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Anti-diabetic and acute toxicity studies of *Annona squamosa* L. ethanolic leaves extract.

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Abstract

Aim of this research work was to explore the anti-diabetic activity and acute toxicity of *Annona squamosa* L. leaves ethanolic extract in albino rats and mice respectively.
Diabetes was induced by Alloxan (120 mg/kg). Seven rats were taken in each group. Glibenclaimide (0.25 mg/kg) was taken as the standard drug. *A. squamosa* was administered orally in 100mg, 200mg and 400mg doses in three different groups of diabetes- induced rats. *A. squamosa* leaves extracts were found to have significant anti-diabetic activity.
Acute toxicity study was carried out on administration of 800mg/kg, 1600mg/kg and 5000 mg/kg body weight. No acute toxicity was observed at 800mg/kg and 1600mg/kg doses. At 5000mg/kg body weight dose 100% fatality was recorded within 24 hours.
Our research work revealed the safe and effective anti-diabetic activity of *Annona squamosa* ethanolic leaves extract.

Keywords: Alloxan, Glibenclaimide, custard apple, hypoglycaemic, toxic effects.

Introduction

Diabetes mellitus is a disorder of pancreas. It is a set of ailments that occurs from either lack of insulin or the factors which interfere with the action of insulin. In animals, it can be induced by partial pancreatectomy or by the administration of diabetogenic drugs such as alloxan, streptozotocin, ditizonx and anti-insulin serum. The disease is progressive and is associated with high risk of atherosclerosis, kidney and nerve damage as well as blindness. Abnormalities in the regulation of peroxide and transition metal metabolism are postulated to result in the development of the disease as well as its long term complication [1].
*Annona squamosa*L. (Family: Annonaceae) is also known as custard apple or sugar-apple. *A. squamosa* leaf contains cardiotonicquinoline alkaloids, flavonoids, volatile oils and glycosides [2]. *A. squamosa* is used in the treatment of various pathologies due to its anti-inflammatory, anti-microbial, anti-oxidant effects in its leaves extract. *A. squamosa* leaves have anti-diabetic activity. It acts by promoting insulin release from the pancreatic islets, increasing utilization of glucose in muscle and inhibiting the glucose output from liver. The *A. squamosa* ethanolic leaf extract is also known for its liver protective action [3-4].

Material and Method

Annona squamosa L. leaves were purchased from local supplier. The leaves were identified and authenticated by Prof. Dr. Mansoor Ahmad at the Institute of Pharmaceutical Sciences, University of Karachi-Karachi, Pakistan. Alloxan, Glibenclaimide, ethanol and glucometer was purchased from Merck supplier.

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White Albino rats (180-200 gm) and mice (20-25 gm) were purchased from animal house, DUHS. Animals were fed food and water *ad libitum*. Animals were maintained under standard conditions (12 h light and 12 h dark cycle; 25±30◦C; 35–60% humidity). Animal studies were carried out according to the NIH guide for the care and use of laboratory animals [5].

Preparation of plant extract

The leaves of *A. squamosa* were thoroughly cleaned with water to remove dust particles and shade-dried at room temperature. The fresh leaves were soaked in ethanol for 15 days. Then leaves extract was obtained using rotary evaporator. The extract was stored in a refrigerator at 4 C until used [6].

Induction of Non-Insulin Dependent Diabetes (NIDDM)

NIDDM was induced in overnight fasted adult wistar strain albino male rats by a single intra-peritoneal injection of Alloxan (120 mg/kg) body weight (Merck Pharmaceuticals, Karachi). Hyperglycemia was confirmed by the elevated glucose levels in plasma, determined at 72 hours and then on day 7 after injection. The rats found with Permanent NIDDM were used for the anti-diabetic study [7].

Grouping of Animals for anti-diabetic activity

Six groups were made (n=7). Group I – Control, group II – Diabetic control, group III – Glibenclaimide, group IV – treated with 100 mg/kg body weight *A. squamosa* extract, group V – treated with 200 mg/kg body weight *A. squamosa* extract, group VI - treated with 400 mg/kg body weight *A. squamosa* extract. All drug administrations were done orally at 24 hours interval for 16 days. Blood glucose level was checked on alternate days.

Grouping of animals for acute toxicity studies

For acute toxicity study, four groups were made: Group I - Control, Group II – treated with 800 mg/kg body weight of *A. squamosa* extract, Group III - 1600 mg/kg body weight of *A. squamosa* extract, Group IV - 5000 mg/kg body weight of *A. squamosa*extract. All drug administrations were done orally at 24 hours interval for 7 days.

Acute Toxicity Studies

Acute toxicity studies of ethanolic extract of *A. squamosa* were carried out on albino mice. Before administration of single dose of test samples the mice were deprived of food for 3 hours. Doses of 800, 1600 and 5000 mg/kg were orally administered to group II, III and IV mice respectively. All the mice were observed for general behavioural changes, symptoms of toxicity and mortality for first two hours (critical), then over a period of 24 hours thereafter daily for 7 days. Effects observed included breathing, paralytic effects of hind limb, passivity, gripping strength, salivation, climbing slide, sense, over activity, aggressiveness, fits, touch response, allergy and time of death if any were recorded.

Statistical Analysis

Results of the study were presented as mean plus minus standard error of mean (M±SEM). Differences between control and treatment groups were analyzed by student-t test [8].

Results

Significant anti-diabetic effect was observed at the dose of 400 mg/kg body weight of *A. squamosa* ethanolic leaves extract (Tables 1 & 2 Figures 1 & 2). Our research work exhibited the safety profile of ethanolic leaves extract at the dose of 16000 mg/kg body weight (See tables 3-5).

Table 1: Fasting blood sugar levels of control and treated groups of rats during the study period.

	2^{ND} Day	4^{TH} Day	6^{TH} Day	8^{TH} Day	10^{TH} Day	12^{TH} Day	14^{TH} Day	16^{TH} Day
Control	74.14±1.72	72.42±2.06	72.57±1.54	74.14±1.64	73±0.88	73.71±1.32	73.85±1.78	74±1.178
Diabetic (C)	344.42±67.61	361.85±70.82	372.14±75.07	158.14±84.24	152.14±82.27	155±80.609	87.28±60.87	99.28±69.42
Glibenclamide	345.28±22.07	332.28±20.05	211.14±7.65	143.42±13.62	134.14±2.29	103.28±6.04	76.14±3.15	80.28±1.09
A.S 100mg	391.28±19.31	296.71±51.21	259±43.78	163.14±37.26	119.71±35.56	107.57±21.23	107.42±14.26	96.28±10.05
A.S 200 mg	306.14±75.22	287.57±85.62	211.57±65.80	216.28±37.49	163±25.68	131.14±20.06	104.85±11.38	94.42±9.85
A.S 400 mg	335.28±47.04	248.71±27.78	274.28±48.60	130.57±37.50	161.71±67.65	222.28±83.80	112.14±55.38	75.71±35.98

Table 2: Random blood sugar levels of control and treated groups of rats during the study period.

	2ND Day	4TH Day	6TH Day	8TH Day	10TH Day	12TH Day	14THDay	16TH Day
Control	100.42±3.34	112.71±2.86	114.71±3.05	111.7±11.29	113.42±2.48	114.28±2.45	113.14±2.36	110.57±2.01
Diabetic(C)	72.42±78.23	151.28±105.47	72.85±78.69	145.57±101.74	145.14±101.19	146.71±102.34	70.28±75.91	68.57±74.06
Glibenclamide	364.85±65.95	439.85±20.07	292±14.27	248.14±13.93	218.42±5.84	184.57±6.21	168±7.09	160.57±4.97
A.S 100mg	337.28±100.74	379.85±92.85	305.57±76.61	361.14±69.59	220.28±53.17	252.85±54.45	234.28±23.51	218.57±19.01
A.S 200 mg	254.71±82.91	262.14±86.23	227.71±73.60	339.14±40.28	283.14±28.71	245.14±20.18	211.42±12.27	191.57±9.52
A.S 400 mg	169.57±91.82	361±70.52	339.28±97.81	272.57±78.28	183±74.49	267.71±106.52	221.14±89.93	191.28±76.51

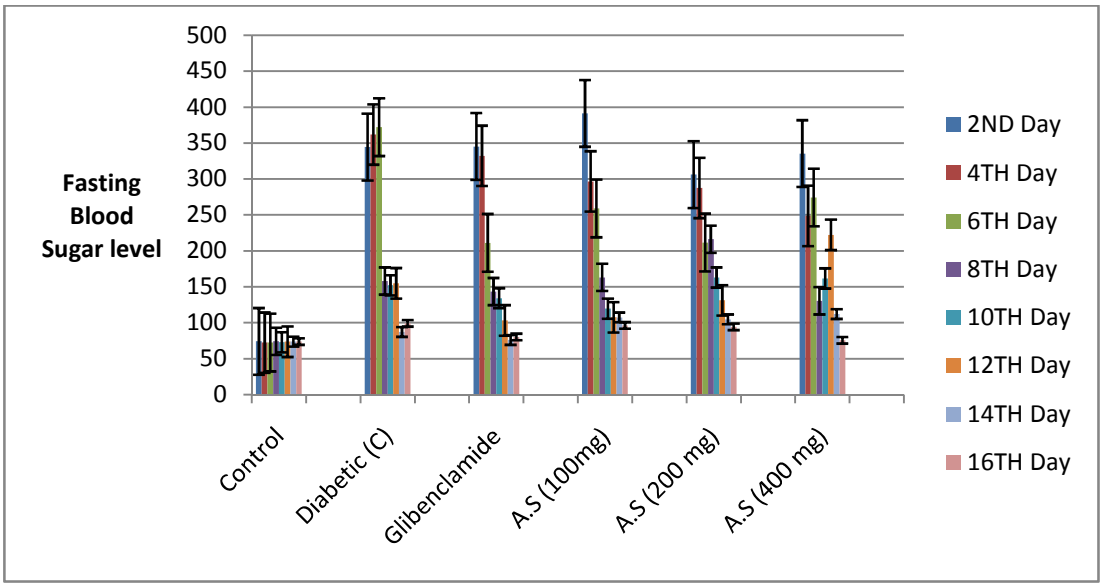

Figure 1: Fasting blood sugar levels of control and treated groups of rats during the study period.

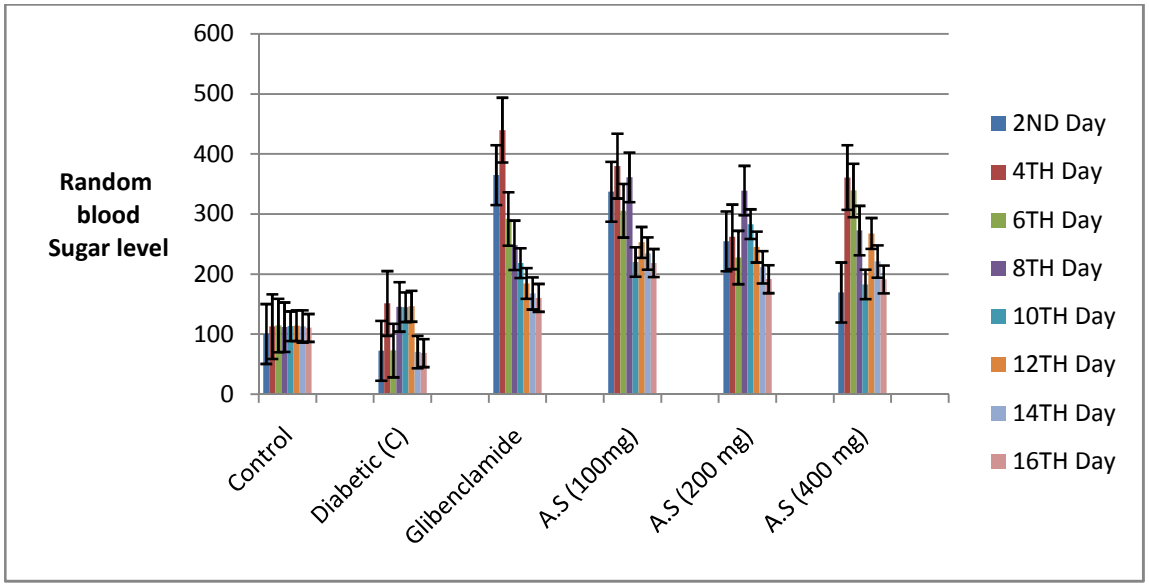

Figure 2: Random blood sugar levels of control and treated groups of rats during the study period.

Table 3: Acute toxicity study of *A. squamosa* (800mg/kg) on albino mice

EFFECTS	RESPONSE	5 MINS	30 MINS	1 HR	2 HRS	24 HRS	48 HRS	7 DAYS
Breathing	Normal Fast Slow	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Paralytic effects of hind limbs	No Yes	No	No	No	No	No	No	No
Passivity	No Yes	No	No	No	No	No	No	No
Grip	Normal Strong Weak	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Salivation	No Yes	No	No	No	No	No	No	No
Climbing slide	No Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Sense	Normal Senseless	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Over activity	No Yes	No	No	No	No	No	No	No
Aggressiveness	No Yes	No	No	No	No	No	No	No
Fits	No Yes	No	No	No	No	No	No	No
Touch response	Normal Pain	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Allergy	No allergy Redness Swelling	No	No	No	No	No	No	No
Died	No Yes	No	No	No	No	No	No	No

Table 4: Acute toxicity study of *A. squamosa* (1600mg/kg) on albino mice

EFFECTS	RESPONSE	5 MINS	30 MINS	1 HR	2 HRS	24 HRS	48 HRS	7 DAYS
Breathing	Normal Fast Slow	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Paralytic effects of hind limbs	No Yes	No	No	No	No	No	No	No
Passivity	No Yes	No	No	No	No	No	No	No
Grip	Normal Strong Weak	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Salivation	No Yes	No	No	No	No	No	No	No
Climbing slide	No Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Sense	Normal Senseless	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Over activity	No Yes	No	No	No	No	No	No	No
Aggressiveness	No Yes	No	No	No	No	No	No	No
Fits	No Yes	No	No	No	No	No	No	No
Touch response	Normal Pain	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Allergy	No allergy Redness swelling	No	No	No	No	No	No	No
Died	No Yes	No	No	No	No	No	No	No

Table 5: Acute toxicity study of *A. squamosa* (5000mg/kg) on albino mice

EFFECTS	RESPONSE	5 MINS	30 MINS	1 HR	2 HRS	24 HRS	48 HRS	7 DAYS
Breathing	Normal Fast Slow	Fast	fast	slow	slow	Die	-	-
Paralytic effects of hind limbs	No Yes	no	no	no	yes	Die	-	-
Passivity	No Yes	no	no	no	Yes	Die	-	-
Grip	Normal Strong Weak	Normal	normal	weak	weak	Die	-	-
Salivation	No Yes	no	no	no	no	Die	-	-
Climbing slide	No Yes	yes	yes	no	no	Die	-	-
Sense	Normal senseless	Normal	normal	normal	senseless	Die	-	-
Over activity	No Yes	no	no	no	no	Yes	-	-
Aggressiveness	No Yes	no	no	no	no	Yes	-	-
Fits	No Yes	no	no	no	yes	Die	-	-
Touch response	Normal Pain	Normal	Normal	Normal	normal	Die	-	-
Allergy	No allergy Redness swelling	no	no	no	no	die	-	-
Died	No Yes	no	no	no	no	Yes	-	-

Discussion

The result of the present study on blood glucose level revealed that administration of ethanolic extract of *A. squamosa* L. to Alloxan-induced diabetic control rats produced a significant reduction in blood glucose level compared to diabetic control group. The hypoglycemic activity of the extract was sustained throughout the monitored period almost comparable to that of the reference drug, Glibenclamide. The present study indicates the scientific basis for its use. Diabetes mellitus is a metabolic disorder characterized by insufficient insulin secretion and/ or insensitive target tissues to metabolic action of insulin though, insulin is presently one of the most important therapeutic agent known to medicine. Efforts are being done to seek insulin substitutes from synthetic or plant sources for treatment of diabetes and reported to have hypoglycemic effect [9-14]. Panda &Kar (2007) revealed in their research work that quercetin present in *A. squamosa* is responsible for its anti-diabetic activity [15]. Gupta *et al.* (2005) reported the safe and effective hypoglycaemic and anti-diabetic effects of water extract of *A. squamosa* leaves [16].

No toxicity effects were observed in mice treated with 800 and 1600 mg/kg doses respectively. Toxic effects were observed in mice treated with 5000 mg/kg dose. Fatality was also observed at the end of 24 hours in the group treated with 5000 mg/kg of *A. squamosa* extract. Our results of *A. squamosa* ethanolic leaves extract were in conformity with that of the research work carried out by El Banna *et al.* (2016). El Banna *et al.* (2016) reported LD_{50} of the *A. squamosa* leaves and seeds extract were found to be 5 g/kg body weight and 520 mg/kg body weight, respectively [17]. Onwusonye *et al.* (2014) reported that oral LD_{50} of methanolic leaf extract was 5000 mg/kg body weight [18]. Safety profile of aqueous leaf extract of *A. squamosa* was explored to be more than 2000 mg/kg body weight of mice by Sharma and Goray (2009) and Madhu *et al.* (2012) [19-20]. LD_{50} of more than 2 g/kg body weight of ethanolic leaf extract was reported by Richa Mishra *et al.* (2012) and Kumar *et al.* (2015) [21-22]. Masimba *et al.* (2016) reported that LD_{50} was more than1.5 g/kg body weight for aqueous-ethanol fraction [23].

Conclusion

The present investigation revealed that the ethanolic leaf extract of *A. squamosa* L. has hypoglycemic activity. The acute toxicity studies results confirmed the safety of *A. squamosa* extract in low doses. Further, comprehensive chemical and pharmacological investigation should be carried out to isolate the active compound and appropriate elucidation of its mechanism of action.

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