/l KCl, 2.5 mmol/l EGTA, 2.5 mmol/l $MgCl_2$, and 1.0 mmol/l G-6-P. The released phosphate was measured by adding 300 µl of 1 N HCl containing 10 mg/ml ammonium molybdate and 0.38 mg/ml malachite green. After 15 min incubation at room temperature, the absorbance was measured[32] at 620 nmol/l. Then 50, 100 and 150 µg of water extract was added before the addition of the enzyme and the same amount of water was used as control.

LD_{50} experiment

Four groups of rats of both sexes (6 animals per group, 3 females and 3 males) and weighing about 150–180 g were administered orally a single dose of either 2.5, 5, 10 or 15 times of effective dose of water extract of leaves of *A. squamosa*. The rats were observed for gross behavioural, neurologic, autonomic, and toxic effect continuously. Food consumption, faeces and urine were also examined at 2 h and then at 6 h intervals for 24 h, and once daily up to a week.

Results

Effect of water extract on normal healthy rats

In order to study the optimum effective dose of water extract of *A. squamosa* on the FBG level and glucose tolerance, different doses (50–500 mg/kg bw) of water extract were given to overnight-fasted healthy rats. Doses of 50 and 100 mg/kg bw did not have any effect on FBG and GTT. It was found that doses of 200 and 300 mg/kg bw of water extract did not have any appreciable effect on FBG, but there was slight improvement in glucose tolerance of about 9.4 and 11.2% at peak value up to 2 h respectively. A dose of 350 mg/kg bw of water extract brought about a slight fall (6.5%) in FBG after 1 h of extract administration and improved glucose tolerance by about 15% at 1 h peak value, compared to control rats which received equal volume of water in place of the extract. The higher dose of 400 mg/kg bw had more or less the same effect as that with 350 mg/kg bw. It therefore appears that 350 mg/kg bw of water extract of *A. squamosa* is the effective dose on FBG and GTT of healthy rats. One of the reasons why even with the optimal dose of 350 mg/kg bw, the fall was only 15% could be that normal regulatory mechanism operates to prevent hypoglycaemia in normal animals. The fall in FBG was maximum after 1 h and was maintained up to 2 h during fasting. So in further experiments, effect of the extract on FBG was observed after 90 min of extract administration, considering that it takes a minimum of 1 h to act and then GTT was performed by giving glucose solution.

Effect of water extract on diabetic rabbits and rats during GTT

In order to choose optimum dose of water extract which would show improvement in GTT in the diabetic animals, different doses of water extract (200, 300, 350 and 400 mg/kg bw, not 50, 100 and 500 mg/kg bw for reasons mentioned above) were tried on glucose tolerance in diabetic rabbits along with the standard drug tolbutamide. As mentioned earlier, GTT pattern of the untreated diabetic animals was assessed. The same rabbits were treated with water extract and GTT pattern was determined again after treatment. A dose of 200 and 300 mg/kg bw of water extract reduced FBG by 8.1 and 13.3% within 90 min of extract administration (0 h of GTT), while after 1 h of glucose administration (1 h of GTT), the BGL was reduced by 17 and 32% respectively. A reduction of 18 % was noted in 2 h of GTT with 200 mg/kg bw, while the reduction was 20% with 300 mg/kg bw of the extract. A dose of 350 mg/kg bw reduced FBG significantly ($P < 0.001$) by 24% during 0 h, and 37% fall was observed in 1 h and 40% fall in 2 h during GTT. The higher dose of 400 mg/kg bw had more or less the same effect as that with 350 mg/kg bw. It therefore appears that 350 mg/kg bw of water extract of *A. squamosa* is the effective dose on FBG and GTT of alloxan-induced diabetic rabbits (Table 1, group a–c). The higher dose of 400 mg/kg bw had more or less the same effect as that with 350 mg/kg bw (Table 1, group e). In similar studies carried out in STZ-induced diabetes in rats, optimum effective dose was found to be 350 mg/kg bw. Therefore, results of GTT were shown with effective dose only (Table 1, group d). A significant ($P < 0.001$) reduction of 24 and 67%

during 1 h and 2 h of GTT was observed with the dose of 350 mg/kg bw (Table 1, group d). A dose of 350 mg/kg bw of tolbutamide reduced FBG by 12.3% during 0 h, 32% in 1 h and 11.5% in 2 h during GTT (Table 1, group f), which is almost equal to that with 300 mg/kg bw of the extract.

Effect of extract on blood glucose levels and urine sugar of diabetic rats

After ten days of treatment of diabetic rats with the optimum dose of 350 mg/kg bw, the FBG value returned from 246 ± 6.0 to the normal value of 65 ± 10 mg/dl (75% fall). To check the effect of the extract on PPG level of diabetic rats, PPG was estimated regularly during the treatment period. At the end of the treatment, there was 51% fall in PPG, from 446 ± 5.6 to finally 261 ± 8.2 mg/dl. No urine sugar was observed after the treatment, while initially it was 2.0 g/l (Table 2). In the untreated animals urine sugar was 2.0 g/l at the end.

Effect of extract on blood glucose levels, lipid profile, glycosylated haemoglobin and urine sugar of severely diabetic rabbits

Since the water extract of *A. squamosa* showed good improvement in FBG and GTT of diabetic animals, it was intended to assess the effect of long-term treatment of the extract on BGL, glycosylated haemoglobin, urine sugar and associated abnormal lipid profile in alloxan-induced severely diabetic rabbits also. Rabbits were treated with 350 mg/kg bw of water extract once a day in the morning for 15 days. At the end of the treatment, the animals when compared with their own initial values showed significant ($P < 0.001$) reduction from 373 ± 6.2 to 191 ± 5.4 mg/dl (48.7% fall) in FBG. Water extract significantly reduces the glycosylated haemoglobin from 10.6 ± 1.0 to 7.4 ± 0.8 (30.0% fall), with 10.8% increase in the total haemoglobin level. The urine sugar was 4.0 g/l before treatment, but only 1.0 g/l after treatment (Table 3). In untreated animals, there was no fall in FBG and no reduction in urine sugar. It would be useful to study the effect of the extract on the reuptake of glucose by glomeruli of the kidneys of diabetic animals treated with the plant extract. Since GTT cannot be performed in severely diabetic animals (because they die if glucose is given), only PPG after giving food was estimated after treatment with water extract; there was 49.4% fall in PPG from 568 ± 5.8 to 287 ± 5.6 mg/dl in the treated rabbits, while in the untreated animals PPG was still high 456 ± 5.6 mg/dl. The various parameters of blood lipid profile of severely diabetic rabbits were tested before and after treatment. The enhanced levels of TC, LDL, VLDL cholesterol and TG were brought down significantly ($P < 0.001$) after the treatment period. Nearly 41.3% fall in TC, 76.6% fall in LDL cholesterol and 25.6% fall in TG were noted in treated diabetic rabbits. There was also an increase of 29.2% HDL cholesterol in the treated diabetic rabbits (Table 3). In untreated rabbits, there was a fall in HDL and slight increase in all the above-mentioned lipid parameters. At the end of the experiment, food intake and body weight were normal in the treated animals.

Studies on the mechanism of action of water extract of leaves of A. squamosa

Effect of water extract on serum insulin levels of normal and diabetic rats: Single dose of 350 mg/kg bw enhanced serum insulin level in overnight-fasted diabetic rats from 22.2 ± 2.8 to 26.4 ± 2.6 pmol/l in 2 h (18.9%). In case of normal animals, fasting serum insulin level was increased by 6.5% from 79.04 ± 4.2 to 84.24 ± 4.4 pmol/l in 2 h. There was no change in fasting insulin levels of untreated normal and diabetic animals. During GTT with a dose of 350 mg/kg bw of water extract, serum insulin level of diabetic animals was increased from 26.4 ± 2.6 to 54.28 ± 2.4 pmol/l in 1 h (3 h in Figure 1), after administration of glucose. This was 27.6% more enhanced compared with the diabetic untreated control animals. In case of normal animals, insulin level was increased from 84.24 ± 4.4 to 101.93 ± 4.4 pmol/l in 1 h during GTT. It was only 9.6% increase compared with normal untreated animals (Figure 1). Glucose-induced insulin released was studied under *in vitro* experiments.

Table 2. Effect of treatment for ten days with water extract of *A. squamosa* on FBG, PPG and urine sugar (US) of STZ diabetic rats. Group 1, Normal healthy control; group 2, Untreated diabetic control; group 3, Treated diabetic-animals

	Initial			Final			
Group	FBG	PPG	US	FBG	PPG	US	FBG % change
Group 1	68 ± 7.4	104 ± 7.6	–ve	79 ± 5.4	102 ± 7.4	–ve	nil
Group 2	242 ± 5.8	438 ± 4.8	2+	288 ± 5.6	523 ± 5.8	2+	nil
Group 3	246 ± 6.0*	446 ± 5.6	2+*	65 ± 10*	261 ± 8.2	nil*	75%↓in FBG

$N = 5$ in each group; FBG, in mg/dl, mean ± SD, PPG, in mg/dl, mean ± SD; US, in g/l. *$P < 0.001$ when compared with initial (before treatment) values.

Table 3. Effect of treatment for 15 days with water extract of *A. squamosa* on FBG, PPG, HbA1c, urine sugar and lipid profile of severely diabetic rabbits. Group 1, Normal healthy control; group 2, Untreated diabetic control; group 3, Treated diabetic animals

	Group 1		Group 2		Group 3		
Parameter[#]	Initial	Final	Initial	Final	Initial	Final	Percentage change
FBG (mg/dl)	68 ± 5.0	72 ± 4.2	285 ± 6.2	315 ± 5.8	373 ± 6.2	191 ± 5.4*	48.7
PPG (mg/dl)	100 ± 8.0	99 ± 8.2	523 ± 5.8	456 ± 5.6	568 ± 5.8	287 ± 5.6*	49.4
US (g/l)	–ve	–ve	+4	+4	+4	+1*	75
TC (mg/dl)	45 ± 5.6	48 ± 4.2	182 ± 5.0	178 ± 4.2	213 ± 6.5	125 ± 5.6*	41.3
HDLC (mg/dl)	18 ± 4.6	20 ± 4.2	38 ± 5.4	20.2 ± 5.0	26 ± 6.0	46 ± 5.2*	29.2
LDLC (mg/dl)	10 ± 5.0	11.6 ± 5	117.4 ± 4	128 ± 4.4	157.4 ± 5.0	56 ± 4.5*	76.6
VLDLC (mg/dl)	17 ± 4.2	16.4 ± 4	26.6 ± 4	29.8 ± 5	29.6 ± 5.6	22 ± 5.8*	25.6
TG (mg/dl)	85 ± 5.6	82 ± 5.0	133 ± 5.4	149 ± 5	148 ± 6.0	110 ± 8.0*	25.6
HbA1c (%)	8.7 ± 0.8	8.3 ± 0.8	11.3 ± 1.4	11.5 ± 1.2	10.6 ± 1.0	7.4 ± 0.8*	30.0
Total Hb (g/dl)	12.3 ± 1.2	12.6 ± 1.2	12.8 ± 1.6	12.3 ± 1.6	12.0 ± 1.4	13.3 ± 1.4*	10.8

$N = 6$ in each group; [#]mean ± SD. *$P < 0.001$ when compared with initial values.
Initial values are those before treatment and final values are those after treatment.

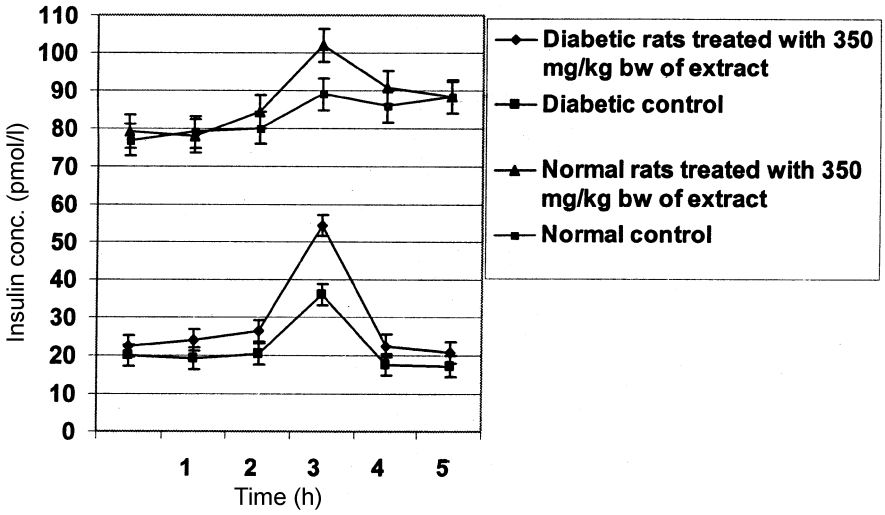

Figure 1. Effect of single dose of 350 mg/kg bw of water extract on serum insulin levels of normal and diabetic rats during fasting and GTT.

Effect on the release of insulin from pancreatic islets: Effect of water extract of *A. squamosa* on glucose-induced insulin release *in vitro* in pancreatic islets was investigated. Pancreatic islets from rat pancreas were incubated with 11.1 mM glucose at different concentrations (50, 100 and 150 µg) of water extract along with standard drug tolbutamide as positive control. The concentration of 100 µg showed maximum release of insulin from the pancreatic islets. Islets were isolated from the pancreas of both normal as well as diabetic rats. In the islets isolated from normal rat pancreas, there was increase of insulin release from 52.0 ± 4.2 to 62.4 ± 4.2 pmol/l in 1 h of incubation with water extract (100 µg), while incubation of islets with tolbutamide (100 µg) enhances insulin release from 59.4 ± 4.6 to 74.6 ± 4.6 pmol/l in 1 h. Incubation of islets with water extract enhanced insulin release by 20.0%, while with tolbutamide by 32.8% in normal rats. There was no enhanced insulin release in control incubation which has the same amount of water in place of extract/tolbutamide (Figure 2 *a*). Islets isolated from STZ-induced diabetic rat pancreas and incubated with 100 µg of water extract enhanced insulin release from 24.9 ± 2.4 to 34.4 ± 2.8 pmol/l in 30 min and 34.4 ± 2.8 to 38.8 ± 2.4 pmol/l within 1 h compared with control incubation. Similarly, incubation with 100 µg of tolbutamide enhanced insulin release from 27.2 ± 2.8 to 38.2 ± 2.8 pmol/l in 30 min and 38.2 ± 2.8 to 44.3 ± 2.8 pmol/l in 1 h of incubation (Figure 2 *b*). It was found that incubation of islets with water extract enhanced insulin release by 38.1% in 30 min and 55.8% in 1 h, while with tolbutamide by 40.4% in 30 min and 62.8% in 1 h in diabetic rats. Results are shown only with 100 µg of water extract (Figure 2 *a* and *b*).

Effect on uptake of glucose by psoas muscle: Effect of water extract of *A. squamosa* on the uptake of glucose by psoas muscle of rat in the presence and absence of insulin

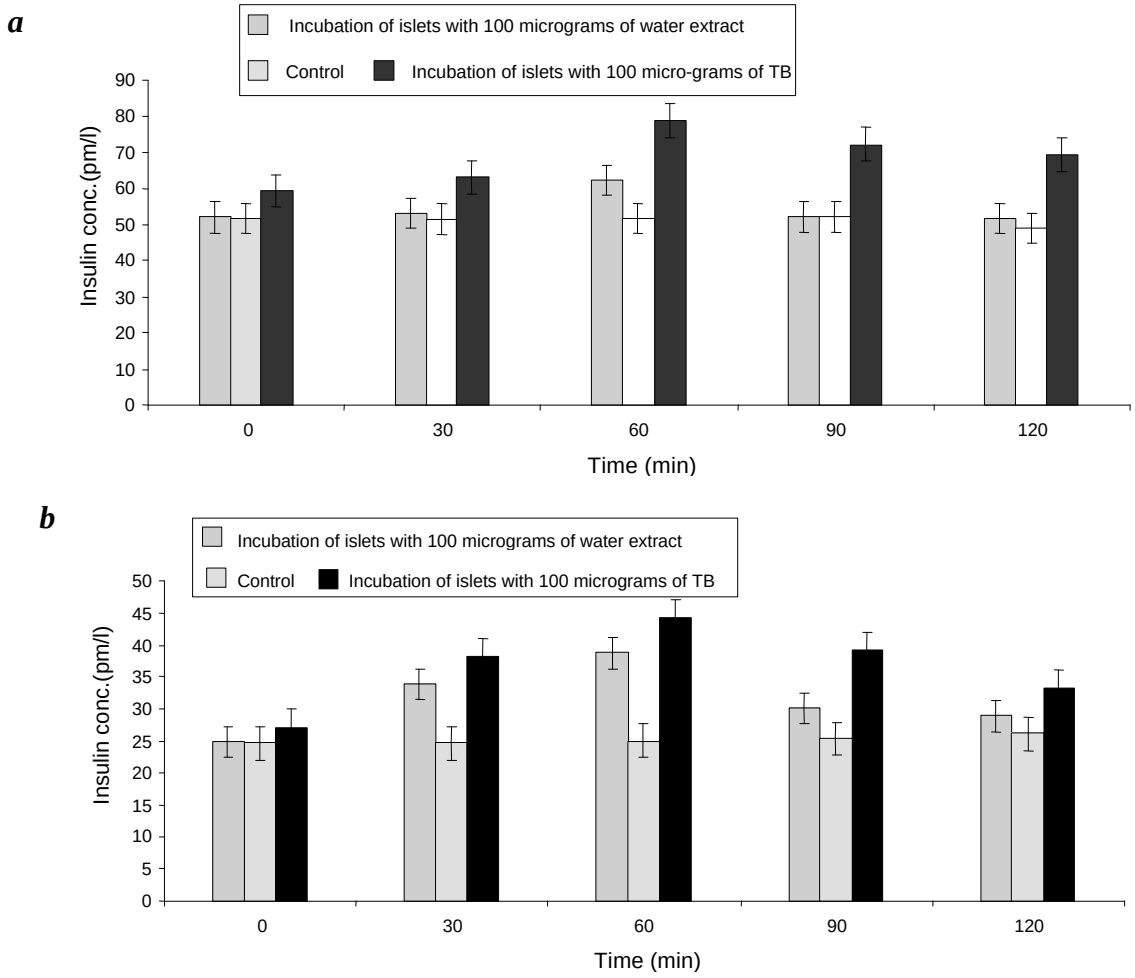

Figure 2. Effect of water extract on release of insulin from pancreatic islets isolated from normal and diabetic rats. ***a***, Normal rat pancreas; ***b***, Diabetic rat pancreas.

was studied by measuring the decrease in glucose concentration in the incubation medium with time. With 50, 100 and 150 µg concentration of the extract, maximum effect was observed with 100 µg of water extract. Thus results are shown only with 100 µg of the water extract. It is clear from the results (Table 4) that 100 µg of the water extract by itself increased glucose uptake by about 20% up to 1 h, after which the effect gradually decreased to zero by 2.5 h. However, in the presence of insulin, increase in glucose uptake was 57% in 30 min, but it decreased to 21% in 1 h and 13% in 2.5 h.

Effect on glucose absorption through the intestine: Isolated intestinal segments were incubated with the extract and glucose absorption was measured by observing the fall in glucose concentration in the medium with time. It was 23.2, 25.3, 40.3 and 49.7% in 30, 60, 90 and 120 min respectively, when no extract/drug was added to the medium (serves as control). In the presence of 100 µg of water extract of *A. squamosa*, the rate of glucose absorption by the intestine was 12.8, 18.9, 22.2 and 31.7%, while with 100 µg of acarbose it was 9.3, 13.5, 17.9 and 20.7% in 30, 60, 90 and 120 min respectively. It is clear from the above results that water extract inhibits the glucose absorption through intestine indicated by the reduced glucose absorption from the medium. This was also true in the case of the standard drug acarbose compared with control. Addition of 100 µg of water extract inhibits glucose absorption gradually from 30 to 90 min; it was 18.1% in 90 min and continues up to 120 min, while with 100 µg of acarbose the inhibition was 22.4% in 90 min and 29.0% in 120 min compared with control (Table 5).

Effect of water extract on activity of G-6-Pase: The activity of G-6-Pase in isolated rat microsomes was inhibited in a concentration-dependent manner by the water extract. It was found that 50, 100 and 150 µg of water extract inhibited the activity of G-6-Pase activity by 23.1, 32.2 and 34.3% respectively, compared with control. Results are shown in Table 6.

Table 4. Effect of water extract on glucose uptake by psoas muscle isolated from rat. Fall in glucose concentration of the medium indicates glucose uptake by muscle tissue

Set	Glucose uptake (mg/dl)				
	30 min	60 min	90 min	120 min	150 min
Muscle tissue (MT)	19 ± 4.3	30 ± 4.6	34 ± 5.2	37 ± 4.5	48 ± 4.8
MT + extract (50 µg)	23 ± 5.4* (21%)[#]	37 ± 5.2 (23%)	40 ± 4.8 (18%)	43 ± 5.6(10%)	48 ± 5.5
MT + insulin (25 µg)	42 ± 4.4	60 ± 4.6	68 ± 4.8	74 ± 4.2	78 ± 5.2
MT + insulin (25 µg) + extract (50 µg)	66 ± 5.4* (57%)	73 ± 4.8 (20%)	78 ± 5.6 (15%)	86 ± 5.8 (16%)	88 ± 4.8 (18%)

[#]Values in brackets indicate per cent increase when compared with muscle tissue alone in the case of muscle tissue with extract and muscle tissue plus insulin in the case of muscle tissue with insulin and extract.
*$P < 0.001$ when compared to control (without extract).

Table 5. Effect of water extract of *A. squamosa* extract on intestinal glucose absorption in isolated intestinal segments of rat (decrease in the glucose concentration of medium indicate the absorption of glucose through intestine)

Time (min)	Glucose conc (mM) in the medium		
	Control	Incubation with water extract (100 µg)	Incubation with acarbose (100 µg)
0	10.5 ± 2.4	10.80 ± 2.8	10.67 ± 2.0
15	70.89 ± 2.2	10.08 ± 2.4	10.58 ± 2.2
30	8.06 ± 1.6 (23.2%)[#]	8.79 ± 2.6 (12.8%)[#]	9.68 ± 1.6 (09.3%)[#]
45	8.12 ± 1.8	8.73 ± 1.4	9.41 ± 1.2
60	7.84 ± 2.0 (25.3%)[#]	8.17 ± 1.2 (18.9%)[#]	9.22 ± 1.4 (13.5%)[#]
75	6.88 ± 1.4	7.67 ± 1.8	8.98 ± 1.2
90	6.27 ± 1.2 (40.3%)[#]	7.84 ± 1.6 (22.2%)[#]	8.76 ± 1.6 (17.9%)[#]
105	5.86 ± 1.2	6.94 ± 1.2	8.52 ± 1.4
120	5.28 ± 1.6 (49.7%)[#]	6.88 ± 2.0 (31.7%)[#]	8.46 ± 1.2 (20.7%)[#]

[#]Percentage fall in glucose conc. of the medium with initial values.

Table 6. Effect of water extract of *A. squamosa* on G-6-Pase activity measured in intact isolated diabetic rat microsomes. G-6-Pase activity was measured in the presence of varying concentrations of water extract. Data are mean ± SD of triplicate measurements

Conc. of extract (µg)	Activity of G-6-Pase (units[†]/mg protein)	Percentage inhibition
50	0.186 ± 0.011	23.1
100	0.164 ± 0.008*	32.2
150	0.159 ± 0.013	34.3
Control	0.242 ± 0.023*	0.0

[†]µmol of Pi liberated/min.
*$P < 0.001$ when compared to control without extract.

LD_{50} experiment

Experiment was carried out on normal healthy rats. The behaviour of the treated rats appeared normal. No toxic effect was seen even with 10 and 15 times the effective dose of water extract and there were no deaths in any of the groups. Only the consumption of food was increased by 20% with 10 and 15 times the effective dose initially for 2 days, but came back to normal afterwards. Body weight was normal.

Discussion

Although *A. squamosa* is reported to possess varied medicinal properties such as insecticidal, antiovulatory and antitumour activities[33], there is no previous report about the hypoglycaemic and antidiabetic activity of this plant. The present investigation reports the hypoglycaemic and antidiabetic effect of water extract of leaves of this plant. The observation and preliminary idea of the mechanism of its action reported here offer scientific explanation for the potential use of this plant for the treatment of diabetes mellitus.

Experiments with different doses of water extract (50–500 mg/kg bw) on FBG and GTT in normal healthy rats indicated that the optimum dose is 350 mg/kg bw. Even this optimum dose produced only 6.5% fall in FBG and improved glucose tolerance by 15%, while a still higher dose of 400 mg/kg bw produced insignificant decrease in FBG (7.2%), but no more improvement in glucose tolerance. Hence the dose of 350 mg/kg bw was used in the experiments. This study also revealed that the maximum hypoglycaemic effect was produced only 1 h after giving to the fasted animals. This indicates that it takes about 1 h for the active ingredient(s) or its (their) metabolites in the water extract to enter into the circulation and reach target tissues to bring about hypoglycaemic effect, which is maintained for at least 2 h. In the GTT experiments in alloxan diabetic rabbits, reduction in blood glucose level was maintained at least for 2 h. Treatment of rats with STZ-induced diabetes for 10 days (Table 2) and rabbits with alloxan induced diabetes (Table 3) for 15 days brought down the elevated blood glucose levels, ranging from 250 to 350 mg/dl to nearly normal range. Alloxan not only de-

stroys the pancreatic *b*-cells but causes kidney damage, which is however reversible, while STZ selectively destroys pancreatic insulin secreting *b*-cells[34–36], causing diabetes close to type-II in humans. The elevated blood glucose levels in the diabetic animals used by us were in the range of 150–350 mg/dl, which resembles both type-II diabetes (150 to about 250 mg/dl) with partially functional pancreas as well type-I (above 300 mg/dl) with considerable amount of pancreas damaged. This shows that the water extract of *A. squamosa* might be useful both in type-II and type-I diabetes, irrespective of whether the pancreas is partly functional or almost totally destroyed. This is an advantage, keeping in mind that the present-day sulphonylurea drugs act only when there is a functional pancreas[37,38]. Our preliminary studies on the mechanism of action of the water extract reveal many interesting observations (Figures 1 and 2). It increased serum insulin level by 18.9% in overnight-fasted diabetic rats and 27.6% during GTT, but in the normal case it was only 9.6% during GTT. This observation indicates that water extract of *A. squamosa* enhances insulin release from destroyed pancreatic beta cells, either by regenerating the partially destroyed pancreatic beta cells or by the release of insulin stored in the granules. So as is to be expected, during GTT there was increase in serum insulin level (Figure 1), which in turn improves glucose tolerance. It is well known that in uncontrolled type-II diabetes mellitus, there will be increase in TC, LDL, and VLDL cholesterol and TG with decrease in HDL cholesterol, all of which contribute to the coronary artery disease[39,40] seen in some diabetic patients. From this point of view, it is interesting that the water extract brought down the elevated levels of TC, LDL and VLDL cholesterol and TG in diabetic animals to nearly normal level after treatment (Table 3) for 15 days. There was increase in HDL cholesterol also, which is a desirable feature.

In order to know whether the increase in serum insulin level after administration of water extract was due to the release of insulin stored in the granules or due to direct effect on the pancreatic islets of *b*-cells, *in vitro* effect of water extract was studied on the islets isolated from rat pancreas. Results showed that the water extract brought about an increase in the release of insulin (Figure 2 *a* and *b*) from the islets, which was more from the diabetic rat pancreas than from the normal pancreas. Even though the release of insulin from the pancreatic islets is small, it is an indication of one of the mechanisms by which water extract from leaves of *A. squamosa* act.

In type-II diabetes, more often the cause is the lack of insulin sensitivity or resistance to insulin action at the receptor or post-receptor level[41,42], rather than lack of insulin. New drugs are required for treatment of type-II diabetes, which increase insulin sensitivity or decrease insulin resistance. Our studies on isolated psoas muscle indicated that the water extract of *A. squamosa* enhanced the uptake of glucose in muscle tissue (Table 4) in a short time of 30 min in the absence of insulin, i.e. directly and the effect was more (57%) in the presence of insulin. Direct effect (20% increase in 30 min) in the absence of insulin indicates that the extract has either insulin-like effect on psoas muscle (skeletal muscle) or direct stimulatory effect on the enzymes involved in the metabolism of glucose. Increase of glucose uptake in the presence of insulin suggests the possibility of increased binding of insulin to receptor in the muscle or increase in the number of insulin receptors. The enhanced uptake of glucose would lead to increased utilization of glucose from the blood.

Water extract inhibits the activity of G-6-Pase (34.3%), which plays a central role in the homeostatic regulation of blood glucose levels (Table 6). When blood glucose levels fall, the liver is capable of rapidly releasing glucose into the circulation, where it serves as a fuel for other tissues that lack the ability to make glucose. The two metabolic pathways by which the liver can produce glucose are gluconeogenesis and glycogenolysis. A single enzyme, G-6-Pase, catalyses the final step of both of these pathways. Increased hepatic glucose output is a major cause of the fasting hyperglycaemia that characterizes diabetes.

The water extract could also inhibit uptake of glucose by the isolated rat intestine slightly, i.e. by 18.1% (Table 5). Even though the inhibition is less one has to keep in mind that the water extract may also act at a different site, namely the intestine. The above are preliminary indications and further detailed studies are necessary to find out whether the action of the water extract is due to one or more of the above-mentioned possible mechanism or not.

Thus the water extract of *A. squamosa* seems to be useful in controlling elevated blood glucose levels in diabetes induced by both the agents, alloxan and STZ in two species of animals, namely rabbits (non-rodents) and rats (rodents). The exact mechanism of action needs further studies, but the present investigation gives some preliminary idea that the water extract from leaves of *A. squamosa* acts at more than one site, namely pancreas (release of hormone insulin), muscle and intestine (uptake of glucose through specific receptor). The LD_{50} of the extract is high (no death even with 15 times the effective dose), indicating the high margin of safety. These results indicate that it is worth undertaking further studies on possible usefulness of the water extract of the leaves of *A. squamosa* in diabetes mellitus.

Statistical calculations

Data were expressed as mean ± SD for all experiments, and statistical significance was calculated according to Student's two-tailed *t* test. Values corresponding to $P < 0.001$ were considered statistically significant.

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