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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