Acute And Sub-Acute Toxicological Evaluation of Aqueous and Ethanol Fractions of *Annona Squamosa* Root A Traditional Medicinal Herb

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Summary

The study was designed to elucidate acute and sub acute toxicity of aqueous and ethanolic extract of *Annona squamosa* root in mice and rats respectively. In acute study aqueous and ethanolic extract of *Annona squamosa* root were administered to mice at a dose level starting from 5, 50,300 and 2000 mg/kg and in sub-acute toxicity study aqueous extract was administered at a dose of 200 mg/kg per day for 28 days. Acute toxicity data revealed that aqueous extract did not show any toxic related clinical signs, while ethanolic extract showed toxic clinical signs at dose of 300 and 2000 mg/kg. Sub-acute toxicity study with aqueous extract at dose of 200mg/kg showed no significant change in body weight, vital organs weight, biochemical and hematological parameters. Histopathological valuation has not shown any abnormalities in aqueous treated group when compared to the control group. Aqueous extract treated animal has not shown any signs of toxicity in acute and sub acute toxicity studies in rats, while ethanolic extract of *Annona Squamosa* roots was found to be toxic in acute toxicity studies.

Key words: Annona Squamosa, Acute toxicity studies, Sub-acute toxicity study

Introduction

Annona squamosa L. is commonly known as custard apple and is a native of West Indies and is cultivated throughout India, mainly for its edible fruit. *Annona squamosa* a common plant with many folklore claims and is reported to have medicinal properties which include antifertility and anti-tumour activities in mice and rats [1,2]. *Annona squamosa* roots are used for mental depression and spinal cord disorders [3].There was no scientific reports on toxicity data of *Annona Squamosa* roots. So the study has been conducted for the evaluation of acute and subacute toxicity study on *Annona Squamosa* roots.

Materials And Methods

Plant material and extraction

Roots of *Annona squamosa* were collected from the regions of Porur, Chennai, India and were identified and authenticated by Prof. P Jeyaraman, Botanist - Plant Anatomy Research Centre, Chennai, India. A voucher specimen (Rt-Sp-03/11) has been preserved at the department for future reference. Fresh roots were washed well with water. Air-dried roots (500 g) were extracted separately with water and ethanol for 7 days and filtered on muslin cloth, the filtrate were centrifuged at 10,000rpm for 10min in normal centrifuge at room temperature and the extract was concentrated to half of its volume by slight warming. The resulting dark brown extract was concentrated on rotary evaporator under reduced pressure. The resulting crude drug (8g for aqueous and 13g for alcoholic) was used for further study.

Experimental Animals

Adult female Albino mice weighing between 25 to 30 g were used for acute toxicity studies and for sub acute toxicity studies both male and female rats weighing between 150-200gm were used. Animals were housed in a well ventilated room with 12 h cycle of day and night light conditions and temperature maintained at around 32°C. The animals were fed with a standard laboratory diet and tap water *ad libitum*. All experiments were carried out in accordance with guide for the care and use of laboratory animals. After one week of habituation, animals were subjected to the experiments. Females were caged separately from the males to prevent mating. All tests were carried out between 9.00 am and 2.00 pm. All efforts were made to minimize animal suffering.

Acute toxicity studies

Acute toxicity studies were carried out according to the OECD (Organization of Economic Co-operation and Development) guidelines 423[4] .Healthy female mice, weighing 25–30 g, were selected and oral administration of the single doses of *Annona squamosa e*xtracts (aqueous and alcoholic) were done aseptically by suspending in Sodium carboxymethyl cellulose. An oral (p.o) dose of 5 mg/kg, 50 mg/kg, 300 mg/kg & 2000 mg/kg was administered step by step according to the guidelines. The general behaviors of the mice were continuously monitored for 1 h after dosing, periodically during the first 24 h (with special attention given during the first 4 h [5], and then daily thereafter, for a total of 14 days. Changes in the normal psychomotor activity and external morphology of mice and their body weights were monitored periodically before dosing and the time at which signs of toxicity or mortality were recorded.

Sub-acute toxicity

Sub-acute toxicity studies were carried out according to OECD TG407[6]and rats were divided into 3 groups of 10 animals (5 male and 5 female). The extracts of Annona squamosa was administered to the first group of rats at the dose of 200 mg/kg/day for 28 days, whereas an equal volume of vehicle was given to the control group. In order to access reversibility, the extracts of *Annona squamosa* at the dose of 200 mg/kg was given once daily to the third group of rats for 28 days, and kept for another 14 days post-treatment. The toxic symptoms such as signs of toxicity, mortality and body weight changes were monitored daily. Rats were anesthetized with ether at the end of the treatment period. All rats were sacrificed after the blood collection.

Blood analyses

The blood sample was carefully collected for blood chemistry and enzyme analysis. Heparinized blood samples were used for determining total red blood cell count, total white blood cell count, platelet count haemoglobin,hematocrit,lymphocytes and neutrophils[7]. Non heparinzed samples were used for the estimation of biochemical parameters like creatinine[8], urea[9], triglycerides, total cholesterol[10], total protein[11], albumin,[12] AST, ALT[13,14], ALP[15] and total bilirubin[16,17].

Histopathological examinations

The internal organs like liver, kidney, heart, lungs, brain and spleen were isolated and weighed to determine relative organs weights and observed for gross lesions. All tissues were preserved in 10% buffered formaldehyde solution for histological examination [18,19].

Statistical analysis

Statistical analysis was carried out by one way ANNOVA and the results were expressed as mean ± SEM. The data obtained from the toxicity study was analyzed using students t- test, p values less than 0.05 were considered as significant.

Results

Acute toxicity studies

Animals treated with aqueous extract of *Annona squamos*a at the dose level starting from 5 mg to 2000mg/ kg has survived the scheduled sacrifice, and there were no test-material-related toxic clinical signs. But one animal among the three animals treated with alcoholic extract at the dose level of 300mg/kg has not survived more than 48 hours and all the animals treated with 2000 mg/kg has not survived more than 12 hours (Table: I). Aqueous extract treated animals has not shown any signs of toxicity provided ethanolic extract treated animals show abnormal symptoms like increased sniffing, seizures, straubs tail and loss of grip strength, righting reflex and spontaneous rearing. (Table: II)

Table: I. Effect on *Annona squamosa* in mice after oral administration of single dose

Treatment	Mice/Sex	Death/ Treated		Mortality latency (hr)	
		Aqueous	Ethanolic	Aqueous	Ethanolic
0 mg/kg(Vehicle)	Female	0/3	0/3	-	-
5mg/kg	Female	0/3	0/3	-	-
50mg/kg	Female	0/3	0/3	-	-
300mg/kg	Female	0/3	1/3	-	<48
2000mg/kg	Female	0/3	3/3	-	<12

Table: II. Symptoms observed for the first four hours for the acute treatment of 2000mg/kg

	I hr		II hr		III hr		IV hr	
Parameters observed	**Aq.**	**Eth.**	**Aq.**	**Eth.**	**Aq.**	**Eth.**	**Aq.**	**Eth.**
Piloerection	-	-	-	-	-	-	-	-
Edema	-	-	-	-	-	-	-	-
Urine stains	-	-	-	-	-	-	-	-
Alopecia	-	-	-	-	-	-	-	-
Loss of righting reflex	-	-	-	-	-	-	-	
Circling	-	-	-	-	-	-	-	-
Nasal sniffing	-	-	-	+	-	+	-	+
Lacrimation	-	-	-	-	-	-	-	-
Seizures	-	-	-	-	-	+	-	+
Righting reflex (Touch)	+	+	+	-	+	-	+	-
Grip strength	+	+	+	-	+	-	+	-
Eye closure at touch	+	+	+	+	+	+	+	+
Rearing	+	+	+	-	+	-	+	-
Straub tail	-	-	-	-	-	+	-	+

Aq. –Aqueous Eth.-Ethanolic

(-) Indicates absence of symptom (+) Indicates presence of symptoms

Sub acute toxicity studies

Annona squamosa extracts at dose of 200mg/kg given orally for 28 days did not produce any mortality changes.

Change in body weight

Body weight changes were monitored at weekly intervals till 28days. Body weights of rats in sub-acute toxicity studies were summarized in (Table: III). Body weights of rats at the dose of 200 mg/kg have not show any significant change over those in the untreated control group. Percentage increase in all the groups including the satellite group is found to be uniform. The extract does not show any reduction in the body weight as an evidence for absence of toxicity.

Table: III. Influence of *Annona Squamosa* on change in body weight (200mg/kg)

Treatment	7th day	14th day	21st day	28th day	% increase
Control	175.92±1.25	176.66±1.25	176.66±1.25	177.00±1.41*	1.04
200 mg/kg	156.16±0.29	157.16±0.79	157.83±0.74	158.16±0.70*	1.07
200mg/kg (Repeat)	155.17±1.20	155.65±1.02	156.98±0.96	157.31±1.09*	1.08

Data are expressed as mean ± SEM
*P<0.05vs value on 7th day

Organ weights

Relative organ weight of rats treated with *Annona squamosa* did not show any evidence of drug-related toxicity. There were no significant changes of weight of extract treated and those of control groups. The satellite group which was kept for further 14 days was also uniform to other groups in organ weight. (Table III)

Table IV. Relative Organ Weight of male and female rats treated with aqueous extract of *Annona squamosa*

Dose	Relative Organ Weight of male and female rats treated with aqueous extract of *Annona squamosa*					
	Liver	Kidney	Brain	Lungs	Heart	Spleen
Control	2.8±0.1	0.66±0.02	0.38±0.22	0.29±0.01	0.29±0.01	0.15±0.01
200mg/kg	2.9±0.1	0.66±0.02	0.40±0.01	0.31±0.02	0.30±0.01	0.16±0.01
200mg/kg (Repeat)	3.0±0.1*	0.67±0.03*	0.43±0.01*	0.32±0.01*	0.31±0.01*	0.17±0.01*

Data are expressed as mean ± SEM
*P<0.05vs control

Pathology—Macroscopic and Microscopic

The histological examination of the various organs was performed in both control and treated groups. Organ weight revealed that at repeated dose administration of extract at dose of 2000mg/kg did not produce any organ swelling, atrophy and hypertrophy. All the sampling tissue sections were within the normal limits and degenerative or infiltrative lesion was not observed in the extract treated group. The results obtained revealed that there were no significant changes in the histology of the internal organs of the aqueous extract treated rats as compared to the control rats (fig-1).

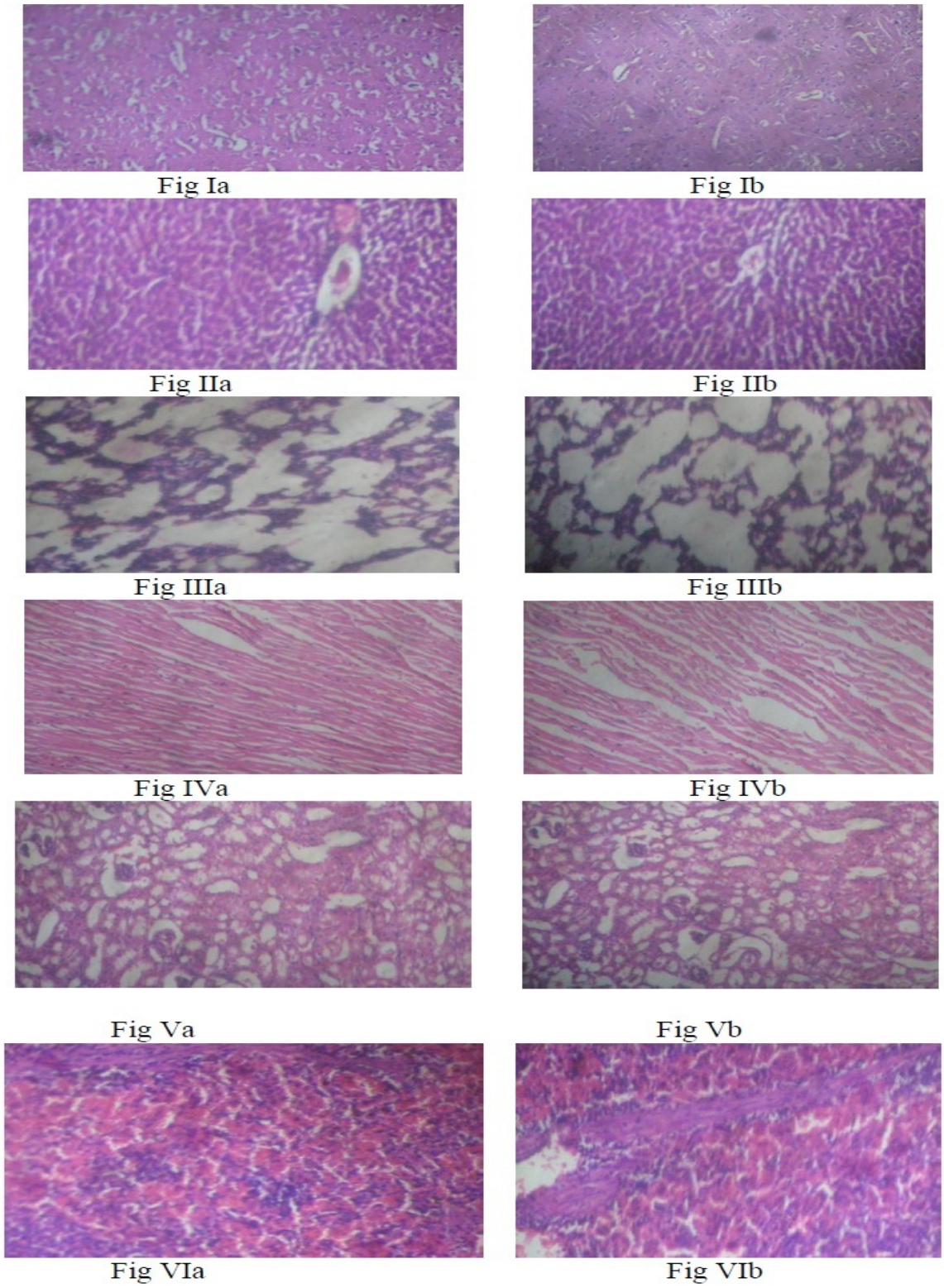

Fig: I Representative photomicrographs of H&E stained sections of rat organs over a time course after *Annona squamosa* administration. Control VS treated at the dose level of 200mg/kg (p.o). Fig I a &I b shows the normal architect of brain, Fig II a &II b shows the normal architect of liver, Fig IIIa &IIIb shows the normal architect of lungs, Fig IV a &IV b shows the normal architect of heart, Fig Va &Vb shows the normal architect of kidney, FigV I a &VI b shows the normal architect of spleen

Hematological and serum biochemical parameters

The haematological analysis shown in the (Table IV), shows no significant change of blood parameters analyzed such as total red blood cell, hematocrit, hemoglobin, total white blood cell, lymphocytes, neutrophils and platelets in the treatment groups compared to the control group.

The biochemical analysis of creatinine, urea, triglycerides, total cholesterol, total protein, albumin, AST, ALT, ALP and total bilirubin (Table V), shows no significant difference in any of the biochemical parameters examined in either of the control or treated groups. All the above said haematological and serum biochemical parameters are within normal limits

Table: V. Effect of *Annona squamosa* Linn, on the Haematological Parameter

Haematological parameter	Control	*Annona squamosa* extract	
		200 mg	200mg (repeat)
Total R.B.C. count (×106 mm−3).	07.27 ± 0.49	07.07 ± 1.86	7.01 ± 1.05
Total W.B.C. Count (×103 mm−3).	07.88 ±1.97	07.54 ± 1.45	7.23 ± 1.88
Haemoglobin (Hb) (g/dl)	11.42 ±1.24	11.41 ± 1.07	11.42 ± 0.72
Hematocrit (%).	37.77±1.96	38.74±0.99	38.14±2.56
Platelets (×103 mm−3).	703±123.4	701±166.74	708±135.67
Lymphocytes(%).	83.43±3.43	76.56±2.45	81±4.55
Neutrophils (%).	16.58±2.33	17.54±4.31	17.09±2.98

Data are expressed as mean ± SEM

Table: VI. Effect of Annona squamosa on the Biochemical Parameters

Biochemical parameter	Control	*Annona squamosa* extract	
		200 mg	200mg (repeat)
Creatinine (mg/dl)	0.72 ± 0.04	0.63±0.02	0.73±0.01
Urea (mg/dl)	47.17 ± 1.92	40.75±1.65	40.17±1.75
Triglycerides (mg/dl)	48.83 ± 4.12	53.25±4.14	54.33±11.83
Total Cholesterol (mg/dl)	68.33 ± 2.03	76.00±2.66	79.25±3.95
Total protein (mg/dl)	8.53 ± 0.24	8.31±0.23	8.47±0.24
Albumin (g/dl)	4.52 ± 0.15	4.17±0.03	4.27±0.19
AST (IU/L)	219.0 ± 17.6	204±19.4	189.6±28.8
ALT (IU/L)	66.00 ± 4.19	66.5±6.36	62.67±7.61
ALP (IU/L)	289.3 ± 28.0	250.4±45.32	267.54±32.76
T. Bilirubin (mg/dl)	0.35 ± 0.15	0.37±0.13	0.35±0.19

Data are expressed as mean ± SEM

Discussion

The results of the acute toxicity study reveal that ethanolic extract treated animal shows toxic symptoms such as change in behavior and mortality at dose of 300mg/kg and 20000mg/kg respectively. But acute toxicity study of aqueous treated animal group did not

show any toxic symptoms in behavior or mortality up to dose level of 2000mg/kg. Hence, we conducted sub acute toxicity study of aqueous extract and it appeared that aqueous extract of *Annona Squamosa* roots at dose level of 200mg/kg did not produce any marked toxic changes in both male and female rats, as evidenced by absence of toxic symptoms, no change in water and food ingestion was noticed. Generally, the reduction in body weight gain and internal organ weights is a simple and sensitive index of toxicity after exposure to toxic substance [20]. In the present study, aqueous extract of *Annona Squamosa* roots does not produce any statistically significant difference among three groups in body weight gain and internal organ weights

All animals survived until the scheduled euthanasia and no gross pathological alteration was found in the internal organs. Histopathological valuation did not find any abnormalities in aqueous treated group compared to the control group. The comparable biochemical results in the control groups and the extract treated groups were consistent with morphological analysis.

In summary , the ethanolic extract of *Annona Squamosa* roots was found to be toxic in acute toxicity studies , while aqueous extract treated animal was found to be non toxic in oral acute and sub acute toxicity studies in rats.

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