ScienSage

Journal of Advanced Scientific Research

Available online through http://www.sciensage.info/jasr

ISSN
0976-9595
Research Article

ORAL ACUTE TOXICITY STUDY OF *ANNONA SQUAMOSA* L. LEAVES EXTRACT AND FRACTIONS IN ALBINO MICE

Pax J. Masimba[1]*, Ester Innocent[1] and Baraka Samwel[2]

[1]*Department of Biological and Preclinical Studies, Institute of Traditional Medicines, Muhimbili University of Health and Allied Sciences, Dar es salaam, Tanzania*

[2]*Department of Natural Products Development and Formulation, Institute of Traditional Medicines, Muhimbili University of Health and Allied Sciences, Dar es salaam, Tanzania*

**Corresponding author: paxmasimba@gmail.com*

ABSTRACT

The medicinal plants have been used traditionally for different purposes such as pest control and treatment of human diseases. Despite these potentials, only few plants have been evaluated for their safety. This study aimed at investigating oral acute toxicity of *Annona squamosa* leaf extract and fractions using albino mice. The aqueous-ethanol, dichloromethane and petroleum ether fractions were prepared and tested for oral acute toxicity. Whereas some toxicity signs occurred for aqueous-ethanol extract at doses ≥1500 mg/kg bwt that of dichloromethane fraction were seen at 1000-5000 mg/kg and that of petroleum ether fraction were at 5000 mg/kg. All the tested materials at a dose above 300 mg/kg bwt had negative effects on the growth of the mice. The LD_{50} were estimated to be >1000 mg/kg bwt for dichloromethane, >1500 mg/kg bwt for aqueous-ethanol extract and >3000 mg/kg bwt for petroleum ether fraction. All mice dissected had normal organs except one among six females treated with aqueous-ethanol extract at 5000 mg/kg bwt which had pus in the uterus. Relative organ weight (ROW) suggested effects in kidney and heart.

The LD_{50} in this study indicated that the dichloromethane fraction and aqueous-ethanol extracts are harmful if swallowed while petroleum ether fraction may be harmful if swallowed hence people should be warned against oral use of these products. Since only behavioral and gross organ examinations were done, further studies involving histological and hematological examinations are suggested.

Keywords: *Annona squamosa,* acute toxicity, albino mice

1. INTRODUCTION

For thousands of years plants have been used traditionally for health care provision. According to a World Health Organization (WHO) survey, about 80% of inhabitants in developing countries depend on traditional medicine for their primary health care [1]. Medicinal plants contain chemical substances that are responsible for biological activities in human body. In order to determine their medication potential, studies of safety and efficacy is compulsory. However, there is a limited data on efficacy and safety for the majority of the used herb remedies. The *A. squamosa* commonly known as custard apple belongs to the Annonaceae family. Different parts of the plant such as the fruits, leaves, barks, roots and the seeds are used for different preparation of traditional medicines. Powdered seeds are used to kill head-lice and fleas, however, when it comes in contact with the eyes it causes great pain [2]. The leaf and seed of *A. squamosa* have been reported to exhibit brine shrimp toxicity [3] and insecticidal activity against mosquito larvae [4,5] while fruit are used as anti-dysenteric [6]. The Previous studies on *A. squamosa* indicated the presence of various classes of phytochemicals including acetogenins, alkaloids, terpenoids flavonoids and [7-8] and saponins [9]. A recent report on *A. squamosa* ethanolic extract against some invertebrate water habiting species revealed that this extract was non toxic [10]. However no study has been done to investigate in-vivo toxicity of *A. squamosa* leaf extract on albino mice so as to guide its potential use.

2. MATERIALS AND METHODS

2.1. Ethical clearance and handling of experimental animals

Ethical clearance was provided by Muhimbili University of Health and Allied Sciences (MUHAS). Handling of animals during this study was assisted by a Veterinary scientist. Few mice were kept in each cage to enable mice to express their normal behaviors. Clean water and food were given to mice *ad lib* and mice were kept at the temperature of 25^0C. Prior to dissection of the mice, diethyl ether was used to induce anesthesia and death (euthanasia).

2.2. Plant materials, extraction and fractionation of extract

The leaves of *Annona squamosa* L. (Annoneceae) were collected in September 2012 from Bagamoyo district, Cost region in Tanzania. Identification of plant species at the site was done by a botanist. Authentication of the plant species was done at the herbarium of Institute of Traditional Medicine, Muhimbili

University of Health and Allied Sciences, Tanzania, where the voucher specimen number ASS-2 is deposited. Air dried plant materials were ground to powders which were soaked thrice with 20% aqueous-ethanol. The filtrate was concentrated using rotary evaporator (*HEIDOLPH,* Germany) then completely dried using a freeze drier (EDWARDS, England). The extract was fractioned by using vacuum liquid chromatography (VLC) technique. During the fractionation process, petroleum ether and dichloromethane were used to separate polarity dependent compounds on silica gel, size 230-400 mesh.

2.3. Acute toxicity study in mice

Albino mice were deprived of food but not water for 24 hrs before experiment. The mice were then randomly divided into three main treatment groups (aqueous-ethanol extract, dichloromethane fraction or petroleum ether fraction) and one control group. Each of the three treatment groups had seven sub-groups of five mice each. Different sample concentrations were prepared by dissolving in 1% Carboxymethyl Cellulose (CMC) appropriate amount of aqueous-ethanol extract, dichloromethane fraction or petroleum ether fraction. Each of the treatment groups orally received 100, 300, 1000, 1500, 3000 and 5000 mg/kg bwt of the respective sample while the control group orally received 1% CMC. After oral drug administration, feeding was withheld for about two hours while observing for signs of toxicity and death once in every 30 minutes. Observation was made periodically during the first 24 hours and then daily for 14 days. Mice were weighed on day zero before drugs administration (on day zero) and day fourteen.

2.4. Pathological examination

All mice used in the experiment were dissected to examine their organs such as liver, kidney, intestines, lung and heart. These organs were removed, blotted to free off blood and weighed immediately on electronic balance.

2.5. Statistical analysis

Analysis was carried out using STATA program (USA, version 10.1). Relative organ weights (ROW) were calculated as per Arthur and others, 2011 [11]. Values were expressed as mean ± S.D. Mean values of $P<0.05$ were considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. Acute toxicity study in mice

The parameters observed in mice upon oral administration of aqueous-ethanol extract and fractions are shown in Table 1. Administration of aqueous-ethanol extract at 1500, 3000 and 5000 mg/kg in mice resulted in breathing difficulties and eye lid closure. These toxicity signs, in general, were shown 40 minutes following drug administration and lasted for 24 hours. Mortalities of up to 50% were recorded at a dose of between 3000 and 5000mg/kg bwt.

For mice which received dichloromethane fraction at doses from 1000 to 5000 mg/kg bwt, abnormal signs like breathing difficulties and eye lid closure were observed. Changes in back hair, diarrhea and comma were observed from the second to eighth day for mice which received 5000 mg/kg bwt; and most of them (67%) died within 48 hours after expression of these signs. Petroleum ether fraction was less toxic compared to the other two extracts. However, at the highest dose (5000 mg/kg bwt) abnormal behaviors like breathing difficulties and comma as well as mortality of 17% were observed.

Table 1: Acute toxicity signs observed in mice within 14 days post oral administration of 80% ethanolic aqueous extract and fractions at different concentrations

Treatment	Dose mg/kg bwt	Change in back hair	Excitement	Difficulty breathing	Diarrhea	Sleep/ comma	Eyelid closure	Death
80% ethanolic aqueous extract	100	x	x	x	x	x	x	x
	300	x	x	x	x	x	x	x
	1000	x	x	x	x	x	x	x
	1500	x	x	√	x	x	x	x
	3000	x	x	√	x	√	√	√
	5000	x	x	√	x	√	√	√
DCM fraction	100	x	x	x	x	x	x	x
	300	x	x	x	x	x	x	x
	1000	x	x	√	x	x	x	x
	1500	x	x	√	x	√	√	√
	3000	x	x	√	x	√	√	√
	5000	√	x	√	√	√	√	√
PE fraction	100	x	x	x	x	x	x	x
	300	x	x	x	x	x	x	x
	1000	x	x	x	x	x	x	x
	1500	x	x	x	x	x	x	x
	3000	x	x	x	x	x	x	x
	5000	x	x	√	x	√	x	√

√ = Toxicity sign observed; x = No sign of toxicity observed; DCM = Dichloromethane; P.E = Petroleum Ether; bwt = body weight

The deaths observed in the treated groups occurred in a dose-dependent manner; being higher for the highest dose (Table 2). The Globally Harmonized System (GHS) of classification and labeling of chemicals classify values for acute oral toxicity LD50 as follows; LD50 ≤ 5 mg/kg – Category 1 (danger), fatal if swallowed; LD50 >5 but ≤ 50 mg/kg – category 2 (danger), fatal if swallowed; LD50> 50 but ≤ 300 mg/kg – category 3 (danger), toxic if swallowed; LD_{50} > 300 but ≤ 2000 mg/kg -category 4 (warning)- harmful if swallowed and LD50 >2000 but ≤ 5000 mg/kg – category 5 (warning)- may be harmful if swallowed [12].

Table 2: Effect of test substances on mortality in the acute toxicity test

Dose (mg/kg bw)	% Mortality		
	Aqueous-ethanol extract	*Dichloromethane fraction*	*Petroleum ether fraction*
Control (1% CMC)	0	0	0
100	0	0	0
300	0	0	0
1000	0	0	0
1500	0	17	0
3000	33	50	0
5000	50	67	17

According to the results of the present study the LD_{50} were estimated as follows; LD_{50} > 1000 mg/kg bwt, LD_{50} > 1500 mg/kg bwt and LD_{50} > 3000 mg/kg bwt for dichloromethane fraction, aqueous-ethanol extract and petroleum ether fraction respectively, hence be classified as GHS category 4 for dichloromethane fraction and aqueous-ethanol extract and GHS category 5 for petroleum ether fraction. This means that the dichloromethane fraction and aqueous-ethanol extracts are harmful if swallowed while petroleum ether fraction may be harmful if swallowed.

Biological activities of plant are due to the presence of natural compounds with different mode of action. These compounds usually occur in the plant extract at different levels depending on factors including polarity of solvent used for extraction which therefore leads to different levels of toxicity of extracts. According to Westendorf [13], saponins are detergents which are weakly absorbed by the intestine where they can be concentrated on gastric and intestinal epithelium hence cause damage. When saponins are absorbed systemically they cause damage to red blood cells, haemolysis and kidney failure. In addition, reflexes via the autonomic nervous system may produce disturbances to heart function and circulatory system. Severe fluid and electrolyte loss may result into a shock reaction and death. Since saponins have been reported in *A. squamosa* [9], it is likely that such kind of compounds may have contributed to the death of mice in this study.

3.2. Effects of aqueous-ethanol extract and fractions on growth of albino mice

The body weight measurements on days zero and fourteen are indicated on Table 3. The groups that received the dose of ≤ 300 mg/kg of test substances and the control groups showed a significant difference (P>0.05) in body weights between day 0 and day 14. Although there were increases in body weights between day zero and day 14 in all groups which received doses above 300 mg/kg bwt, the increases were not statistically significant (Table 3).

Table 3: Effect of *A. squamosa* 80% ethanolic aqueous extract and fractions on bwt changes in mice

Treatment	Dose(mg/kg bwt)	Time	
		Day 0	Day 14
1% CMC	-	20.4±1.14	25.5±2.42*
80% ethanolic aqueous extract	100	23.3±1.23	26.1±0.53*
	300	20±1.51	26.25±3.86*
	1000	20.25±0.63	22.75±2.10
	1500	21.6±2.07	26±3.67
	3000	21.33±1.51	24.33±2.50
	5000	19.8 ±1.3	22.6 ± 2.07
DCM fraction	100	23±0.55	27.1±1.46*
	300	20±2.3	24±2.91*
	1000	20.25±2.5	24.33±2.78
	1500	19.5±1.98	23.5±2.56
	3000	22.25±2.35	26.67±2.45
	5000	21.6±1.89	25.25±2.5
P. E fraction	100	21.0±1.0	23.88±1.18*
	300	24.0±2.63	25.25±2.36*
	1000	18.4±2.19	21.2±0.84
	1500	18.75±1.71	20.5±4.80
	3000	18.5±1.89	20.75±3.70
	5000	18.67±0.58	21.3±2.52

*Data are expressed as Mean ± Standard deviation: *Means which indicated significant difference of increase in body weight between day 0 and 14*

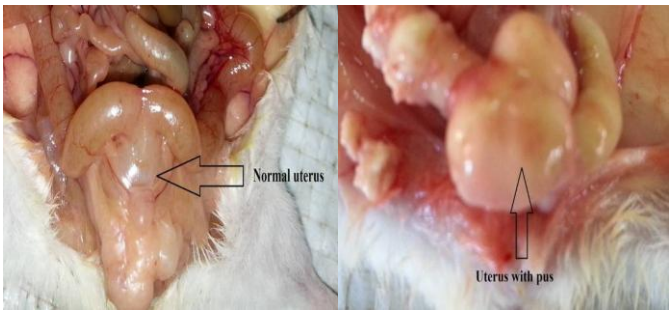

Fig. 1: Mice with normal (left) and abnormal uterus (right) in group of mice which received 5000 mg/kg of aqueous-ethanol extract

Some of effects of saponins include reduced feed intake due to the astringent and irritating taste of saponins [14], reduction in protein digestibility [15] and inhibition of active mucosal transport of nutrients [16]. Previously, annonaceae acetogenins which are cytotoxic were reported from the family Annonaceae [17]. Acetogenins are one of the most potent inhibitors of mitochondrial respiratory chain complex I, hence lowers ATP levels [18]. So, this also lowers the energy generation in organism which is essential for growth. In the present study, the mice which received test substances at the doses above 300 mg/kg bwt did not show significant increase in body weights between day zero and day 14 when compared to the dose below 300 mg/kg bwt and control groups. These differences might indicate effects caused by presence of higher concentration of growth inhibitors in the higher doses as compared to the lower doses.

3.3. Pathological examinations

On day fourteen, organs like liver, kidney, lungs, spleen and heart were examined. All mice that were examined had normal organs except at 5000 mg/kg bwt whereby one among six female mice treated with aqueous-ethanol extract had pus in the uterus (Figure 1). Previous studies reported some effects of *A. squamosa* including anti-ovulatory [19] and abortificant [20]. In this study, pus in the uterus (pyometra) of one among six mice treated with aqueous-ethanol extract may be due to the presence of compound(s) with abortive property. Since only one mouse was affected, this finding cannot be conclusive and therefore further studies are suggested.

Table 4: Effect of *A. squamosa* leaves 80% ethanolic aqueous extract and fractions on relative organ weight (ROW) in acute toxicity study

Treatment	Dose (mg/kg bwt)	Organ				
		Liver	Kidney	Lung	Heart	Spleen
1% CMC		5.59±0.07	1.26±0.06	0.46±0.08	0.53±0.04	0.4±0.04
80% ethanolic aqueous extract	100	4.91±0.52	1.47±0.23	0.54±0.04	0.55±0.08	0.47±0.06
	300	5.41±0.49	1.46±0.33	0.61±0.09	0.46±0.05	0.5±0.05
	1000	5.57±1.29	1.45±0.17	0.52±0.08	0.52±0.21	0.57±0.3
	1500	5.5±1.07	1.32±0.43	0.53±0.06	0.49±0.04	0.57±0.14
	3000	5.49±0.41	1.31±0.39	0.46±0.12	0.42±0.06	0.4±0.09
	5000	4.58±0.54	1.69±0.13*	0.58±0.1	0.51±0.07	0.43±0.15
DCM fraction	100	5.21±1.24	1.51±0.04	0.51±0.14	0.52±0.03	0.4±0.14
	300	5.78±0.59	1.15±0.18	0.59±0.05	0.44±0.03	0.5±0.1
	1000	5.57±0.67	1.31±0.04	0.55±0.07	0.52±0.07	0.51±0.12
	1500	6.21±0.43	1.52±0.04	0.65±0.06	0.49±0.05	0.54±0.06
	3000	7.13±0.37	1.37±0.24	0.56±0.06	0.49±0.02	0.51±0.06
	5000	6.54±0.77	1.68±0.11*	0.49±0.1	0.43±0.07*	0.43±0.06
P.E fraction	100	5.88±0.86	1.45±0.42	0.46±0.06	0.43±0.1	0.65±0.28
	300	4.71±0.76	1.69±0.52	0.54±0.08	0.5±0.06	0.63±0.27
	1000	4.59±0.55	1.2±0.27	0.67±0.38	0.5±0.23	0.44±0.09
	1500	4.56±1.33	1.5±0.27	0.63±0.12	0.48±0.09	0.64±0.27
	3000	4.32±1.15	1.4±0.15	0.49±0.1	0.45±0.04	0.56±0.19
	5000	4.65±0.83	1.58±0.03*	0.55±0.09	0.49±0.05	0.57±0.15

*Data are expressed as Mean ± Standard deviation: Down the table; * Means with significant difference compared to the 1%CMC (Control)*

In this study, the relative organ weight of kidney at 5000 mg/kg bwt for all extracts and of heart at 5000 mg/kg bwt (dichloromethane extract), revealed significant changes compared to that of the control group, suggesting that the kidney and the heart were the organs affected by the constituents of these extracts at the stated doses. The other organs were not significantly affected. Similar findings were reported on *A. squamosa* root extract which showed insignificant relative organ weights changes at 200 mg/kg as compared to the control [21]. Previous oral acute toxicity study indicates that seeds extract of *A. squamosa* at a dose of 2000 mg/kg were not safe to rats [22]. This study has generally indicated the toxicity of kidney was by all extracts at higher dose of 5000 mg/kg bwt and toxicity of heart was by dichloromethane fraction at the same higher dose. This may be suggesting that the toxicity of organs was exhibited at relatively higher concentration of the toxic substance(s).

4. CONCLUSION

In this study, all tested substances at a dose above 300 mg/kg bwt had negative effects on the growth of the mice. In addition, the relative organ weight of kidney at 5000 mg/kg bwt for all tested extracts and of heart for dichloromethane extract, revealed significant changes compared to that of the control group, suggesting that the kidney and the heart were the organs affected by the constituents of these extracts at this dose. The LD_{50} in this study can be classified as GHS category 4 for dichloromethane fraction and aqueous-ethanol extract and GHS category 5 for petroleum ether fraction. This means that the dichloromethane fraction and aqueous-ethanol extracts are harmful if swallowed while petroleum ether fraction may be harmful if swallowed. People should therefore be warned against using these products orally. Since only behavioral and gross organ examinations were done, further studies that will involve histological and hematological examinations are suggested

5. ACKNOWLEDGEMENT

Authors wish to acknowledge the Ministry of Health and Social welfare, Tanzania and the British Council through Development Partnership in Higher Education Program for funding this study.

6. REFERENCES

1. Gias U. M. Standardization of Herbal Preparation, The independent, 1998; pp: 13
2. Nadkarni KM and Nadkarni AK. Indian Materia Medica. Publisher: Bombay Popular Prakashan reprinted, 2000; 37-39.
3. Sirintorn P, Pinyupa P, Nisit P, Siriluk R et al. *Naresuan University Journal,* 2004; **12:**13-18.
4. Magadula JJ, Innocent E, Otieno NJ. *Journal of Medicinal Plant Research,* 2009; **3:**674-680.
5. Daniel B, Innocent E, Mbwambo ZH, Musharraf SG. *International Journal of Phama and Bio Sciences,* 2011; **2:**557-565.
6. Churchill AJ, Bentley R, Trimen H. Medicinal Plants. London Publishers, 1880; 158-196.
7. Chang FR. Chen JL, Chiu HF, Wu MJ et al. *Phytochemistry,* 1998; **47:** 1057-1061.
8. Fossen T, Larsen A, Kiremire BT, Andersen OM. Flavonoids from blue flowers of *Nymphaea caerulea*. *Phytochemistry,* 1999; **51:** 1133-1137.
9. Vijayaraghavan K, Ali MS, Maruthi R. *International Journal of Innovative Research in Science, Engineering and Technology* 2013; **2:** 2319-8753.
10. Innocent E, Kisanga AC, Gamba N, Kisinza WN, Hassanali A. *Journal of Entomology and Zoology Study*, 2014; **2:**214-219.
11. Arthur FKN, Woode E Terlabi EO, Larbie C. *Europian Journal of Experimental Biology*, 2011, **1:**115-124.
12. Globally harmonized system of classification and labeling of chemicals (GHS). United Nations, 2011.
13. Westendorf J. Natural Compounds, Chapter 40. *Department of Toxicology, University Medical School, Hamburg, Germany, Academic Press* 1999; 985-988.
14. Oleszek W, Nowacka J, Gee JM, Wortley G, Johnson IT. *Journal of the Science of Food and Agriculture*, 1994; **65**:35-39.
15. Shimoyamada M, Ikedo S, Ootsubo R, Watanabe K. *Journal of Agricultural and Food Chemistry*, 1998; **46**:4793-4797.
16. Gee JM, Price KR, Ridout CL, Johnson IT, Fenwick GR. *Toxicology In Vitro,* 1989; **3**:85-90.
17. Nkunya, MHH. *Pure Appl Chem*, 2005; **77:**1943-1955.
18. Coloma AG, Guadan˜ oa A, Ine′ sa C, Martı′nez-Dı′azb et al. *Zeitschrift fur Naturforsch*, 2002; 1028-1034.
19. Vohora SB, Kumar I, Nagvi SAH. *Plant Medica*, 1975; **28:**96-100.
20. Mishra A, Dogra JV, Singh JN, Jha OP. *Planta Medica*, 1979; **35:**283-285.
21. Dawning CR, Vijaya C, Sujith K, Sadhavi K et al. *Pharmacology online,* 2011; **2:**36-43.
22. Aneela S, Somnath de, Kanthal L, Choudhury NSK. *International journal of Research in Pharmacy and Chemistry*, 2011; **1:**820-824.