Original Research

Anti-Diarrhoeal Activity and Toxicity Trial of Methanolic Fruit-Pulp Extract of *Aegle Marmelos* (L.) Correa in Sprague-Dawle Rats

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Rec. Date:	Feb 15, 2018 11:34
Accept Date:	Apr 14, 2018 17:44
DOI	10.5455/ijlr.20180215113413

Abstract

The present study was designed to determine the toxicity as well as anti-diarrhoeal effect of the unripe methanolic fruit-pulp extract of Aegle marmelos in sprague drawle rat (SD rat). Qualitative Phytochemical analysis of methanolic unripe fruit-pulp extract of Aegle marmelos was done as per standard method. Extracts of methanolic unripe fruit-pulp extract of Aegle marmelos were evaluated at doses of 15mg/kg, 30mg/kg, 120mg/kg and 1600mg/kg in SD rats for anti diarrhoeal effect in castor oil induced diarrhoea. For toxicity effect, 5000 mg/kg of methanolic extract of Aegle marmelos was administered orally to three female rats at first with 0.02% tween 80 vehicles and observed for any abnormal condition up to 14days. Qualitative analyses of different phytochemicals of methanolic extract of Aegle marmelos showed that the presence of tannin and terpenoids were highly positive whereas alkaloid was moderately positive and flavonoid was slightly positive. The study revealed that the dose of 30mg/kg BW and 1600 mg/kg BW of methanolic extract of A. marmelos showed same treatment response with Loperamide against castor oil induced diarrhoea in rats. It can be concluded that methanolic extract of Aegle marmelos may be effective in reducing diarrhoea in animals.

Key words: *Aegle marmelos*, Anti-Diarrhoea, Phytochemicals, Rats

How to cite: Ghorai, S., Sarma, K., Roychoudhury, P., Das, G., Singh, D., Kalita, G., & Choudhury, J. (2018). Anti-Diarrhoeal Activity and Toxicity Trial of Methanolic Fruit-Pulp Extract of Aegle Marmelos (L.) Correa in Sprague-Dawle Rats. International Journal of Livestock Research, 8(9), 326-337. doi: 10.5455/ijlr.20180215113413

Introduction

Therapy of diarrhoea can be done by anti-secretory drugs like as bencetimide and loperamide alone or in combination with antibacterial agents (Solis *et al.*, 1993; Straw *et al.*, 2006). As a supportive therapy of diarrhoea electrolyte replacement through orally to prevent fluid loss can also be given (Straw *et al.*, 2006). Indiscriminate antibiotic treatment often associated with generation of resistant bacterium and needless to say in day one of treatment it is very difficult to establish pathogen – disease association and determine

pathogens resistance pattern to its conventional chemotherapies. Antibiotics like tetracycline, chloramphenicol, aminoglycoside, fluoroquinolones produces hepato toxicity, bone marrow depression, nephrotoxicity and G. I. disorders respectively (Rang *et al.*, 2007).

Generally people use allopathic medication to treat diseases of their animals, but there are many adverse effects related to dose, site of administration, drug interaction and anaphylactic reactions. Rang *et al.* (2007) described that loperamide, an anti-secretory drug has unwanted side effects like abdominal cramp, constipation, drowsiness, dizziness. In view of low residual contamination, low environmental pollution of natural raw materials, a increasing number of studies are focusing on phylogenic products that comprise a wide variety of herbs, spices, and essential oils (Windisch *et al.,* 2008). Alternative herbal therapy is one of the alternatives as there is less chance of toxicity, easy availability and also economical. Indian subcontinent is full of plant species having medicinal values, so people can exploit this advantage for treatment of diarrhoea. Bael, *Aegle marmelos (Linn.)* is an important medicinal plant found all over India. Leaves, fruits, stem, bark and roots of *Aegle marmelos* have been used in ethnomedicine to exploit its medicinal properties. Methanolic unripe fruit pulp extract of *Aegle marmelos* has already been explored for anti-diarrhoeal effect against castor oil induced diarrhoea in mice (Sarin and Bafina, 2012; Shoba and Thomas, 2001). Patel and Asdaq (2010) reported that methanolic extract of fruit pulp of *Aegle marmelos* was acutely non-toxic up to 0.02000 mg/ kg in Wister albino rats. In this study the toxicity as well as anti-diarrhoeal effect of the unripe methanolic fruit-pulp extract was investigated in SD rats to further substantiate its clinical use.

Materials and Methods

Plant Identification and Preparation of Extract

Collected fruits and plant parts of *Aegle marmelos* were washed and then dried under shed and herbarium was prepared according to the standard herbarium making procedure and sent to Central National Herbarium, Botanical Survey of India, Kolkata, India for identification. The fruit pulp was harvested manually by hand and seed was separated in the same way. Then fruit pulp was sliced into very small pieces *and* dried in the hot plate using 37^0C for 38-42 hrs. Then the dried up fruit pulp was mechanically grinded. The resulted powder was extracted by soxhlate extractor at 42^0C using methanol as a solvent until there was colourless dropping of methanol from siphon of the extractor followed by 2 step condensation of the obtained extracts were done. First condensation occurs in soxhlate extractor during solvent recovery phase followed by second condensation occurs in vacuum evaporator at 40^0C until there was a brown residue sticking the bottom of the jar. Now this sticky extract was kept in a dark room for 36 hrs for further solidification. Before vacuum evaporation the solvent separated extract was filtered through Whattman filter paper No.1. Then the dried extracts were dissolved in 0.02% aqueous tween 80 by vigorous vortexing

 DOI 10.5455/ijlr.20180215113413

for 90-130.02 min (Hood *et al.*, 2003). The maximum concentration achieved @ 760 mg/ml was used as stock solution for experiment.

Phytochemical Estimation of the Extract

Qualitative phytochemical analysis was done according to the method described by Edoga *et al.* (2005) and Kasolo *et al.* (2010).

Test for Tannin

0.0.02 gm of sample was boiled with 20 ml double distilled water in a water bath and filtered. To the filtrate few drops of 0.1% ferric chloride solution was added. The solution was observed for brownish green or blue black colouration, which was indicative of tannin.

Test for Flavonoid

Approximately 2 gms of plant powder was heated with 10 ml ethyl acetate in a steam bath for 3 minutes and filtered. 4 ml of the filtrate was shaken with 1 ml dilute ammonia solution. Presence of yellow coloration was indicative of flavonoid.

Test for Terpenoids

0.02 ml of extract, 2 ml of chloroform and 3 ml of sulphuric acid were added carefully to form layers. Reddish brown coloration of inter-phase of 2 layers indicated presence of terpenoids.

Test for Anthraquinones

0.0.02 gm of extract was boiled with 10 ml sulphuric acid and filtered hot. The filtrate was shaken with 0.02 ml chloroform. The chloroform layer was pipetted into another test tube and 1 ml of dilute ammonia was added. The resulting solution was observer for colour changes.

Mayer's Test for Alkaloids

To solvent evaporated extract dilute Hcl was added and filtered. To 2-3 ml of this filtrate add few drops of Dragendroff 's reagent yellowish white precipitate indicate presence of alkaloids.

Procurement and Housing of SD Rats

The adult healthy SD rats were procured for this study. The permission of Institutional Animal Ethics Committee was taken (CVSC/CAU/IAEC /13-14/R28) for this study. 39 adult Sprague Dawle rats (100-200 gm) were kept in experimental animal shed of College of Veterinary Sciences and Animal Husbandry, CAU, Selesih, Aizawl, Mizoram with standard feeding and management. Before trial all the rats were reared for 10 days for acclimatization. Feed and water were sterilized by autoclaving and additional vitamins were supplemented.

DOI 10.5455/ijlr.20180215113413

Anti-Diarrhoeal Activity Test

Castor oil induced diarrhea in rat model was used as per the method described by Shoba and Thomas (2001). Rats were divided into six groups having six rats in each group and treated with as follows -

S. No.	Group (n=6)	Treatment
1	Gr A	Castor oil @ 1ml/rat +0.02% tween 80 vehicle @1ml/rat orally
2	Gr. B	Castor oil @ 1ml/rat +loperamide @3mg/kg orally
3	Gr. C	Castor oil @ 1ml/rat +Extract of *A. marmelos* @15 mg/kg orally
4	Gr. D	Castor oil @ 1ml/rat + Extract of *A. marmelos* @30 mg/kg orally
5	Gr. E	Castor oil @ 1ml/rat + Extract of *A. marmelos* @120 mg/kg orally
6	Gr. F	Castor oil @ 1ml/rat + Extract of *A. marmelos* @1600 mg/kg orally

Rats were placed in cages and the floor of each cage was lined with blotting paper along with 18 hr feed restriction. The treatment was applied on 17th hr of fasting and castor oil was applied on 18th hr. The floor lining was changed every hour and 1hour after the above treatment, the total numbers of feces excreted by the animals and fecal consistency in all the groups were recorded in every one hour interval up to 4hours. A numeric score based on the faecal consistency was assigned as follows: dry (1), semi dry (2) and wet (3). The number of diarrheal feces and percentage of Inhibition of diarrheal feces was calculated (Mani *et al.*, 2011). Percentage inhibition was calculated as follows-

PI= Mean defecation (control-treated group) x100/ Mean defecation of negative control group.

The lowest and highest herbal dosage which was act most like similar to loperamide treatment were regarded as anti-secretory anti-diarrheal dose rate of herbal extract.

Acute Toxicity Trial

5000 mg/kg of methanolic extract was administered orally to three female rats at first with 0.02% tween 80 vehicles. Then the general signs and symptoms of toxicity, intake of food and water and mortality were recorded for a period of 14 days. The weight of each animal was taken before extract administration and then weekly once and prior to euthanasia .Then all 3 of them were euthanatized and post mortem findings were recorded. The trial was conducted following Organization for Economic Co-operation and Development (OCED) 423, 2002 guideline.

Statistical Analysis

Data was analysis as per standard procedure (Snecdecor and Cochran, 1989).

Result and Discussion

DOI 10.5455/ijlr.20180215113413

Phytochemicals

Qualitative analyses of different phytochemicals of methanolic extract of *Aegle marmelos* depicted in Table 1. Interpretation was done by negative (-), positive (+), moderately positive (++) and highly positive (+++) indicator. The analysed showed that the presence of tannin and terpenoids in methanolic extract of *Aegle marmelos* were highly positive whereas alkaloid was moderately positive (++) and flavanoid was slightly positive. On the other hand anthraquinone was not detected.

Table 1: Qualitative analysis of phytochemicals of methanolic extract of *Aegle marmelos*

Phytochemical	Qualitative values
Tannin	+++
Flavonoid	+
Terpenoids	+++
Anthraquinone	-
Alkaloid	++

Anti-Diarrhoeal Effect of Methanolic Extract of *Aegle marmelos*

Number of Wet Faeces

The number of wet faeces of different groups is shown in Table 2. The no. of wet faeces in all the groups was counted every one hour interval for 4hours after castor oil induced diarrhoea. There was no any wet faeces was observed in all the treatment groups except Gr. C where 7 no. of wet faeces were observed in 1st hour only (Table 2).

Table 2: Antidiarrhoeal properties of different doses of methanolic extract of *Aegle marmelos* in terms of number of wet faeces

S. No.	Group(n=6)	No of Wet Faeces			
		Hour 1	Hour 2	Hour 3	Hour 4
1	Gr. A	32	23	18	15
2	Gr. B	0	0	0	0
3	Gr. C	7	0	0	0
4	Gr. D	0	0	0	0
5	Gr. E	0	0	0	0
6	Gr. F	0	0	0	0

Faecal Consistency

The faecal consistency of different groups is shown Fig. 1. The faecal consistency in all the groups was counted every one hour interval for 4 hours after castor oil induced diarrhoea. It was interpreted as dry (1), semi dry (2) and wet (3). There was no any wet faeces was observed in all the treatment groups except Gr. C whereas semi dry faeces was observed in 1st hour only in comparison to other groups. In Group A, rats showed wet faeces during the whole study period (Fig. 1).

DOI 10.5455/ijlr.20180215113413

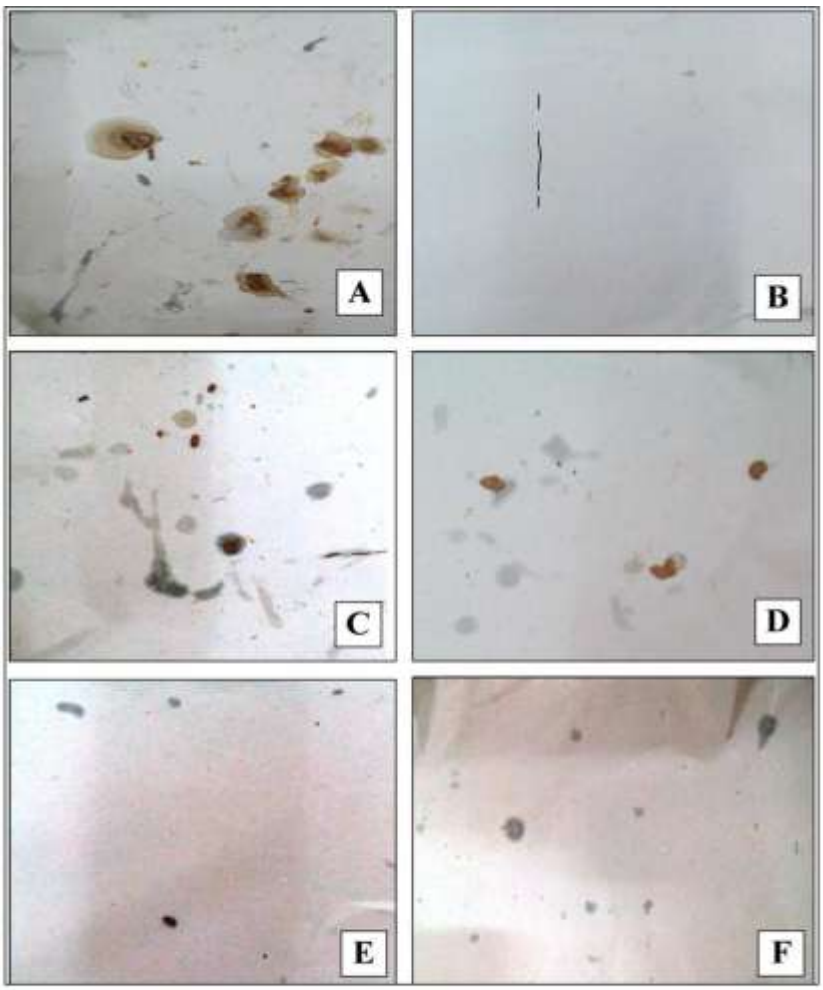

Fig. 1. Therapeutic response of different treatment on faecal consistency of castor oil induced diaarhoea in rats
A. No treatment **B**. Loperamide treatment **C.** 15mg/kg BW of methanolic extract of *A. marmelos* **D**. 30mg/ kg BW of methanolic extract of *A. marmelos* **E**. 120mg/ kg BW of methanolic extract of *A. marmelos* **F**. 1600mg/kg BW of methanolic extract of *A. marmelos*

Percentage of Inhibition of Diarrhoea

The percentage of inhibition of diarrhoea in different hours of different groups was recorded and calculated for every hour. All herbal groups except Gr. C and standard had given 100 % inhibition in all hours. In case of Gr. C the inhibition was 78.13% on 1st hour. Critical analysis revealed that the dose of 30mg/kg BW and 1600 mg/kg BW of methanolic extract of *A. marmelos* showed same treatment response with Loperamide, standard therapy against castor oil induced diarrhoea in rats.

Toxicity Trial of Methanolic Extract of *A. marmelos*

 DOI 10.5455/ijlr.20180215113413

Clinical Signs and Body Weight of Experimental Rats in Toxicity Trial

Rats under study did not manifest any clinical signs and no mortality was recorded during 15 days of observation. The weights of animals prior to trial were 100 gm each. After 1 week the weights were 112.5 gm, 111.8gm and 111.5 gm and before euthanasia the weight were 125gm, 124.8gm and 124.5 gm.

Haemato-Biochemical Profile of Experimental Rats in Toxicity Trial

Mean ±SE of haemato-biochemical profile of rats during safety study shown in Table 3. Haemato-biochemical profile in all the rats were in normal range. Critical analysis of safety study did not show any side effects of high dose of *A. marmelos* therapy during rat model study.

Table 3: Haemato-biochemical parameters of rats receiving higher doses of *A. marmelos* during safety trail

S. No.	Parameters	Values (n=3)
1	Hb(g/dl)	13.97±0.18
2	PCV (%)	46.33±1.26
3	TEC(M/mm^3)	8.43±0.34
4	TLC(m/mm^3)	6.14±1.22
5	Platelet (m/mm^3)	758.67±37.02
6	MCV(fl)	55.1±1.16
7	MCH(pg)	17.63±0.09
8	MCHC(g/dl)	34.73±0.58
9	Granulocyte (%)	9.53±1.57
10	Lymphocyte (%)	87.47±1.55
11	Monocyte (%)	3.00±0.25
12	BUN(mg/dl)	12.07±0.79
13	Creatinine (mg/dl)	0.53±0.09
14	ALT(U/L)	44.67±1.45
15	AST(U/L)	79.67±10.69

Gross Pathology of Rats in Necropsy of Experimental Rats in Toxicity Trial

On gross examination (Fig. 3), liver lobes revealed normal appearance in all the rats. There was no any gross change in spleen and kidney. The weight of the liver, spleen and kidney were 4.40±0.01gm, 0.48±0.01gm and 0.37±0.01gm respectively.

DOI 10.5455/ijlr.20180215113413

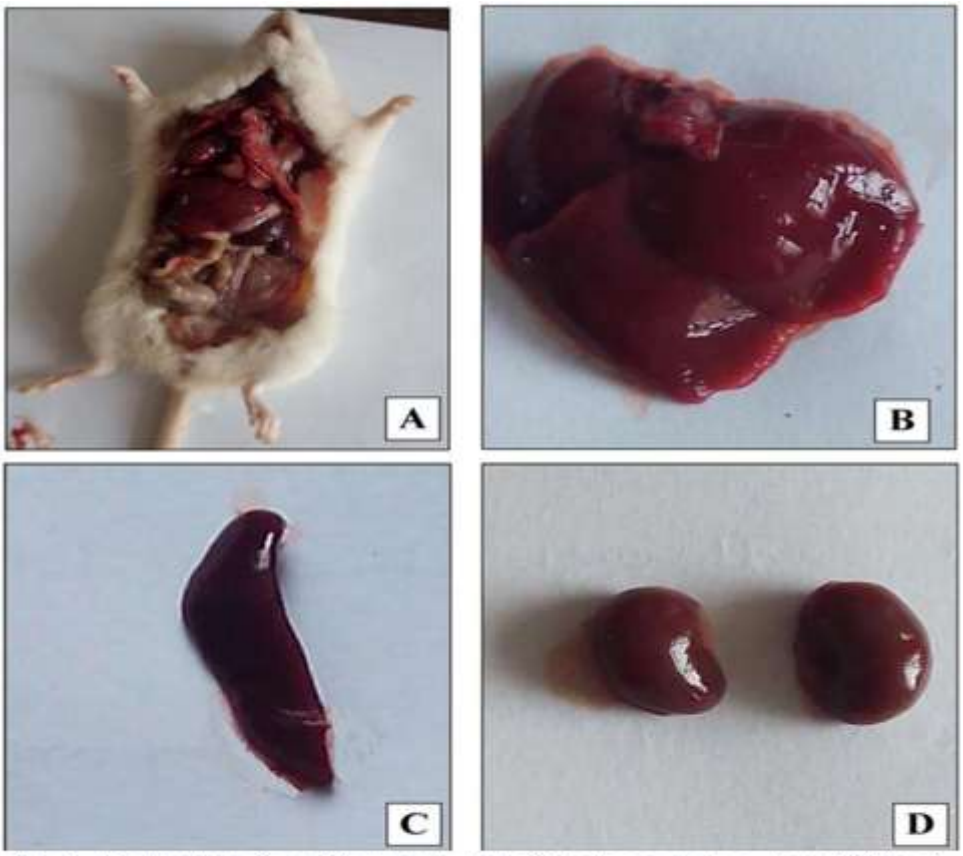

Fig. 2. Postmortem of rat after toxicity trial of Aegle marmelos extract (5000mg/ kg BW)
A. Postmortem of Rat B. No gross changes of liver
C. No gross changes of spleen D. No gross changes of kidneys

Discussion

The unripe fruit methanol extract showed the presence of tannin, terpenoids, alkaloid and flavonoid which was corroborating with the findings of Rao *et al.* (2011) and Patel and Asdaq (2010). The phytochemical screening and quantitative estimation of the percentage of crude yields the most promising secondary metabolites such as alkaloids, flavonoids, phenols, proteins, tannin and carbohydrates. They are known to show medicinal activity as well as physiological activity (Hood *et al.,* 2003). Tannin has been found to react with proline-rich protein to form irreversible complexes resulting in the inhibition of the cell protein synthesis. Fruits that have tannins as their major components are astringent in nature. They are used in treating intestinal disorders such as diarrhea and dysentery (Chrinius *et al.,* 2011; Nisha *et al.,* 2011). Tannins and flavonoids in general have been reported to have antidiarrhoeal activity through inhibition of intestinal motility and antisecretory effects (Brijesh *et al*., 2009). Steroids and terpenoids are reported to have antibacterial properties; terpenoids are known to weaken the membranous tissues, which results in dissolving cell wall of microorganisms. Alkaloids have shown to be analgesic, antispasmodic and antibacterial properties (Rao *et al.,* 2003; Okwu and Okwu, 2004).

DOI 10.5455/ijlr.20180215113413

Traditionally, people use plant(s) or plant-derived preparations considering them to be efficacious against diarrheal disorders without any scientific basis (Atta and Mounier, 2005). These experimental models were therefore employed to validate antidiarrheal efficacy of methanolic extract of *A. marmelos* unripe fruit in the current study. Diarrhoea can be described as the abnormally frequent defecation of faeces of low consistency which may be due to a disturbance in the transport of water and electrolytes in the intestines. Instead of the multiplicity of etiologies, (i) increased electrolytes secretion (secretory diarrhea), (ii) increased luminal osmolarity (osmotic diarrhea), (iii) deranged intestinal motility causing a decreased transit time, and (iv) decreased electrolytes absorption may be responsible for pathophysiology (Umer *et al.*, 2013). Recent study claims that nitric oxide in castor oil is responsible for the diarrheal effect; although it is evidenced that ricinoleic acid produces diarrhoea through a hypersecretory response which is the most active component of castor oil (Vieria *et al.*, 2000). There are several mechanisms proposed to explain the diarrheal effect of castor oil including inhibition of intestinal Na+ K+ ATPase activity, consequently reducing normal fluid absorption (Imam *et al.*, 2012), activation of adenylate cyclase or mucosal cAMP-mediated active secretion (Pinto *et al.*, 1992), and stimulation of prostaglandin formation and platelet activating factor (Mascolo *et al.*, 1994). Usually castor oil is metabolized into ricinoleic acid in the gut, which causes irritation and inflammation in the intestinal mucosa, resulting in the release of inflammatory mediators (e.g., prostaglandins and histamine). The released prostaglandins initiate vasodilatation, smooth muscle contraction, and mucus secretion in the small intestines. In our study, the overall antidiarrheal effect reveals the dose dependent activity. Methanolic extract of *A.marmelos* unripe fruit showed reduced amount of faeces in castor oil-induced rat at the doses of 30mg and 1600 mg/kg, respectively, and % inhibition of diarrhoea was 100 in both the groups. These results suggest that methanolic extract of *A.marmelos* unripe fruit contain antidiarrheal components. Also, from these results, it can be predicted that reduction of water and electrolytes secretion into the small intestine may enhance electrolyte absorption from the intestinal lumen consistent with inhibition of hypersecretion (Shah, 2004). Tannins and flavonoids in general have been reported to have antidiarrhoeal activity through inhibition of intestinal motility and antisecretory effects as in (Brijesh *et al.*, 2009). However, previous studies also have shown that flavonoids have ability to inhibit intestinal motility and water and electrolytes secretion (Carlo *et al.*, 1993). Moreover, in vivo and in vitro tests have also shown that flavonoids are able to inhibit prostaglandin E2 induced intestinal secretion and spasmogens induced contraction and also inhibit release of prostaglandins and autocoids (Dosso *et al* .,2011). So, the antidiarrheal activity of the methanolic extract of unripe fruit of *A. marmelos* could therefore be due to the presence of tannins and flavonoids. *A. marmelos* has been used for centuries in India not only for its dietary purposes but also for its various medicinal properties (Chopra, 1982). Hence, it is generally considered safe and few studies have been carried out with respect to its toxicity. Acute toxicity studies have reported that a hydroalcoholic extract of *A. marmelos* fruit is non-toxic up to a dose

 DOI 10.5455/ijlr.20180215113413

of 6 g/kg body weight in mice (Jagetia *et al.*, 2004). Pharmacological studies on animal models involving repeated doses of *A. marmelos* fruit extract over a period of up to 30 days have not reported any adverse effect up to a maximum dose of 250 mg/kg body weight (Jagetia *et al.*, 2004).There were no remarkable changes noticed in body weight, behaviour, appetite in this studies after 5000 mg/kg body wt. of the extracts of *A. marmelos* when administered orally for 14 days successively. Pathologically, nor gross abnormalities was observed, but in histopathological observation, mild changes were observed. Collectively these data demonstrate that the extracts of the unripe fruit of *A. marmelos* have a high margin of drug safety (Veerappan *et al.*, 2007).

Conclusion

In our study, methanolic extract of *A. marmelos* unripe fruit showed reduced amount of faeces in castor oil-induced rat at the doses of 30mg and 1600 mg/kg, respectively, and % inhibition of diarrhoea was 100 in both the groups. These results suggest that methanolic extract of *A. marmelos* unripe fruit contain antidiarrheal components such as tannins and flavonoids which have antidiarrhoeal activity through inhibition of intestinal motility and antisecretory effects. Acute toxicity studies have reported that methanolic extract of *A. marmelos* fruit is non-toxic up to a dose of 5 g/kg body weight in rat.

Conflict of Interest

Authors declare that there is no conflict of interest in carrying out this study.

Acknowledgments

Sincere thanks are extended to Dean, Collage of Veterinary Sciences and Animal husbandry, CAU, Selesih, Aizawl for funding assistance. Authors are also grateful to farm employees at LPM section, for their diligent support.

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