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ORIGINAL ARTICLE

Acute Oral Toxicity Studies of Methanolic Extract and Chloroform Fraction of Methanolic Extract of Seeds of *Annona muricata*

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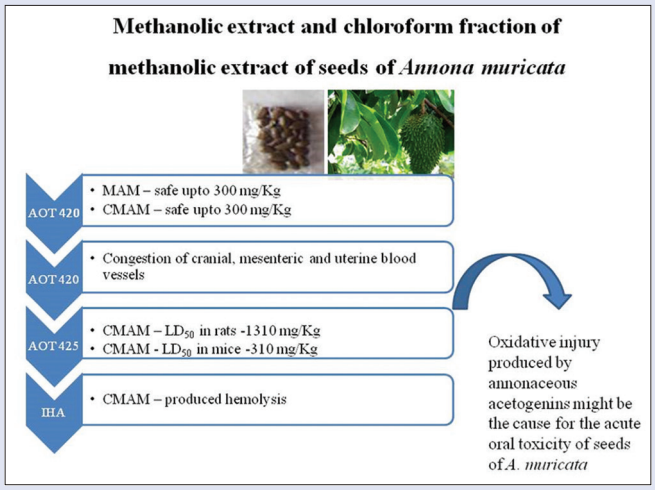
ABSTRACT

Background: Natural products are considered to be safe and efficacious. Adequate proof of the safety of these herbal drugs is lacking. **Objectives:** The present study was undertaken to evaluate the acute oral toxicity of methanolic extract of seeds of *Annona muricata* (MAM) and chloroform fraction of MAM and to derive the lethal dose 50 (LD_{50}) of chloroform fraction of methanol extract of seeds of *A. muricata* (CMAM). **Materials and Methods:** Acute toxicity studies of MAM and CMAM were conducted as per the Organisation for Economic Co-operation and Development (OECD) guidelines 420. LD_{50} of CMAM in rats and mice was estimated as per OECD guidelines 425. The toxic response was evaluated *in vitro* using hemolytic assay in rat and human erythrocytes. **Results:** The acute toxicity study done using OECD 420 guideline revealed that MAM and CMAM were safe at 300 mg/kg and produced mortality at 2000 and 5000 mg/kg. On gross examination, congestion of cranial, mesenteric, and uterine blood vessels was observed. As per OECD guidelines 425, CMAM was safe up to 980 mg/kg in rats and up to 175 mg/kg in mice and LD_{50} value was estimated to be 1310 mg/kg in rats and 310 mg/kg in mice. *In vitro* hemolytic assay showed that the plant fraction produced hemolysis. **Conclusion:** The present study concluded the toxicity produced by *A. muricata* seeds in the vascular system might be due to oxidative injury caused by the phytochemical constituents and it is essential to derive the safe pharmacological dose whenever the plant material is tested for efficacy studies.

Key words: Acute oral toxicity, *Annona muricata,* hemolysis, Organisation for Economic Co-operation and Development 420, Organisation for Economic Co-operation and Development 425

SUMMARY

- There are increased interventions of herbal extracts for many ailments, but adequate proof of the safety of these herbal drugs is lacking. Hence, the present study was designed to evaluate the acute toxicity of to evaluate the acute toxicity of MAM and CMAM as the seeds are proven to have ethnomedical and pharmacological activities. Acute toxicity studies of MAM and CMAM were conducted as per the Organisation for Economic Co-operation and Development (OECD) guidelines 420. The lethal dose 50 of CMAM in rats and mice was estimated as per OECD guidelines 425. The toxic responses were evaluated *in vitro* using hemolytic assay in rat and human erythrocytes.
- The study conducted using OECD guidelines 420 revealed that MAM and CMAM showed mortality at 5000 mg/kg and 2000 mg/kg and no mortality at 300 mg/kg. As per OECD guidelines 425, CMAM was safe up to 980 mg/kg in rats and up to 175 mg/kg in mice. According to the globally harmonized system of classification and labeling of chemicals recommended by OECD, CMAM can be assigned as category 4. As per Hodge and Sterner scale, CMAM can be considered as moderately toxic in mice and slightly toxic in rats. *In vitro* hemolytic assay also revealed that the fraction was able to induce hemolysis in normal erythrocytes which might be due to annonaceous acetogenins producing oxidative injury and associated changes. Thus, the present study concluded that it would be essential to derive a safe pharmacological dose whenever the plant material is tested for efficacy studies.



Abbreviations used: MAM: Methanolic extract of seeds of *Annona muricata*; CMAM: Chloroform fraction of methanolic extract of seeds of *Annona muricata;* Tris-HCl: Tris-hydrochloric acid; NaCl: Sodium chloride; DPBS: Dulbecco's phosphate-buffered saline; $CaCl_2$: Calcium chloride; DMSO: Dimethyl sulfoxide; AOT: Acute oral toxicity; IHA*: In vitro* hemolytic assay; OECD: Organisation for Economic Co-operation and Development; LD_{50}: Lethal dose 50; ATP: Adenosine triphosphate; ROS: Reactive oxygen species.

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INTRODUCTION

Natural products play a dominant role in the discovery of novel drugs. Investigations on plants provide evidence for the presence of phytochemicals that offer potential human health benefits. These phytochemicals have relied since they are considered to be safe and

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efficacious. And also, the efficacy of these phytochemicals has been studied at the tissue, cellular, and molecular levels. At the same time, the evaluation of toxic property of plant products is also crucial due to hazardous and adverse effects that it may cause on human beings.

Annona muricata L. (soursop and paw-paw) belonging to the family Annonaceae consists of 130 genera and 2300 species.[1] The plant and its fruit are having numerous ethnomedical values such as antiparasitic, antispasmodic, astringent, anticancer, sedative, hypotensive, insecticide, piscicide, vermifuge, and febrifuge activities.[2,3] The crushed seeds have been reported to have vermifuge and anthelmintic activities against internal and external parasites and worms.[3] Extracts from *A. muricata* have shown promising medicinal values.[4-7] However, it has been reviewed that no safety studies have been conducted to assess the efficacy of the extracts of *A. muricata* as the phytochemical constituents may vary according to the part of the plant, the extraction method, the location where the plant is grown, and the time of harvest.[8] It has been reported that the major health benefits exerted by this plant are primarily due to the presence of annonaceous acetogenins besides alkaloids, flavonoids, sterols, and other active principles.[8,9] *In vitro* analysis conducted by the authors has revealed the anticancer activity of methanolic extract of seeds of *A. muricata* (MAM) and CMAM against triple-negative breast cancer cell lines (data under publication). Hence, the present study was aimed at the evaluation of acute oral toxicity (AOT) of MAM and CMAM so that the plant extract and fraction could be further evaluated for their therapeutic efficacy.

MATERIALS AND METHODS

Plant materials

The fruits of *A. muricata* were collected from Kaliyikkavila and Palayam Market, Trivandrum, Kerala. The seeds were separated and authenticated by the Raw Material Herbarium and Museum Department of National Institute of Science Communication and Information Resources, New Delhi (NISCAIR/RHMD/Consult/2012-13/2096/103/02 dt. 13/9/2012). The voucher specimen was deposited at Fr. Gabriel Herbarium specialized in medicinal plants at Amala Ayurveda Hospital and Research Centre, Amalanagar, Thrissur.

Experimental animals

Adult female Sprague Dawley rats of 8–12 weeks of age weighing around 120 g and adult female Balb/c mice of 6–8 weeks of age weighing around 25 g were procured from Small Animal Breeding Station, Mannuthy of Kerala Veterinary and Animal Sciences University. Sprague Dawley rats and Balb/c mice were housed separately in different rooms. These animals were housed individually in polypropylene cages with stainless steel grill tops and bedding of paddy husk. The animals were acclimatized for 1 week before the start of the experiment. The animals were fed with pelleted feed from M/s Krish Scientist's Shopee, Bangalore, India, and purified water *ad libitum*. The temperature was maintained between 22 ± 2°C and 70% relative humidity with optimal air changes per hour and illumination cycle set to 12 h light and 12 h dark. The work was approved by the Institutional Animal Ethics Committee of College of Veterinary and Animal Sciences, Mannuthy, with reference number as IAEC-12-01.

Preparation of methanolic extract of seeds of *Annona muricata* and separation of CMAM

The seeds of *A. muricata* were air-dried at room temperature. It was coarsely powdered using an electrical pulverizer, and the powders obtained were extracted using the Soxhlet apparatus with methanol. The methanolic extracts were then concentrated on a rotary vacuum evaporator under reduced pressure and temperature and kept under refrigeration for the complete evaporation of the solvent. The MAM was partitioned with chloroform and water (1:1) ratio to yield chloroform fraction and water fraction. The process was repeated three times, with 500 mL of the chloroform water mixture. The chloroform solvent was reduced by air drying followed by freeze-drying.[10]

In vitro hemolytic assay of CMAM

The hemolytic effect of CMAM on rat and human erythrocytes was evaluated using washed erythrocytes. Blood samples were collected from Sprague Dawley rats (weighing about 150 g) in citrated tubes. The cells were then washed three times with 20 mM tris (hydroxymethyl) aminomethane hydrochloric acid (Tris-HCl) containing 144 mM sodium chloride (NaCl) (pH 7.4) and 2% erythrocyte suspension was prepared. Human erythrocytes were obtained from the peripheral blood (A+ve) of the first author. The blood was used within 24 h after bleeding and washed three times in nine volumes of sterile 0.85% NaCl solution. After each washing, cells were centrifuged at 150 g for 5 min and the supernatant was discarded. The final pellet was diluted to 1:9 (v/v) in sterile 0.85% NaCl solution and then in 1:24 (v/v) sterile Dulbecco's phosphate-buffered saline, pH 7.0, containing 0.5 mM boric acid and 1 mM calcium chloride ($CaCl_2$).

The hemolytic activity of CMAM was tested under *in vitro* conditions in 96-well plates. Each well was loaded with 100 µL of 0.85% NaCl solution containing 10 mM $CaCl_2$. The first well served as a negative control containing the only solvent. In the second well, 100 µL of CMAM at various concentrations of 2.5, 5, 10, 50, 100, 250, and 500 µg/mL dissolved in dimethyl sulfoxide (DMSO) was added. The last well served as a positive control containing 20 µL of 0.1% Triton X-100 in 0.85% saline. Each well was then loaded with 100 µL of a 2% suspension of rat and human erythrocytes in 0.85% saline containing 10 mM $CaCl_2$. After 30-min incubation at room temperature, cells were centrifuged and the supernatant was used to measure the absorbance of the liberated hemoglobin at 540 nm using microplate reader (Varioskan Flash, Thermo Fisher Scientific, Finland). The average value was calculated from triplicate assays.[11] All the chemicals used for the study were procured from Sigma-Aldrich, Bangalore, India.

Selection of vehicle for the oral administration of methanolic extract of seeds of *Annona muricata* and CMAM

In the present study, MAM and CMAM were mixed in various vehicles for solubility. All the vehicles used were nontoxic, as shown in Table 1.[12]

Acute toxicity study of methanolic extract of seeds of *Annona muricata*, CMAM, and estimation of oral lethal dose 50 of CMAM in rats and mice

AOT study of MAM and CMAM was performed as per the Organisation for Economic Co-operation and Development (OECD) guidelines for testing of chemicals, test no. 420, AOT – Fixed-dose procedure using seven female Sprague Dawley rats. Methanolic extract and CMAM solubilized in peanut oil were administered orally in a sequential manner in one 8-week-old female Sprague Dawley rat, each at 5000, 2000, and 300 mg/kg (limit test for sighting study), and four female rats each were dosed at 300 mg/kg in the main study for MAM and CMAM. On the day of dosing, all the rats were observed for mortality and clinical signs, such as behavioral pattern and changes in the various body systems like skin, mucous membrane, respiratory, cardiovascular, central nervous system

and somotomotor functions. The mortality and clinical signs were observed for the first 10 min, 30 min, 1 h, 2 h, 4 h, and 6 h after dosing and thereafter twice daily for mortality and once a day for clinical signs. The study period for the acute toxicity was 14 days. The body weight of the rat was recorded on day 0 (after overnight fasting), weekly thereafter, and at the termination of the study before sacrifice and subjecting the animals for complete gross pathology examination.[13]

The lethal dose 50 (LD_{50}) of CMAM was performed as per the OECD guidelines for testing of chemicals, test no. 425, AOT – Up-and-down procedure using 12 female Sprague Dawley rats and 6 female Balb/c mice. The fraction solubilized in peanut oil was administered orally to single female Sprague Dawley rat at 5000 mg/Kg (limit test dose for sighting study), and the main study was conducted in 11 female Sprague Dawley rats by dosing them sequentially at 175, 233, 310, 410, 550, 740, 980, 1310, and 1750 mg/kg. The test was stopped when the LD_{50} criterion was met. Similarly, the fraction was administered orally to a single female Balb/c mouse at 5000 mg/kg (limit test dose for sighting study), and the main study was conducted in five female Balb/c mice by dosing them sequentially at 175, 310 and 550 mg/kg. The test was stopped when the LD_{50} criterion was met.

On the day of dosing, all the animals (Sprague Dawley rats and Balb/c mice) were observed for mortality and clinical signs for the first 10 min, 30 min, 1 h, 2 h, 4 h, and 6 h after dosing and thereafter twice daily for mortality and once a day for clinical signs for 14 days. The body weight of the animal was recorded on day 0 (after overnight fasting), weekly thereafter, and at the termination of the study before sacrifice and subjecting the animals for complete gross pathology examination. LD_{50} was estimated by AOT425StatPgm (version: 1.0) statistical program.[14]

RESULTS

Preparation of methanolic extract of seeds of *Annona muricata* and CMAM

The yield of the MAM was 11.41% concerning the dry starting material. The partitioning of the methanolic extract yielded two fractions, CMAM, and aqueous fraction (WMAM). The yield of the fractions was 42.6% and 20.8%, respectively.

Table 1: Vehicles selected for oral administration of methanolic extract of seeds of *Annona muricata* and chloroform fraction of methanol extract of *Annona muricata*

Vehicle
Demineralized water
Saline (0.9 % sodium chloride solution)
Tween 20 (1%–5%)
Tween 80 (1%–5%)
1% Tween 20 + 1% Tween 80
DMSO (1%)
1% DMSO + 1% Tween 20
CMC (0.1%–1%)
Ethanol (2%)
4% Tween 20 + 2% ethanol
Polypropylene glycol (10%) Peanut oil (10 g/kg)

DMSO: Dimethyl sulfoxide; CMC: Carboxymethyl cellulose

In vitro hemolytic assay of CMAM

In vitro hemolytic assay of CMAM in rat and human erythrocytes demonstrated that the plant fraction produced concentration-dependent hemolytic activity at 2.5, 5, 10, and 50 µg/mL. The plant fraction did not show any hemolytic activity in rat and human erythrocytes at 100, 250, and 500 µg/mL. The results of the *in vitro* hemolytic assay are shown in Figure 1.

Selection of vehicle for the oral administration of methanolic extract of seeds of *Annona muricata* and CMAM

The plant extract and fraction showed different insolubility characteristics, such as clumps and oil droplets, suspending at the bottom of the beaker in different vehicles except for peanut oil. The test substances were soluble in peanut oil at a volume of 10 g/kg body weight of test animal.

Acute toxicity study of methanolic extract of seeds of *Annona muricata*, CMAM, and estimation of oral lethal dose 50 of CMAM in rats and mice

In the sighting study, the animals administered with MAM at a dose level of 5000 mg/kg and 322000 mg/kg showed mortality within 24 h of oral administration of test substance on day 0. On gross examination, it was found that all the blood vessels including brain, mesenteric, and uterine blood vessels were congested. Animals treated at a dose level of 300 mg/kg body weight survived throughout the study period and showed moderate abnormal clinical signs (diarrhea during the initial 3 h) following dosing and were to be normal during the observation period of 14 days post treatment. In the main study with 300 mg/kg, the observation was the same as in the sighting study. The results are presented in Tables 2 and 3.

The sighting study showed mortality at the dose levels of 5000 and 2000 mg/kg body weight within 18 h on day 0 after oral administration of CMAM. Congestion of cranial and mesenteric blood vessels and multifocal ecchymosal hemorrhagic lesions were also observed [Figure 2]. The sighting study at 300 mg/kg showed moderate abnormal clinical signs of diarrhea during the initial 3 h

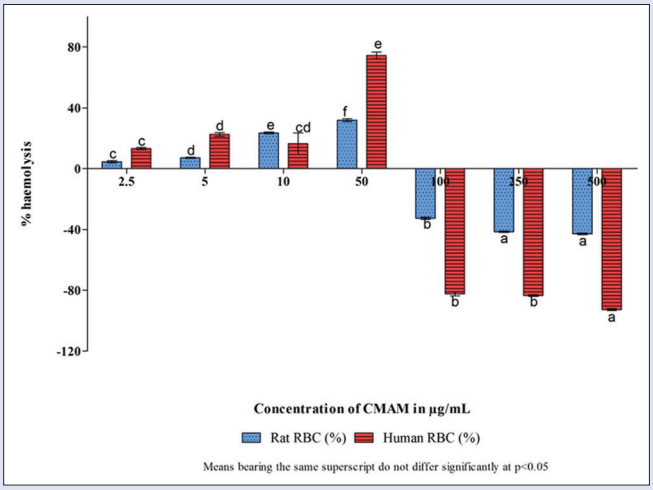

Figure 1: The hemolytic effect of chloroform fraction of methanolic extract of seeds of *Annona muricata* on rat and human red blood corpuscles. Values are expressed as mean ± standard error, $n = 3$, triplicate experiments

following dosing and was found to be normal during the observation period of 14 days post treatment at a dose level of 300 mg/kg, and the animal survived throughout the study period. The treated animal showed overall normal body weight gain during the 14-day observation period. In the main study, all the animals survived throughout the study period. The treated animals showed moderate diarrhea during the initial 3 h after oral administration of CMAM and were found to be normal during the observation period of 14 days post treatment at a dose level of 300 mg/kg body weight. The overall weight gain of the animals during the observation period was found to be normal. The gross pathological examination did not reveal any major abnormalities at 300 mg/kg. The results of mortality, clinical signs, gross pathology findings, and body weight of AOT study of CMAM are furnished in Tables 4 and 5.

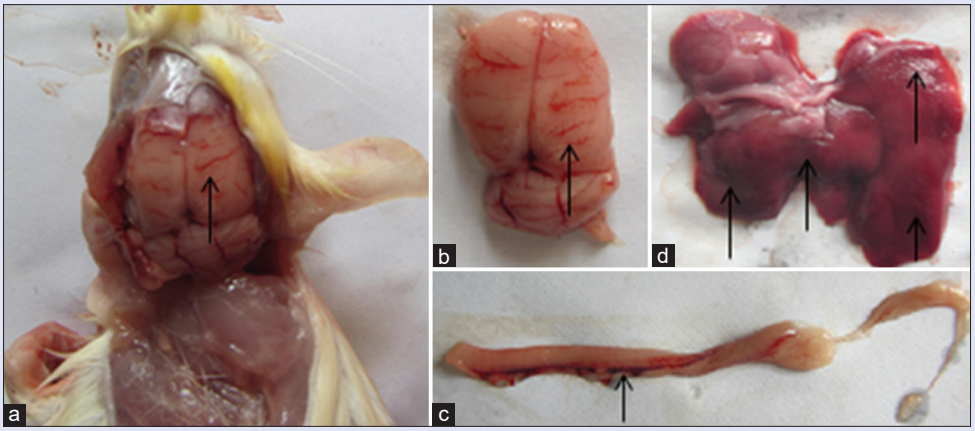

Figure 2: Vascular changes observed after the administration of CMAM at 5000 mg/kg and 2000 mg/kg. (a and b) Congestion of cranial blood vessels. (c) Congestion of mesenteric blood vessels. (d) Multifocal ecchymosal hemorrhagic lesions in liver

Table 2: Mortality, clinical signs, and gross pathology findings of acute toxicity study of methanolic extract of seeds of *Annona muricata* as per the Organisation for Economic Co-operation and Development guidelines 420

Study	Dose (mg/kg)	Sex	Mortality	Cage side observations		Gross pathology findings
				Signs	Period	
Sighting (n=1)	5000	Female	1/1	Diarrhea	Died within 24 h	Congestion of cranial and mesenteric blood vessels
Sighting (n=1)	2000	Female	1/1	Diarrhea	Died within 24 h	Congestion of cranial and mesenteric blood vessels
Sighting (n=1)	300	Female	0/1	Diarrhea	No mortality	No abnormality detected
Main (n=4)	300	Female	4/4	Diarrhea	No mortality	No abnormality detected

Table 3: Effect of methanolic extract of seeds of *Annona muricata* on body weight (g) and body weight gain (%) as per the Organisation for Economic Co-operation and Development guidelines 420

Study	Dose (mg/kg)	Sex	Body weight Day 0	Body weight Day 7	Percentage body weight gain Day 0–7	Body weight Day 14	Percentage body weight gain Day 7–14	Percentage body weight gain Day 0–14
Sighting (n=1)	5000	Female	155	-	-	-	-	-
Sighting (n=1)	2000	Female	156	-	-	-	-	-
Sighting (n=1)	300	Female	155	188	21.29	211	12.23	36.13
Main (n=4)	300	Female	160.7	192.75	19.94	214.25	11.12	33.32

Table 4: Mortality, clinical signs, and gross pathology findings of acute toxicity study of chloroform fraction of methanol extract of *Annona muricata* as per the Organisation for Economic Co-operation and Development guidelines 420

Study	Dose (mg/kg)	Sex	Mortality	Cage side observations		Gross pathology findings
				Signs	Period	
Sighting (n=1)	5000	Female	1/1	Diarrhea	Died within 18 h	Congestion of cranial and mesenteric blood vessels and multifocal ecchymosal hemorrhagic lesions
Sighting (n=1)	2000	Female	1/1	Diarrhea	Died within 18 h	Congestion of cranial and mesenteric blood vessels and multifocal ecchymosal hemorrhagic lesions
Sighting (n=1)	300	Female	0/1	Diarrhea	No mortality	No abnormality detected
Main (n=4)	300	Female	4/4	Diarrhea	No mortality	No abnormality detected

Table 5: Effect of chloroform fraction of methanol extract of *Annona muricata* on body weight (g) and body weight gain (%) as per the Organisation for Economic Co-operation and Development guidelines 420

Study	Dose (mg/kg)	Sex	Body weight Day 0	Body weight Day 7	Percentage body weight gain Day 0–7	Body weight Day 14	Percentage body weight gain Day 7–14	Percentage body weight gain Day 0–14
Sighting (n=1)	5000	Female	150	-	-	-	-	-
Sighting (n=1)	2000	Female	140	-	-	-	-	-
Sighting (n=1)	300	Female	140	170	21.43	200	17.65	42.86
Main (n=4)	300	Female	100	140	40	160	14.29	60

Rats administered with CMAM at doses of 175, 233, 310, 410, 550, 740, and 980 mg/kg did not show any mortality. These animals did not show any abnormal clinical signs except diarrhea at 550, 740, and 980 mg/kg during the initial 3 h which was later subsided. There were normal weight gain and no major gross abnormalities within these animals after the observation period of 14 days. However, doses of 1310 and 1750 mg/kg showed mortality within 18 h except for one animal dosed at 1310 mg/kg which did not show mortality [Table 6]. The LD_{50} criterion as per OECD 425 was met, and the LD_{50} value was estimated to be 1310 mg/kg.

To find the oral LD_{50} of CMAM in mice, the Balb/c mouse was administered with progressive doses of CMAM starting from 175, 310, and 550 mg/kg as per OECD 425 guidelines. Two animals survived at 175 mg/kg: one animal died out of two at 310 mg/kg and oral administration of CMAM to one animal at 550 mg/kg showed mortality meeting the LD_{50} criterion. The LD_{50} value was estimated to be 310 mg/kg. The results are presented in Table 7. The survived animals did not show any abnormal clinical signs throughout the 14-day observation period. The animals showed normal weight gain, and there were no major gross abnormalities.

Table 6: Estimation of lethal dose 50 of chloroform fraction of methanol extract of *Annona muricata* in rats as per the Organisation for Economic Co-operation and Development guidelines 425

Test sequence	Animal ID present in	Dose (mg/kg)	Short-term result	Long-term result
1	Head	175	O	O
2	Body	233	O	O
3	Tail	310	O	O
4	One forelimb	410	O	O
5	One hindlimb	550	O	O
6	Both forelimb	740	O	O
7	Both hindlimb	980	O	O
8	Dorsal	1310	X	X
9	Ventral	980	O	O
10	Forefingers	1310	O	O
11	Hindfingers	1750	X	X

X: Died; O: Survived

Table 7: Estimation of lethal dose 50 of chloroform fraction of methanol extract of *Annona muricata* in mice as per the Organisation for Economic Co-operation and Development guidelines 425

Test sequence	Animal ID	Dose (mg/kg)	Short-term result	Long-term result
1	Head	175	O	O
2	Body	310	X	X
3	Tail	175	O	O
4	Forelimb	310	O	O
5	Hindlimb	550	X	X

X: Died, O: Survived

DISCUSSION

The desire for herbal interventions is intensifying nowadays, as they are efficacious and generally regarded as safe. Nevertheless, there is inadequate proof of the safety of herbal drugs. Hence, assessment of acute toxicity is vital to identify clinical signs/morality elicited by the herbal remedy under investigation and also for identifying the range of doses that could be used in evaluating the efficacy of substances.

Various cytotoxic studies conducted with the seeds of *A. muricata* had revealed that annonaceous acetogenins possessed cytotoxic activity.[6,15,16] Hence, the extraction procedure was selected in a manner to isolate these annonaceous acetogenins. Previous studies have reported the presence of annonaceous acetogenins using the extraction procedure.[10] The authors have also reported various annonaceous acetogenins in CMAM when analyzed using liquid chromatography–mass spectroscopic analysis in CMAM (data under publication).

Erythrocytes have been used model system to study the interaction of drugs with membranes.[17] Depending on the phytoconstituents, the plants might exert a hemolytic or antihemolytic effect on rat and human erythrocytes. Therefore, many of the plants need to be evaluated for their potential hemolytic activity. Many plant extracts of *Arthrophytum schmittianum, Calotropis procera, Thymelaea hirsuta, Haloxylon scoparium, Tamarix aphylla, Daphne gnidium,* and *Morettia canescens* have been demonstrated to possess hemolytic activity at various concentrations.[17] Studies conducted in various solvent extracts of *Bridelia ferruginea* leaves had inferred that the plant extracts affected the red blood cell membrane and induced adverse effects like hemolytic anemia.[18] Thus, it could be suggested from the data that CMAM was capable of producing the adverse effect by inducing hemolysis.

One of the general mechanisms of production of hemolysis is the destruction of erythrocytes by oxidative injury. The oxidative injury can result in number of changes that may cause a decrease in the viability of erythrocytes. The generation of free radicals by oxidative injury may lead to peroxidation of the membrane lipids which may affect the deformability of erythrocytes and permeability of membrane to potassium which is potentially lethal to the affected erythrocyte.[19] The oxidative injury also impairs the metabolic machinery of the erythrocyte, resulting in a decrease in the concentration of adenosine triphosphate (ATP).[20] Damage to the membrane can also permit the leakage of denatured hemoglobin which could be toxic on its own.[21]

Annonaceous acetogenins have been reported to increment reactive oxygen species (ROS) in cancer cells.[22] However, there are no such reports of increase in ROS in normal cells. Thus, it could be inferred that the hemolysis produced in normal erythrocytes by MAM and CMAM

might be due to annonaceous acetogenins producing oxidative injury and associated changes.

In the preclinical safety assessment of potential new drugs, it is required that the material of interest must be suitably formulated in a manner that allows adequate administration of the test substance, with little or no effects in test animals that are attributable to the vehicles used in producing such a formulation. The formulation must be suitable for the intended route of administration, maintain the stability of the active ingredient, and preferably maximize the systemic bioavailability of the drug.[12] In the present study, peanut oil was found to be well tolerated at a dose of 10 g/kg body weight for MAM and CMAM.

The present toxicity investigation employed a fixed oral dose method as per OECD test guideline 420 for the preliminary AOT evaluation, which provides information on the health hazards that may arise on acute exposure and classify substances according to the globally harmonized system for the classification of chemicals.[13] In the present study, MAM and CMAM showed mortality at 5000 mg/kg and 2000 mg/kg with congestion of cranial and mesenteric blood vessels and multifocal ecchymosal hemorrhagic lesions. *In vitro* hemolytic assay also revealed that the fraction was able to induce hemolysis. Annonacin has been reported to deplete the ATP supply in rat striatal neurons and interrupted the transportation of mitochondria to the cell soma which caused the cellular perturbations of tau protein and led to similar characteristics of neurodegenerative diseases.[23] It has also been reported that the fruits of *A. muricata* with annonacin, as the major annonaceous acetogenin, showed the potential risk for neurodegeneration.[24] The results of the present study also confirm the neurotoxicity. Besides neurotoxicity, the gastrointestinal tract and liver were also affected probably by annonaceous acetogenins. The study revealed no mortality at 300 mg/kg. Although moderate abnormal clinical signs of diarrhea during the initial 3 h following dosing at 300 mg/kg were observed, it did not produce any deleterious effect on body weight gain and gross pathology. Since the acute toxicity study using 420 guideline revealed that the test substances were safe at 300 mg/kg, it became imperative to find the estimated oral LD_{50} of CMAM to derive the pharmacological safe dose as the plant fraction concentrated maximum annonaceous acetogenins. Estimated oral LD_{50} assessment was done using up-and-down procedure as per OECD test guideline 425. In the study, the LD_{50} value was estimated to be 1310 mg/kg in rats and 310 mg/kg in mice. The animals dosed at 175 mg/kg survived the 14-day observation period.

A remarkable reduction in body weight of an animal is regarded as one of the vital signs of declining health condition, while postmortem evaluation contributes substantially in ascertaining the general and target organ-related toxic manifestations of the test material under investigation.[25-27] The absence of any remarkable change in body weight gain and gross pathological lesions in the organs of the treated rats in the study suggested CMAM to be safe up to 980 mg/kg in rats and CMAM to be safe up to 175 mg/kg in mice. According to the globally harmonized system of classification and labeling of chemicals recommended by OECD, CMAM can be assigned as category 4 status (LD_{50} between 300 and 2000 mg/kg). As per Hodge and Sterner scale (according to the value), CMAM can be considered as moderately toxic in mice (LD_{50} between 50 and 500 mg/kg) and slightly toxic in rats (LD_{50} between 500 and 5000 mg/kg).

Hence, the present study clearly indicated that the maximum dose that could be used in rats for therapeutic evaluation of CMAM is 980 mg/kg while in mice is 175 mg/kg above which the fraction itself would produce mortality of the animals. While extrapolating the dose for human trials using CMAM, the study would be beneficial.

CONCLUSION

The study concluded with the findings that MAM and CMAM to be toxic. The toxicity exerted might be due to the oxidative injury produced by the phytochemical constituents. Therefore, it is essential to derive a safe pharmacological dose whenever the plant material is tested for efficacy studies. The study also recommends the subacute toxicity study with doses lower than 300 mg/kg.

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Conflicts of interest

There are no conflicts of interest.

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