Original article:

Studies to determine antidiarrhoeal and spasmolytic activities of extract of *Aegle marmelos*. L fruit

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Abstract

Objective: To investigate and determine the antidiarrhoeal and spasmolytic potential of fruit of *Aegle marmelos* is due to CCBs compounds and not tannic acid. ***Materials and methods*:** The extract of ripe and dry fruit of *A. marmelos* (Am.Cr) was prepared in methanol: water (70:30). The antidiarrhoeal activities of the extract, loperamide and tannic acid were studied *in vivo;* in castor oil induced diarrhoeal model in mice whereas spasmolytic effect was studied *in vitro;* in isolated mice ileum. Calcium channel blocking (CCB) activity was investigated after pre-incubation of mice ileum by Am. Cr or loperamide and subsequent adding of K^+80 mM. ***Results:*** Am. Cr inhibited castor oil-induced diarrhoea: onset of diarrhoea, total no. of faeces, and total no. of wet faeces dose-dependently, the dose of 800 mg/mL was found statistically significant, total weight of feces and total weight of wet faeces were also inhibited. Concentration-dependent inhibition of spontaneous contraction and contractile effect of K^+80 mM after pre-incubation by Am. Cr were confirmed in isolated mice ileum. The results of Am. Cr are comparable with Loperamide. Tannic acid produced neither antidiarrhoeal effect nor exhibited CCB activity, however relaxant effect was observed in isolated mice ileum. ***Conclusion:*** The antidiarrhoeal and spasmolytic effects of Am.Cr may be mediated through compounds which posses CCB effect. Tannic acid exhibited relaxant effect only which is not sufficient evidence scientifically to classify as antidiarrhoeal.

Keywords: antidiarrhoeal; spasmolytic; calcium channel blocking activity; mice ileum

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Introduction

Each and every part of *Aegle marmelos*. L; is used in traditional medicine to treat wide variety of disorders [1]. The ripe and dry fruit is selected to study because the fruit is being favorably used by the people and herbal healer of this sub-continent. The literature reports the traditional use of fruit in; convalescence after diarrhoea, gastric troubles, constipation, digestive, stomachic, dysentery, ulcer, intestinal parasites, gonorrhea, epilepsy, antiviral and as brain and heart tonic [2]. In Ayurvaedic system of medicine, half and full ripe bael fruit; a common name, are advised to treat diarrhoea and constipation, the ripe fruit is thought to be more useful than raw fruit to prevent

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sub-acute and chronic dysentery [3]; unripe fruit is used as effective therapy in acute and chronic diarrhoea[4-6] additionally, root also possess antidiarrhoeal activity[7]. British Pharmacopeia[4] describes the effectiveness of bael fruit in diarrhoea and dysentery. According to Chopra[4], large quantity of mucilage which acts as demulcent that is effective in diarrhoea is present in the fruit's pulp. *A. marmelos* has been shown to be effective in experimental models of irritable bowel syndrome where diarrhoea is a prominent symptom[6-9]. The unripe fruit of *A. marmelos* being an astringent, is said to be an excellent remedy for diarrhoea, due to tannin or mucilaginous substance[10] however, literature lacks the experimental evidence of tannin as an antidiarrhoeal agent. Brijesh[11] reported limited antibacterial activity of unripe fruit. Phytochemical studies of the fruit have revealed some bioactive compounds like: marmelosin, aureptin, psoralen, luvangetin, marmelide and tannin. Psoralen and tannin are reported as antispasmodic and antidiarrhoeal respectively[12]. Therefore, experiments were designed to evaluate the antidiarrhoeal activity of the crude extract and tannic acid both *in vivo* and *in vitro* experimental model of castor oil induced diarrhoea in mice to determine the therapeutic potential as antidiarrhoeal agent.

Experimental

Plant materials

The ripe and dry Bael fruit was purchased from the indigenous market. The fruit material was recognized by Dr Bina Naqvi, Taxonomist at PCSIR Lab complex, Karachi, Pakistan. The specimen voucher (Pharm-AM-0012/2013) was preserved in the herbarium of Faculty of Pharmacy, Federal Urdu University, Gulshan Iqbal campus, Karachi, Pakistan.

Preparation of crude extract

The ripe and dry fruit was cleaned of dust particle and other adulterants by manual examination and picking. The material (2.0 Kg) was coarse grinded and soaked in mixture of methanol: water (70:30) for eight days with infrequent shaking[13]. The material was passed through double layered muslin cloth to get rid of organic debris and the fluid portion was filtered through what man grade 1 filter paper. The filtrate was subsequently concentrated to thick semi solid mass at 37ºC on a rotary evaporator (BUCHI Rotavapor R210, Switzerland) under reduced pressure and transferred to final containers to keep in refrigerator (-4ºC). The approximate yield was 4.0% this is designated as crude extract of ripe and dry fruit of *A. marmelos* (Am. Cr). Different dilutions of the crude extract or standard were freshly prepared on the day of experiment.

Animals and housing

Balb-c mice of either sex weighing (20-30 g) were purchased from the animal house facility of PCSIR Labs. The animals were kept at standard housing conditions, fed at standard diet and tap water *ad libitum.* The experiments were adhered to the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council[14]. The project was approved by the Institutional Review Board for the use of laboratory animals of Federal Urdu University of Arts, Science and Technology, Karachi campus, Karachi, Pakistan.

Chemicals and salts

Acetylcholine chloride, potassium chloride, sodium chloride, sodium bicarbonate, magnesium sulfate and loperamide were purchased from Sigma Aldrich, calcium chloride was purchased from Fluka, Glucose anhydrous, and potassium dehydrogenate phosphate and castor oil were purchased from Scharlau and Karachi Chemical Industry respectively. Tannic acid was obtained from BDH Chemicals, England.

In vivo experiments

Acute toxicity studies by oral administration

Acute toxicity study of the crude extract of ripe and dry fruit of *A. marmelos* was performed according to the method described previously[15] with some modifications. Swiss albino mice of either sex were randomly divided into six groups (Group 1 to Group 6) comprised of five animals each. Group-1 served as negative control which received normal saline (10 ml/kg) whereas other groups (Group-2, 3, 4, 5 and 6) serving as test groups[16] were given increasing doses of crude extract of *Aegle marmelos* Linn (500, 1000, 2500,5000 and 7500 mg/kg, orally) in 10 mL/kg volume by gastric intubation (No. 24) respectively and provided free access to food and water *ad libitum*. All the test animals were observed continuously for changes in behavior, physiological parameters, restlessness, and seizures for 6 hours, and then intermittently monitored for any morbidity and mortality up to 24 hours[17].

Castor oil-induced diarrhoea

Mice (20–25g) of either sex were used as described earlier[18]. Briefly, the animals were divided into five groups composed of five animals, which were

housed in individual cages. Animals were fasted for 24 h prior to experiments. Totally nine groups of mice were prepared, each group was composed of five animals. The group 1 received saline (10 mL/kg; p.o.) which dealt as the negative control. The doses of Am. Cr was selected after trial experiments, four increasing doses (100, 200, 400 and 800 mg/kg/b.w) were decided for oral administration to the animals to generate dose-response curves. Tannic acid was studied similarly at 10, 50 and 100 mg/kg/b.w. Loperamide was administered at the dose of 10 mg/kg/b.w serving as the positive control. There after one hour, each animal received 10 mL/kg of castor oil orally through a feeding cannula (No. 24). Subsequently, without delay each animal was kept in an individual cage lined with blotting paper and examined for 4 h[19]. The following parameters were observed; 1) onset of diarrhoea, 2) total no. of faeces, 3) total no. of wet faeces, 4) total weight of faeces, 5) total weight of wet faeces.

In vitro experiments

Vasorelaxant activity

The vasorelaxant activity of the Am.Cr was examined in isolated mice ileum[20]. Mice (25-30 g) was fasted for 12 hr prior to experiments. The ileum was isolated and cleaned off the connective tissue and kept in Krebs-Henseleit solution which was bubbled with carbogen (95%O_2 and 5% CO_2 mixture).The segment of 2cm length was suspended in 15mL organ bath in Krebs-Henseleit solution, bubbled with carbogen and maintained at 37°C. Composition of Krebs-Henseleit solution in mM was: KCl4.7, NaCl119.0, $MgSO_4$1.2, $KH_2PO_4$1.2, $CaCl_2$2.5, $NaHCO_3$25.0, and glucose 11.0 (pH 7.4). The rhythmic and spontaneous responses of ileum were recorded via isotonic force transducers (MLT0202) coupled through Bridge Amplifier (Model: FE221) to Power Lab data acquisition system (AD Instruments, Sydney, Australia). A preload of 1.0 g was exercised and the tissues were allowed to equilibrate for 1hr prior to the addition of test sample or drug. Under these experimental conditions, mice ileum exhibits spontaneous rhythmic contractions, allowing to test the relaxant (spasmolytic) effect directly, without adding some agonist [20]. The control responses to sub-maximal concentration of acetylcholine (0.3 µM) were recorded. Reproducible responses determined the stability of the tissues. Am.Cr, tannic acid or loperamide was added in the organ bath in ascending order to produce the concentration-dependent curve exhibiting the dose-dependent relaxant effect [21] to determine the spasmolytic effect. The normal saline, used to prepare different concentrations of the crude extract, had no effect on tissue contractility in the control experiments. The experiments were repeated using loperamide and tannic acid.

Calcium antagonist activity

Calcium antagonist activity was determined by constructing dose-dependent effect of Am.Cr, loperamide or tannic acid on the contractile response induced by K^+80 mM in mice ileum.

K^+80 mM response was studied in the absence and presence of the test samples; the procedure of Mazzolin *et al.,*[22] was adopted .The segments of ileum were pre-incubated by various concentrations of Am.Cr in ascending order (0.1, 1.0, 5.0 and 10mg/mL) for 30 minutes. Subsequently K^+80 mM was added in the organ bath. The same experiment was repeated by using loperamide (0.0001, 0.001 and 0.01µM).Tannic acid was not studied to determine the CCB activity because tannic acid did not relax the spontaneously contractile isolated mice ileum.

Statistical analysis

The data is expressed as standard error of the mean (±SEM). The median effective concentrations (EC_{50} values) were obtained by using Graph Pad Prism version 6 (http://www.graphpad.com). Data was analyzed by two-tailed *t*-test and one way ANOVA, as required, followed by the Tukey-Kramer Multiple Comparisons Test, when significant difference was present $p< 0.05$ was considered statistically significant.

Result

In vivo experiments

Acute toxicity studies

The increasing concentrations of crude extract of *Aegle marmelos* were administered orally in the animals of test groups. There was no change in the behavioural and the physiological parameters of the animals during the observation period of 24 hours. No mortality of the animal among the test groups determined the safety of the test sample at the given doses so LD_{50} dose was not calculated. The crude extract did not show any sign of toxicity at the concentrations of 500, 1000, 2500, 5000 and 7500 mg/kg body weight respectively. The collected data demonstrate that the crude extract of dried fruit of *A. marmelos* have a high margin of drug safety (Table 1).

Table-1: Showing the effect of toxicity study of crude extract of ripe and dry fruit of *Aegle marmelos* after oral administration in Swiss albino mice

S. No	Groups	No. of animals	Dose mg/kg	Animals survived	Animals dead	% Mortality	% Survival
1	Group-1	05	N/S	05	Nil	0%	100%
2	Group-2	05	500	05	Nil	0%	100%
3	Group-3	05	1000	05	Nil	0%	100%
4	Group-4	05	2500	05	Nil	0%	100%
5	Group-5	05	5000	05	Nil	0%	100%
6	Group-6	05	7500	05	Nil	0%	100%

Castor oil-induced diarrhea

Effect of Am. Cr, tannic acid and loperamide on castor oil-induced diarrhoea Am. Cr caused dose-dependent protection against castor oil-induced diarrhoea in mice. The per cent inhibition of faeces and total no. of faeces showed dose-dependent effect whereas a dose of 800mg/kg inhibited maximally. The onset of diarrhoea and total no. of wet faeces also showed dose-dependent inhibitory effect. The doses of 400 and 800 mg/kg showed statistically significant effect. The total weight of faeces was found to be increased at 200 and 400mg/kg, whereas at the dose of 800 mg/kg slight decrease was observed. The total weight of wet faeces however showed inhibitory effect at all doses. Whereas, loperamide showed marked inhibitory effect on onset of diarrhoea, on total no. of faces and no. of wet faeces and statistically insignificant inhibitory effect on weight of faeces and weight of wet faeces.

In vivo experiments tannic acid did not cause delay in the onset of diarrhoea, none of the administered dose induced statistically significant antidiarrhoeal effect in mice. Tannic acid caused increase in total no of faeces, total no of wet faeces and total weight of wet faeces. However the dose of 100 mg increased the total weight of faeces statically significant. Tannic acid showed some secretary effect in this model (Table 2).

Table-2: Effect of crude extract of fruit of *Aegel marmelos. L* (Am.Cr), Loperamide and Tannic acid on castor oil induced diarrhoea in mice

Parameters	Am.Cr (per kg/b.w)					Loperamide (perkg/b.w)
	Control	100mg	200 mg	400mg	800mg	10mg
Onset of diarrhoea	14.80 ±2.55	37.00 ± 5.27	43.40±5.59	*110.80 ± 6.90	**138.0 ±13.29	***172.6 ±45.762
Total no of faeces	12.00±1.924	13.20 ±0.735	11.80 ±0.860	*6.00 ± 0.836	**4.00 ±0.707	***1.600± 1.166
Total no of wet faeces	9.40 ± 1.860	9.20±1.020	7.20 ±1.241	4.200 ± 1.463	*3.60± 0.600	***1.000± 0.775
Total weight of faeces	0.420 ±0.124	0.418 ±0.072	0.5080 ±0.072	0.4660 ±0.108	0.4200 ±0.049	0.1420± 0.107
Total weight of wet faeces	0.34± 0.121	0.306 ±0.049	0.338 ±0.117	0.346±0.094	0.34±0.024	0.0980± 0.084

Tannic Acid (per kg/b.w)				
Parameters	**Control**	**10mg**	**50mg**	**100mg**
Onset of diarrhoea	19.6±3.558	18.8±9.993	21.2 ±16.963	19.2±3.108
Total no of faeces	11.2 ±2.746	12.0±2.000	14.0±1.924	16.2±1.393
Total no of wet faeces	9.2±1.934	7.2±1.562	8.0±1.871	12.0±1.789
Total weight of faeces	0.16 ± 0.024	0.3 ±0.055	0.28 ±0.030	*0.34±0.034
Total weight of wet faeces	0.14 ±0.024	0.22 ±0.058	0.18 ±0.018	0.22±0.037

(* P< 0.05, ** P< 0.01, *** P< 0.001)

In vitro experiments

Vasorelaxant activity

Effect of Am.Cr, tannic acid and loperamide on spontaneous contraction of isolated mice ileum

Am.Cr relaxed the spontaneously contracting isolated mice ileum in concentration-dependant manner. The doses of 0.1, 1.0, 3.0 and 10.0mg/kg were found to be statistically significant (EC_{50}; 2.040 mg/mL, 95% C.I., 1.099 to 3.786). The inhibition of the spontaneous contraction was found to be more than 65%. Whereas, loperamide inhibited the spontaneous contraction by 80%, the doses of 0.01, 0.03, 0.1, 0.3 and 1.0µM caused statistically significant inhibition (EC_{50}; 0.03804 µM, 95% C.I., 0.02000 to 0.07233). Tannic acid was examined in mice ileum. In mice ileum statistically significant relaxant effect was observed (EC_{50}0.1527 µM, 95% C.I., 0.005853 to 3.986). Tannic acid showed less spasmolytic effect (15% relaxation, Figure 1) therefore, further confirmatory studies were abandoned.

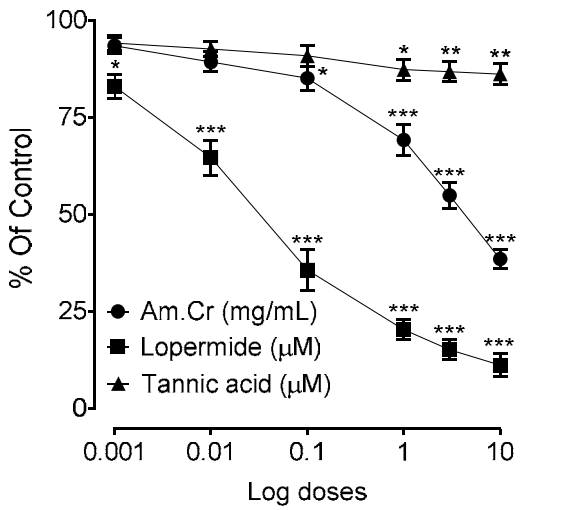

Fig.1: Effect of the crude extract of A.marmelos.L (Am.Cr), loperamide and tannic acid on the spontaneous contrcation of mice ileum (*P<0.05,**P<0.01,***P<0.001)

Calcium antagonist activity

The tissues were pre-incubated by ascending concentrations of Am.Cr which inhibited the contractile effect induced by K^+80 mM. The doses of 1.0, 5.0 and 10.0 mg/mL caused statistically significant inhibition of K^+80 mM contraction. The dose of 10.0 mg/mL caused maximum inhibition (Figure 2). Loperamide inhibited the contractile effect of K^+80 mMconcentration-dependently, 0.01 µM showed maximal and statistically significant effect (Figure 3).

Discussion

The bioactive constituents derived from many plants have led to the development of various efficacious and safe drugs for the management of several disorders [23]. Of particular interest and based on the traditional use of different parts of *A. marmelos* in diarrhoea and dysentery, antidiarrhoeal effect of the fruit of *A. marmelos* was evaluated in the present studies. Tannic acid was also studied to understand whether tannic acid; a tannin has some role to inhibit diarrhoea.

Acute toxicity studies determined the high margin of safety of fruit of *A.marmelos*. The castor-oil-induced diarrhoea model is suitable for the study of both secretory diarrhoea and intestinal motility [24]. Castor oil is known to decrease fluid absorption, increases electrolyte secretion, and produces alterations in intestinal motility [25]. Increase in mucosal secretion, water content and electrolytes which accumulate in lumen, finally moves promptly through the small and large intestines is attributed to castor oil or its active constituent [26]. The ricinoleic acid; an active component of castor oil has been demonstrated [27] to stimulate the production of chemical mediators including prostaglandins, nitric oxide, platelet activating factor and cAMP[28]. Castor oil or its active component, ricinoleic acid, intensifies peristaltic activity and induce changes in the permeability of the intestinal mucosal membrane to electrolytes and water [29]. Therefore, antidiarrhoeal agent should inhibit intestinal secretions and contractions as well. Am.Cr exhibited dose-dependent protective effect on various parameters observed during in vivo experiments. Am.Cr increased the time of onset of diarrhoea and decreased the total no. offaeces showing the significant inhibitory effect on the motility of the intestine. The inhibitory effect on the intestinal secretions was demonstrated by significant decrease in total no. of wet faeces.

The decrease in motility suggested the antispasmodic activity of *A. marmelos*. Therefore, spamsolyitc effect was further confirmed in isolated mice ileum. Am.Cr exhibited concentration-dependant spasmolytic activity in isolated mice ileum. The increase in intracellular Ca^{++} is essential to cause contraction of smooth muscle preparations that activated the contractile elements [30] of course mice ileum is no exception. The increase in intracellular Ca^{++}may be caused by the influx of Ca^{++} either, via voltage dependant calcium channels (VDCCs) or through

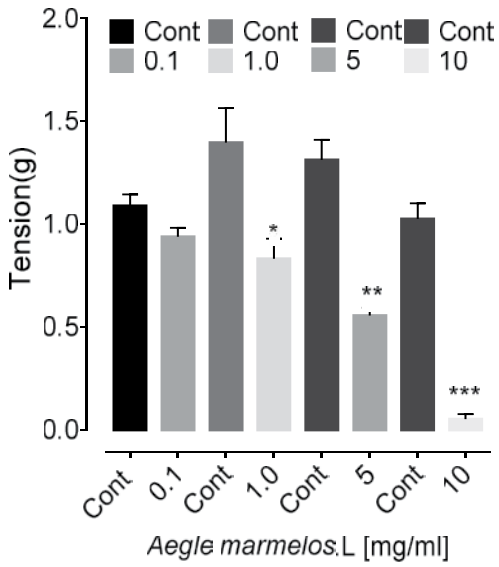

Fig.2: Contractile effect of high K^+ in the absence(cont) and presence of various concentrations of A.marmelos in isolated mice ileum(*P<0.5,**P<0.01,***P<0.00)

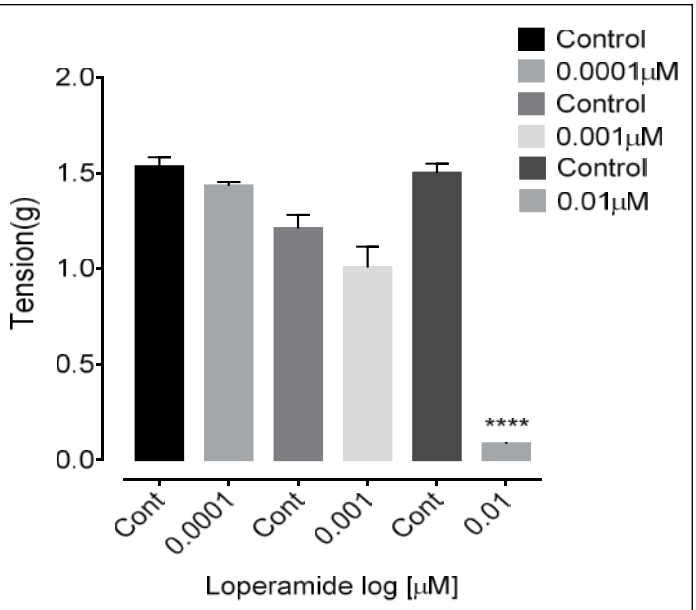

Fig.3: Contractile effect of high K^+ in the absence (cont) and presence of various concentrations of loperamide in isolated mice ileum(****P<0.0001)

the release of Ca^{++} from intracellular stores like sarcoplasmic reticulum. The spontaneous movement of intestine is regulated by periodic depolarization of smooth muscle. At the peak of depolarization rapid influx of Ca^{++} takes place via VDCCs [31].Am.Cr inhibited the spontaneous contraction of mice ileum, most probably either due to intervention with the calcium release or with the calcium influx through VDCCs.

Calcium channel blockade is reported to be the most probable mechanism for the spasmolytic effect in medicinal plants [32,33]. To confirm the involvement of $Ca^{++}$to cause the spasmolytic effect by Am.Cr, the extract was tested on the contractile effect induced by K^+ (80 mM). The ileum was pre-incubated by Am. Cr in ascending order, then K^+ (80 mM) was added respectively. The contractile effect of high K^+ was diminished concentration-dependently, which is comparable with loperamide which mediated spasmolytic activity through calcium channel blockade [34]. In smooth muscle, high K^+ opens VDCCs through which Ca^{++} influx takes place causing contractile effect. Therefore, a test sample which can inhibit high K^+-induced contraction, is considered a blocker of calcium influx [35,36]. The presence of bioactive compound(s) which possess calcium antagonist activity was confirmed in Am.Cr when the contractile effect of K^+80 mM was inhibited concentration-dependently (Figure2) which is comparable with loperamide (Figure3). Earlier there are reports showing that antidiarrhoeals share the calcium antagonist property which is found true in *A. marmelos* Linn.

Tannic acid is present in most of the plants. Several studies report the presence of tannin in the fruit of *A.marmelos*[37], tannic acid is supposed to be responsible for antidiarrhoeal effect of herbal medicines. In this study tannic acid was investigated to examine the antidiarrhoeal activity. Tannic acid did not inhibit the onset of diarrhoea and no. of faeces. In contradiction it showed some secretory effect by increasing weight and no. of faeces. Spasmolytic activity was observed lacking Ca^{++} channel blocking activity. So in this experimental model tannin may add some relaxant effect in the intestine, this may partially add to the antidiarrhoeal effect of Am.Cr.

Conclusion

The results suggest the antidiarrhoeal activity of the ripe and dry fruit of *Aegle marmelos.*L is mediated through calcium channel blockade. Tannin or tannic acid possesses some relaxant activity whereas antidiarrhoeal activity was not found. However, further studies are needed to isolate the bioactive compound(s) which is calcium antagonist in the ripe and dry fruit of *A. marmelos* L.

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