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Research Paper

In vitro antidiarrhoeal activity and toxicity profile of *Aegle marmelos* Correa ex Roxb. dried fruit pulp

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Abstract

The antidiarrhoeal effect of ethanolic extract of the dried fruit pulp of ***Aegle marmelos* Correa ex Roxb.** was studied on various intestinal pathogens. It showed excellent activity against *Shigella boydii, S. sonnei* and *S. flexneri* whereas the activity was found to be moderate against *S. dysenteriae*. The minimum inhibitory concentration against the strains of *Shigella* was recorded between 250 to 500µg/ml. Preliminary phytochemical tests of extract demonstrated the presence of common phytochemicals including phenols, tannins and flavonoids as major active constituents. Spectrophotometric study showed presence of a compound with overlapping spectrum with the marker standard Umbelliferone — a coumarin glycoside which was further confirmed by the TLC and HPTLC studies of the ethanolic extract. LD_{50} of >1250 mg/kg body weight, no change in the behaviour and physiological activity was recorded (at this dose) in the acute oral toxicity test in mice with the ethanolic extract of ***A. marmelos*** dried fruit pulp.

Keywords: *Aegle marmelos*, Antidiarrhoeal activity, Bael tree, HPTLC, Oral acute toxicity, Umbelliferone.

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Introduction

Gastrointestinal infections (GI) encompass a wide variety of symptom complexes and recognized infectious agents. Among the GI infections, diarrhoea is a common symptom of the intestinal disorder and has remained a global threat to human health. Diarrhoeal infection is a leading cause of morbidity and mortality with over 1000 million episodes and over 4 million deaths annually in children under five years of age[1]. Drug-associated diarrhoeal diseases are mainly because of disruption of normal micro flora of gut by broad spectrum antibiotics. In the present scenario, outbreak of GI infections and other diarrhoeal diseases has necessitated a search for new antidiarrhoeal substances from other sources including plants. Medicinal plants used in the traditional Indian system of medicine are known to produce a variety of compounds with acknowledged therapeutic properties[2]. The use of medicinal herbs for curing the diarrhoeal infections has been documented in all civilizations[2]. Increased side effects, high costs of new drugs, increasing microbial resistance and emerging new intestinal pathogens has led to renewed interest and focus in the medicinal plant research.

***Aegle marmelos* Correa ex Roxb.** (Family — Rutaceae), commonly known as Bael tree, is a deciduous tree, 7-8 m in height with trifoliate aromatic leaves and bisexual flowers, indigenous to India, Myanmar and Sri Lanka, often planted in the vicinity of Shiva temples[3, 4]. It grows wild all over the sub Himalayan forests, central India, its west coast and in dry hilly places. Fresh half ripe fruit is a mild astringent, and has been used to cure dysentery, diarrhoea, hepatitis, tuberculosis and dyspepsia whereas roots are reported to have anti-inflammatory and wound healing properties[5-7]. In view of above information and folklore use of roots and fruits as antidiarrhoeal and antimicrobial agents, present study was undertaken to evaluate the antidiarrhoeal activity and acute oral toxicity and to perform chemical characterization of this plant.

Fruiting branch of Bael tree

Materials and Methods

Plant material and extraction

The specimen of ***A. marmelos*** dried fruit was procured from Lallubhai Vrajalal Gandhi, Ahmedabad and authenticated from Botanical Survey of India (BSI), Pune; a herbarium specimen was prepared and deposited in the Pharmacognosy Department of R.C. Patel College of Pharmacy, Shirpur. Coarsely ground powders of fruit pulp pieces were extracted using ethanol as solvent. The ethanolic extraction was carried out by cold maceration. The extract obtained was filtered and concentrated under vacuum using rotary vacuum evaporator (Buchi, Switzerland) and stored desiccated in refrigerator until further use.

Test microorganisms and determination of antimicrobial activity

The antimicrobial activity was evaluated on the following intestinal pathogens, procured from Microbial Type Culture Collection (MTCC), Chandigarh and KLE College of Pharmacy, Belgaum, India. *Shigella flexneri* MTCC 1457, *S. sonnei* MTCC 2957, *S. dysenteriae* LMP 0208U and *S. boydii* ATCC 8700. The agar well diffusion method[8,9] was used for determining the antimicrobial activity. The culture of test organism (Optical Density 0.22 at 600 nm, approximately 10^5 CFU/ml) was prepared in isotonic saline. 0.1 ml of it was spread on Muller and Hinton agar plates. Wells were prepared using sterile 6mm diam. cork borer under aseptic conditions. Solvent blank, standard antibiotics: ciprofloxacin and tetracycline and Tablet O_2 (a combination of ofloxacin and ornidazole), respectively (at the concentrations of 0.5 mg /ml to 4.0 mg /ml) were used as standards. The plates were kept at 4ºC for 15 min for diffusion and then were incubated overnight at 37ºC. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms[9].

Determination of MIC

The micro dilution broth method[10] was used to determine the MIC. The ethanolic extract inhibiting growth of one or more of microorganisms was further tested for minimum inhibitory concentration (MIC). The dilutions were prepared using DMSO in the decreasing order of concentration of extracts or antibiotics to be tested. The least concentration of extract showing inhibition of growth was selected for preparing dilution. The wells were inoculated with 0.1 ml aliquot of test organisms having serial dilution of extract. The micro plate was incubated at 37°±1° C for 24 hours. The highest dilution of each extract corresponding to respective test organism showing no visible growth was considered as MIC (The growth was compared with positive as well as negative controls).

Chemical characterization

(i) Phytochemical analysis of the plant extract for the major phyto-constituents was undertaken using standard qualitative methods as described in literature[11,12]. The plant extract was screened for the presence of biologically active compounds like coumarin glycosides, phenolics, alkaloids, tannins, flavonoids, saponins and steroids.

(ii) The thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC)-densitometry studies[13] were performed on standard silica gel 60 F_{254} plates (Merck, Germany). Automatic sample applicator Linomat 5 (CAMAG, Switzerland) was used for the band application in HPTLC. The sample (10 mg of ethanolic extract) was prepared by refluxing it with 10 ml of methanol for 15 min. The filtrate was evaporated to dryness and the residue dissolved in the 10 ml methanol. A solvent system comprising toluene and ether (1:1) saturated with 10% acetic acid was found to give excellent resolution in our studies. The plates were developed in twin trough chamber (CAMAG, Switzerland) under saturating conditions. After drying, the plates were scanned densitometrically in a TLC scanner 3 (CAMAG, Switzerland) at 366 nm. The plates were derivatized by spraying potassium hydroxide reagent. Umbelliferone was used as a marker standard.

(iii) The spectra of the extract[14] and the standard Umbelliferone (purchased from Plantex India, Vijayawada) were obtained by a scanning double beam spectrophotometer (Shimadzu Model 2450, Japan).

The acute oral toxicity assay

The experiments on animal were performed as per the OECD guidelines 425 (OECD, 2002) with Swiss albino mice. The age, body weight, acclimatization, randomization, accommodation and environmental conditions for the animals during the period of study were as per

standard OECD guidelines. The doses were administered orally by gavage.

In the limit test, the test dose of 2000 mg/kg body weight of ***A. marmelos*** ethanolic extract produced mortality of animals and therefore, main tests were performed to determine acute oral toxicity profile with a group of 6 male and 6 female mice. A group of animals was treated similarly by the vehicle and kept as control.

After administration of the extract (550 mg/kg body weight to Group II, 1250 mg/kg body weight to Group III and vehicle to Group I), the animals were observed frequently for pre-terminal deaths and signs of toxicity on the day of administration and twice daily thereafter for next 14 days[15]. The body weight and food and water consumption were recorded for 14 days. The animals were sacrificed at the termination and subjected to complete necropsy for observing the gross pathological changes as a function of treatment.

Statistical analysis

All experiments were performed in triplicate and the mean and the standard deviation of three experiments were determined. Statistical analysis of the differences between mean values obtained for experimental group was done by analysis of variance (ANOVA).

Results and Discussion

Antimicrobial activity

The extract of fruit pulp of ***A. marmelos*** is given to patients suffering from diarrhoea/dysentery in the traditional folklore medicine in India. The antimicrobial profile of ethanolic fruit extract and its comparison with that of standard drugs against 4 strains of *Shigella* (main causative agent of the disease) was evaluated and presented in Table 1. Out of the 4 strains evaluated *S. dysenteriae* showed the minimum response to the extract. The extract was found to be more effective than the standards at lower end of the concentrations tested (0.5 mg/ml and 1.0 mg/ml). However, the zones of inhibitions were bigger with the drugs at higher concentrations (2 and 4 mg/ml) against all the strains. The MIC and MBC of extract required for different strains of *Shigella* was calculated to be in the range of 250-500 µg/ml and 400-500 µg/ml, respectively (Table 2).

Chemical characterization

The phytochemical analysis of the ethanolic extract showed presence of coumarin glycoside, Umbelliferone along with other active principles like phenolics, alkaloids, tannins, flavonoids, saponins and steroids[3, 4]. The antimicrobial activity of the plant extract could be a combined effect of these active constituents therein. In HPTLC studies, the extract showed presence of fluorescent blue bands with R_f values ranging between 0.39-0.59. Of the various mobile phases tried, the best resolution was obtained with toluene: ether (1:1, v/v) saturated with 10% acetic acid in our studies. The band at R_f 0.49 in the chromatogram of extract matched with band of standard Umbelliferone and the two showed overlapping spectra when scanned in the spectrophotometer (Fig. 1). Umbelliferone is reported to be one of the active ingredients present in the fruits of ***A. marmelos***[3, 4] and our observations seem to confirm this and give

Table 1: Profile of the antimicrobial activity of *Aegle marmelos* fruit pulp ethanolic extract and standard antidysenteric drugs

Organism	Conc. of extract/standard (mg/ml)															
	Fruit extract				Ciprofloxacin				Tetracycline				Tablet O_2			
	0.5	1	2	4	0.5	1	2	4	0.5	1	2	4	0.5	1	2	4
Shigella sonnei	13	13	15	15	0	0	16	16	0	0	10	11	0	17	21	22
Shigella flexneri	9.5	9	10	10	0	0	15	16	0	0	13	10	0	16	20	23
Shigella boydii	13	13	13	14	0	0	16	17	0	0	13	8	0	14	16	22
Shigella dysenteriae	6	6	7	7	0	0	12	11	0	0	8	9	0	10	12	21

Values in Table indicate zones of inhibition in mm including the diameter of hole (6 mm)

Table 2 : The minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of *Aegle marmelos* fruit pulp ethanolic extract

Organism	MIC (µg/ml)	MBC (µg/ml)
Shigella sonnei	250	500
Shigella flexneri	400	500
Shigella boydii	500	500
Shigella dysenteriae	250	400

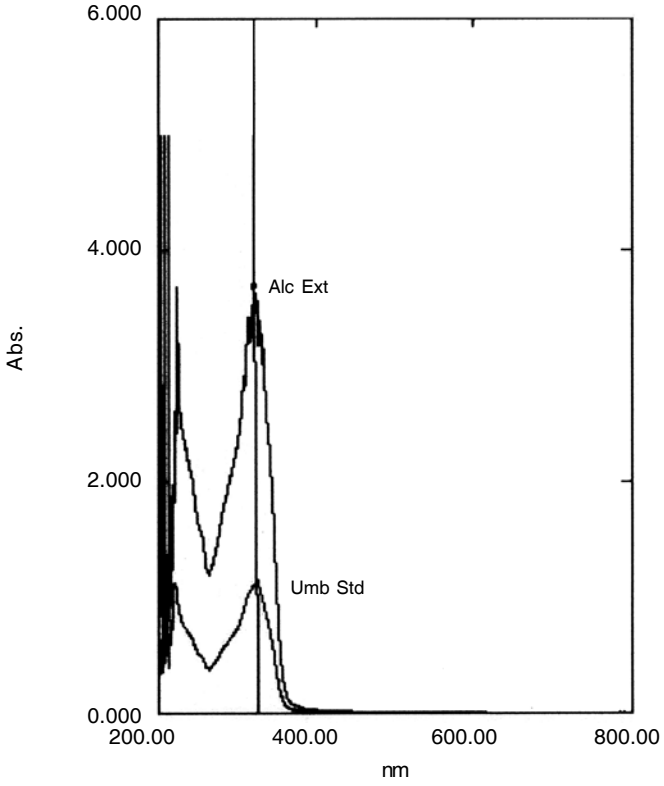

Fig.1 : Absorption spectra of *Aegle marmelos* ethanolic fruit pulp extract and standard Umbelliferone

chemical fingerprint of the ***A. marmelos***.

To rationalize the use of botanicals, the novel concept of marker is gaining momentum for evaluation of medicinal plants. The characteristic finger print together with phyto-chemical evaluation may help to establish the identity and standardize profile of this plant for use as raw material as well as in formulation for effective herbal dosage form.

Toxicological studies

The oral acute toxicity assay of ethanolic extract was conducted in order to evaluate its biosafety in pharmaceutical formulations and for designing further anti-dysenteric experiments like Charcoal motility and Castor oil induced diarrhoea tests. The acute oral toxicity studies provide information on health hazards likely to arise from the short term exposure to the test substance by this route[15]. One of the first steps in this study is to ensure systemic absorption of the test substance when fed orally.

Since mortality of experimental animals was observed at the limit dose of 2000 mg/kg body weight, for the LD_{50} study, a dose regimen of less than this limit dose value, spread logarithmically was planned and done. The results of the toxicological studies showed that administration of ethanolic extract of ***A. marmelos*** by oral route at doses up to 1250 mg/kg body weight did not produce any signs of toxicity or deaths in experimental animals (no observed adverse effect level, NOAEL). The body weight, water and food intake were not affected during 14 days observation. The animals upon the completion of 14 days, when sacrificed terminally and subjected to necropsy did not show any statistically significant differences in organ weights between the treated and control groups. The results indicate no or minimal chance of toxicity of this extract at the likely therapeutic doses in human which are lower by several orders of magnitude than NOAEL.

Conclusion

The ethanolic extract of dried fruit pulp of ***A. marmelos*** was found effective against all tested intestinal pathogens. The inhibition of microbial growth at concentrations as low as 250µg/ml indicates the potent antimicrobial activity of this extract and maximum inhibition is observed with *Shigella sonnei*. The antimicrobial activity of the extract is comparable to standard antibiotic Ciprofloxacin. The qualitative chemo profiling indicated presence of Umbelliferone which has been reported as one of the active ingredients present in the fruits of the plant. The chemical finger

printing can be helpful for standardization of the herb as raw material for commercial purpose. The oral acute toxicity study did not show any toxic symptoms, changes in behaviour or mortality at 1250 mg/kg doses. The extract seemed to have no discernable biologically significant toxic effect on the mice below LD_{50}. Thus, the study provides a stage and preliminary data on the toxicity profile of ethanolic extract of this medicinally important plant for future preclinical and clinical studies.

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