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Original Article

Antihyperglycemic and histological effects on the pancreas of the aqueous leaves extract of *Annona squamosa* L. in normal and diabetic rats

Pornrut Rabintossaporn[1]*, Suphaket Saenthaweesuk [1], Amornnat Thuppia[1], Kornkanok Ingkaninan[2] and Seewaboon Sireeratawong[1]

[1] *Department of Preclinical Science, Faculty of Medicine, Thammasat University, Rangsit Campus, Khlong Luang, Pathum Thani, 12120 Thailand.*

[2] *Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Science, Naresuan University, Muang, Phitsanulok, 65000 Thailand.*

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Abstract

The purposes of our present study were to investigate the antihyperglycemic effect of aqueous leaves extract of *Annona squamosa* L. at different doses and histology of the pancreas in normal and diabetic rats after receiving the extract. The study of antihyperglycemic activities was undertaken by oral administration of vehicle, glibenclamide (3 mg/kg) and water leaves extract of *Annona squamosa* L (125, 250 and 500 mg/kg) for 12 days. Fasting blood glucose levels were recorded on the fifth and twelfth days of administration and the third day after stopping administration. The results showed that diabetic rats receiving the doses of 250 and 500 mg/kg leaves extract had statistically significant ($p<0.05$) reduced fasting blood glucose level on the fifth and twelfth days of administration. In oral glucose tolerance test, the doses of 250 and 500 mg/kg leaves extract were able to reduce blood glucose levels from 60 minutes onward. The histological study showed improvement of diabetic pancreases with a dose-dependent effect in which diabetic pancreases possessed a clearer shape of islet of Langerhans and the cellular shape within the islet was rounder than in the pancreas of diabetic rats receiving vehicle only. The present data suggest that water leaves extract of *Annona squamosa* L. has an antihyperglycemic effect and is able to improve the histological appearance of diabetic rat pancreas.

Keywords: antihyprglycemic effect, *Annona squamosa* L., histology, pancreas

1. Introduction

Annona squamosa L.**,** commonly known as sugar apple or custard apple, is an edible fruit which has been reported to possess various medicinal properties. Young leaves of *A. squamosa* contain steroids, alkaloids, saponins, terpenes, tannins, phenolic substances, carbohydrates, volatile oil, mucilage (Kokate, 1994; Harborne, 1998) and flavonoid (Seetharaman, 1986; Kotkar *et al.*, 2002). Flavonoid and tetrahydroisoquinoline alkaloid isolated from leaves of *A. squamosa* have cardiotonic activity (Wagner *et al.*, 1980). In addition, flavonoid also possesses antioxidative (Saija *et al.*, 1995), antimicrobial and insecticidal activities (Cheema *et al.*, 1985; Kotkar *et al.*, 2002). Methanolic extract from leaves of *A. squamosa* has anti-cancer activity (Bhakuni *et al.*, 1969) whereas aqueous leaves extract has been reported to ameliorate hyperthyroidism (Sunanda *et al.*, 2003). Gupta *et al.* (2005) reported that ethanolic extract from the leave of Northern Indian *A. squamosa* at doses of 200, 300, 350 and 400 mg/kg could reduce blood glucose levels of normal and streptozotocin-induced diabetic rats as well as normal and alloxan-induced diabetic rabbits. Moreover, the dose of 350 mg/kg was the most effective dose for normal rats and alloxan-induced diabetic rabbits.

*Corresponding author.
Email address: Pornrut_r@yahoo.com

Shirwaikar *et al.* (2004) also revealed that cold aqueous extract from the leaves of *A. squamosa* in Northern Indian at doses of 250 and 500 mg/kg has antidiabetic activity in male diabetic rats.

Streptozotocin (2-deoxy-2-(3-(methyl-3-nitrosoureido) -D-glucopyranose), STZ) is one of the most wildly used chemical agents which produce DNA damage followed by consequent event that brings about specific destruction of β-cells leading to diabetes (Szkudelski, 2001). In addition, several pathological changes have been demonstrated in islets of Langerhans in association with diabetes (Kissane and Lacy, 1990). Therefore, histological examination of the pancreas was included in this study to investigate the histological improvement in islets of Langerhans.

Heretofore, there has been no study conducted on the antihyprglycemic activity of *A. squamosa* and histological examination of the pancreas after receiving the extract from Thailand region. Thus, the present study aimed to evaluate the antihyperglycemic effect and histological characters of pancreas in normal and STZ-induced diabetic rats from the aqueous leaves extract of *A. squamosa* in Thailand.

2. Materials and Methods

2.1 Plant material

Young leaves of *A. squamosa* were collected from Phitsanuloke province, Thailand. The voucher specimen (Tothium 022) was kept by Mahidol University Herbarium (PBM), Faculty of Pharmaceutical Sciences, Mahidol University, Bangkok, Thailand.

2.2 Preparation of the plant extract

A. squamosa leaves were washed with water and air-dried at room temperature then put into an oven at 55°C for 3 days. The dried leaves of *A. squamosa* (2500 gram) were finely chopped and put into a stainless boiler. Twenty five liters of hot water was added to the cut leaves, boiled for 2 hours, and then filtered by grid cloth. The clear filtrate was collected and freeze dried in Freeze-Dryers Technology System (FTS) evaporator, Dura Dry® Science Engineer International CO., LTD., USA. The weight of the water extract was recorded. The percentage yield was 10.40.

2.3 Preparation of glibenclamide

Glibenclamide was given to the animals at dose of 3 mg/kg body weight. They were orally administered in an equivalent volume of 0.1 ml/100 g body weight of the rats.

2.4 Laboratory animals

Male Sprague-Dawley rats, weight 250-270 g, were obtained from the National Laboratory Animal Center, Nakorn Pathom, Thailand. They were all maintained in environmentally controlled conditions at 25±1°C under a 12-hour-dark-light cycle, at 60% relative humidity and given a standard diet (20-40 g/day) and water (10-12 ml/100 g/day) throughout the experimental period. All of the experimental protocols in animals were ethically approved by the Animal Ethics Committee of Faculty of Medicine, Thammasat University (No.0008/2007).

After one week stabilization period, STZ was injected into rats at a dose of 45 mg/kg. Blood samples were taken from rat tails for blood glucose analysis on the eighth and eleventh days after injecting STZ. Rats with blood glucose levels of 180 mg/dl or more on both days were selected for the diabetic group.

2.5 Hypoglycemic test

A) Diabetic rats

Thirty male diabetic rats were divided into five groups of six rats each. Groups 1 and 2 served as control group, given vehicle (distilled water) and 3 mg/kg glibenclamide respectively. Groups 3, 4 and 5 served as treated groups and received extract at doses of 125, 250 and 500 s mg/kg respectively.

B) Normal rats

Twenty-four male normal rats were divided into four groups of six rats each. Group 1 served as control groups given vehicle (distilled water). Groups 2, 3 and 4 served as treated groups and received extract at doses of 125, 250 and 500 mg/kg respectively.

Both diabetic and normal rats in treated groups were orally administered a single dose of 125, 250 or 500 mg/kg of the extract for twelve days. They were fasted overnight and then blood samples taken for testing blood glucose on the fifth and twelfth days of the experiment and three days later after withdrawal the extract. At the end of the experimental period, all animals were sacrificed for histological examination.

2.6 Oral glucose tolerance test (OGTT)

A total of fifty-four male rats were divided into two groups: thirty diabetic rats and twenty-four normal rats, similarly to the hypoglycemic test.

Thirty diabetic rats were divided into five groups of six rats each. Groups 1 and 2 served as control diabetic groups and received vehicle and 3 mg/kg glibenclamide respectively. Groups 3, 4 and 5 served as treated groups and received the extract at doses of 125, 250 and 500 mg/kg respectively.

Twenty-four male normal rats were divided into four groups of six rats each. Group 1 served as control normal group and received vehicle. Groups 2, 3 and 4 served as treated groups and given the extract orally at doses of 125,

250 and 500 mg/kg respectively.

All rats were fasted for 18 hours before being orally administered vehicle, glibenclamide or the plant extract. Thirty minutes later, their blood glucose levels were recorded and defined as basal blood glucose levels. After that they were given glucose orally at the dose of 3 g/kg body weight. All rats were tested for blood glucose levels at 30, 60, 90, 120, 150 and 180 minutes.

2.7 Histological sample preparation

After sacrifice, the body of pancreas was dissected, collected and fixed in 10% Neutral Buffered Formalin (NBF). The samples were processed in graded series of alcohol and embedded in paraffin wax, sectioned at 5 µm and stained with hematoxylin and eosin (H&E) for histological examination.

2.8 Statistical analysis

The results of the study were expressed as mean ± standard error of mean. Statistical significance was tested by one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test. *P* values less than 0.05 were considered significant.

3. Results

3.1 Hypoglycemic test in normal rats

No hypoglycemic effect of *A. squamosa* leaves extract was observed in normal rats as shown in Table 1.

3.2 Hypoglycemic test in diabetic rats

In diabetic rats, the extract at doses of 250 and 500 mg/kg significantly reduced fasting blood glucose level on the fifth and twelfth days of administration; however, after withdrawing the extract for three days, blood glucose levels of both groups were almost similar to the levels before receiving the extract (Table 2).

3.3 Oral glucose tolerance test in normal rats

Table 3 shows blood glucose levels when normal rats were orally given glucose load. All groups except the 250 mg/kg group reached their peaks at 60 minutes and gradually decreased until the end of the experiment. The glucose lowering responses are significant for the 250 and 500 mg/kg groups from 60 minutes until the end of the experiment when compared to normal rats receiving vehicle.

Table 1. Effect of *Annona squamosa* aqueous leaves extract on the fasting blood sugar in normal rats.

Experimental groups	Fasting Blood Sugar: FBS (mg/dl)			
	Day 0	Day 5	Day 12	3 day withdrawn
Vehicle	100.17±4.13	96.5±2.39	98.83±3.31	97.67±2.87
AS 125 mg/kg	101.17±5.04	96.50±9.49	96.67±7.83	92.50±6.92
AS 250 mg/kg	104.67±6.41	99.67±10.40	110.17±9.81	100.00±7.03
AS 500 mg/kg	94.17±2.18	97.50±8.38	104.50±10.12	114.33±8.33

Values are expressed as mean ± S.E.M., n = 6.
*Significantly different from control, $p<0.05$.
AS stands for *Annona squamosa*

Table 2. Effect of *Annona squamosa* aqueous leaves extract on the fasting blood sugar in diabetic rats.

Experimental groups	Fasting Blood Sugar: FBS (mg/dl)			
	Day 0	Day 5	Day 12	3 day withdrawn
Vehicle	330.17±12.06	336.33±9.21	346.83±4.24	333.83±4.13
Glibenclamide	312.67±19.76	188.83±29.76*	166.00±12.80*	158.83±12.40*
AS 125 mg/kg	349.50±13.74	292.67±42.98	319.71±25.71	326.50±21.98
AS 250 mg/kg	318.00±33.94	202.33±20.94*	170.17±17.94*	316.17±39.67
AS 500 mg/kg	331.50±12.16	218.83±27.20*	260.17±36.96*	307.83±27.15

Values are expressed as mean ± S.E.M., n = 6.
*Significantly different from control, $p<0.05$.
AS stands for *Annona squamosa*

Table 3. Effect of *Annona squamosa* aqueous leaves extract on oral glucose tolerance test in normal rats.

Experimental groups	Blood glucose concentration (mg/dl) (min)							
	-30	0	30	60	90	120	150	180
Vehicle	87.50±2.59	106.17±2.74	161.67±9.82	191.67±1.16	150.33±7.68	140.83±3.48	123.83±2.35	120.83±5.87
AS 125 mg/kg	92.67±2.62	111.83±12.18	157.50±6.54	169.83±13.14	169.67±21.36	124.50±11.74	111.00±7.26	115.17±5.05
AS 250 mg/kg	91.83±2.17	101.33±6.42	139.33±5.13	130.00±4.77*	109.83±4.07*	105.00±8.28*	96.33±5.05*	91.00±6.47*
AS 500 mg/kg	90.67±4.48	123.50±7.9	137.83±13.54	144.33±7.65*	92.17±4.94*	91.00±4.43*	91.00±5.76*	89.33±4.13*

Values are expressed as mean ± S.E.M., n = 6.
*p<0.05 when compared with control group at the same time
AS stands for *Annona squamosa*

Table 4. Effect of *Annona squamosa* aqueous leaves extract on oral glucose tolerance test in diabetic rats.

Experimental groups	Blood glucose concentration (mg/dl) (min)							
	-30	0	30	60	90	120	150	180
Vehicle	337.50±48.64	394.67±45.18	519.33±31.14	560.17±5.59	544.83±22.56	517.33±24.21	498.17±34.64	469.83±17.5
Glibenclamide	346.83±18.91	351.00±43.07	491.33±55.46	453.67±29.66*	424.00±19.59*	409.33±21.44*	374.00±36.13*	348.33±21.20*
AS 125 mg/kg	387.67±11.59	423.83±18.23	507.33±15.10	535.67±2.85	499.33±26.72	504.71±23.22	467.00±53.04	448.5±33.04
AS 250 mg/kg	381.83±13.68	422.33±22.42	410.17±25.51	481.00±16.36*	434.67±29.74*	411.67±19.9*	380.00±24.90*	349.67±21.5*
AS 500 mg/kg	355.67±19.92	412.00±25.92	440.00±8.56	456.00±13.35*	431.83±21.53*	391.00±18.20*	384.50±21.21	339.00±30.43*

Values are expressed as mean ± S.E.M., n = 6.
*p<0.05 when compared with control group at the same time
AS stands for *Annona squamosa*

3.4 Oral glucose tolerance test in diabetic rats

As shown in Table 4, at 60 minutes after oral glucose loading, blood glucose levels of diabetic rats that received doses of 250 and 500 mg/kg were significantly decreased when compared to diabetic rats receiving vehicle. Subsequently, blood glucose levels of both groups continued to reduce significantly until reaching the pre-glucose load level at 150 and 180 minutes, respectively.

3.5 Histological study of pancreas in normal and diabetic treated rats

Pancreatic histology was observed in normal and diabetic rats as shown in Figure 1. The pancreas of all groups of normal rats showed normal histological features and islet structure whereas those of diabetic rats receiving vehicle showed apparently irregular shaped islets of Langerhans crowded with small oval-shaped cells. All groups of diabetic treated rats showed improvement of pancreatic histological features. In addition, there was a dose-dependent histological response in which increasing doses of the extract led to a rounder-shaped islets of Langerhans as well as larger and rounder cells within the islets of Langerhans than those of diabetic rats receiving vehicle.

4. Discussions

The hypoglycemic test showed that the extract of *A. squamosa* at doses of 250 and 500 mg/kg could significantly reduce blood glucose level in STZ-induced diabetic rats when compared to diabetic rats receiving vehicle. Normally, the role of pancreatic islets of Langerhans is to moderate cellular nutrition, therefore their abnormal functions will result in disturbances of nutrient homeostasis (Masharani and German, 2007) including glucose homeostasis, which leads to diabetes. Streptozotocin, used in this study, inhibits free radical scavenger-enzymes causing β-cell cytotoxic effect (Kanter *et al.*, 2003). Young leaves of *A. squamosa* contain phenol and flavonoid (Seetharaman, 1986; Kotkar *et al.*, 2002), which are known to possess antioxidant and free radical scavenging ability (Saija *et al.*, 1995; Shirwaikar *et al.*, 2004). Therefore, the antihyperglycemic activity of *A. squmosa* may be due to antioxidant activities of its phenol and flavonoid (Saija *et al.*, 1995; Shirwaikar *et al.*, 2004) which reduce β-cell cytotoxicity and may also restore β-cell function. This assumption was confirmed by our histological examination which revealed histological recovery of islets of Langerhans and cells within the islets of diabetic rat pancreas.

Another mechanism may be due to mucilage obtained from the extract which was claimed to absorb blood glucose well (Riyad *et al*., 1988; Ajabnoor, 1990). Our finding of an antihyperglycemic effect of *A. squamosa* corresponds to the study of Gupta *et al.* (2005), who conducted experiments on the ethanolic extract of Northern Indian *A. squamosa* but their effective dose was 350 mg/kg whereas that of our study and of Shirwaikar *et al.* (2004) was 250 mg/kg. The difference in effective dose for reducing blood glucose level may

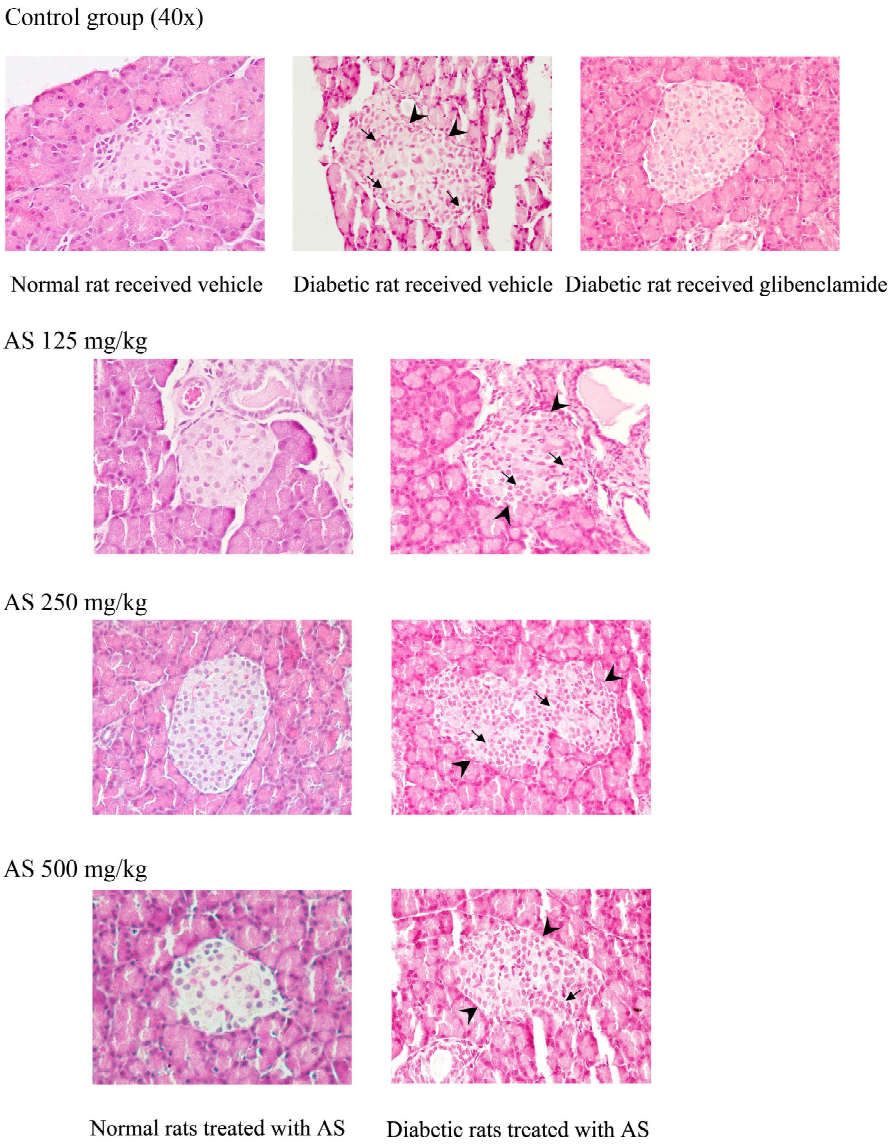

Figure 1. Rat pancreatic histological sections stained with hematoxylin and eosin of control and treated groups (the 40x magnification). Arrowheads represent the boundary of islets of Langerhans, arrows represent characteristic of cells inside the islets.

be due to variation in amount of active ingredients among two sources of plant or difference in employed solvent. Normal blood glucose level of normal rats receiving the extract in the hypoglycemic test may be explained by functional β-cell activity that decreases high blood glucose levels until reaching homeostasis.

In OGTT, our results showed that the extract at doses of 250 and 500 mg/kg significantly reduced blood glucose level in both normal and diabetic rats from 60 min onwards. Theoretically, blood glucose level after glucose loading depends on insulin secretion, glucose utilization, intestinal glucose absorption and intestinal motility (Peungvicha *et al.*, 1996), therefore additional hypoglycemic responsive mechanisms may be due to stimulation of insulin secretion, glucose utilization as well as inhibition of intestinal glucose absorption and intestinal motility.

In conclusion, the aqueous extract of leaves of Thai *A. squamosa* exhibits antihyperglycemic activity in STZ-diabetic rats by anti-oxidative effect which improves the histology of cells within the islets of Langerhans, and possibly their function in glucose homeostasis control. Moreover, other possible mechanisms are stimulation of blood glucose absorption, insulin secretion, glucose utilization, inhibition of intestinal glucose absorption and intestinal motility. However, this study was unable to identify β-cells in the histological improved islet of Langerhans and evaluate β-cell function, therefore further study by β-cell- specific staining and insulin level measurement should be done. Furthermore, this study and previous studies of Indian *A. squamosa* revealed some differences in effective dose for reducing blood glucose level, therefore further study to elucidate the amount of their active ingredients and the most appropriate

solvent for *A. squamosa* leaves extract should also be done. Such information would become very useful leading to the development for a potent antidiabetic drug.

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