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Asian Journal of Plant Science and Research, 2011, 1 (2):57-62

ISSN : 2249 – 7412

Evaluation of the antimicrobial activity of *Punica granatum* peel against the enteric pathogens: An *invitro* study

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ABSTRACT

Punica granatum (pomegranate) is commonly used in India as a traditional medicine for the treatment of pathogenic bacteria. The present study investigates the antibacterial activity of Pomegranate rind extracts (alcoholic and aqueous) against various enteric pathogens. Both Standard strains and clinical isolates of Vibrio cholerae, Entero toxigenic E. coli, Entero pathogenic E. coli, Entero aggregative E.coli, Salmonella and Shigella species along with few strains of Candida were used in the study . Antimicrobial susceptibility testing was performed following standard procedure (Kirby-Bauer's diffusion method) by the punch well technique. The results obtained were encouraging as the ethanolic extract showed greater zones of inhibition against the various enteric pathogens tested in comparison with the aqueous extract. Most significant inhibitory effect was seen against Shigella flexneri and Aeromonas hydrophila.

Key wprds : *Punica granatum* ,peel, antimicrobial activity, enteric pathogens.

INTRODUCTION

Infections caused by enteric pathogens are an important cause of morbidity and mortality worldwide and have a major impact on public health [1] .They are clinically important bacterial pathogens that cause food poisoning and gastroenteritis in millions of people worldwide every year .The Centre for Disease Control and Prevention(CDC), Atlanta, estimates that there are nearly 4.4 million enteric food-borne bacterial infections annually in India. Enteric pathogens are thought to cause systemic disease by ordered dissemination from the intestine to local lymph nodes and then to distant organs[2]. Ingestion of these enteric pathogens could cause a variety of infections which may be confined to the gastrointestinal tract or initiated in the gut before

spreading to other parts of the body[3]. Gastrointestinal symptoms include diarrhea, dysentery, enterocolitis etc. Enteric bacteria such as *Salmonella, Shigella, Proteus, Klebsiella, E.coli, Pseudomonas, Vibrio cholerae* and *Staphylococcus aureus* are major etiologic agents of sporadic and epidemic diarrhea in both children and adults[4,5].

Diarrhea is a major public health problem in developing countries and is said to be endemic in many regions of Asia and is the leading cause of a high degree of morbidity and mortality ,contributing to the death of 3.3 to 6 million children annually [6]. However, antibiotic resistance is a major clinical cause of concern in treating infections caused by these microorganisms. Many readily available plants in India are used as traditional folklore medicine for the treatment of gastrointestinal disorders such as cholera, and dysentery. However, several of them have not been investigated from a pharmacological point of view to demonstrate their antibacterial properties, which could support their use as antidiarrhoeal remedies in traditional medicine [7]. Multi drug resistance among enteropathogens in various geographic regions present as a major threat in the control of diarrhea[8,9]. The use and misuse of antimicrobials in human medicine and animal husbandry over the past 70 years has led to a relentless rise in the number and types of microorganisms resistant to these medicines [19].The rise in antibiotic-resistant pathogens has led to the development of new therapeutic agents that are effective against these bacteria. Recently, there has been considerable interest in the use of various plant materials as an alternative medicine to treat some of the enteric infections and many compounds of plant products have been specifically targeted against resistant pathogenic bacteria [10]. Therefore the use of indigenous medicinal plants as an alternative to antibiotics are being extensively evaluated these days and are considered to play a significant role[11].

Punica granatum (pomegranate) is one of the oldest known edible fruits . It has been widely used in traditional medicine in America, Asia, Africa and Europe for the treatment of different types of diseases. In addition to its ancient historical uses, pomegranate is used in several systems of medicine for a variety of ailments [12]. In Ayurvedic medicine ,pomegranate is considered “a pharmacy unto itself ” and is used as an antiparasitic agent, a “blood tonic,” and to heal aphthae, , and ulcers [13].However, to date, very few studies have been conducted on the antimicrobial activity of *P. granatum* peels. Therefore, the present study was aimed to evaluate the antimicrobial activity of the ethanolic (EtOH) and aqueous (Aq) extract of *P. granatum* peel against various enteric pathogens in vitro .

MATERIALS AND METHODS

Plant collection

Fresh fruit peels of *Punica granatum* were collected randomly from the gardens and villages of Udupi district, Karnataka, India. The taxonomic identities of the plant were confirmed by Dr.Richard Lobo, Department of Pharmacognosy **,** Manipal College of Pharmaceutical Sciences,Manipal.The collected fruit peels were washed under running tap water, air dried, homogenized to a coarse powder and stored in air-tight bottles at 4^{o}C.

Preparation of the plant extract:
Coarse powdered Pomegranate peel was extracted by ethanol using Soxhlet apparatus [14]. Aqueous extract was prepared by steeping plant material in chloroform-water mixture in the ratio 1:99 for 5-7 days by Maceration process [15].

Bacterial strains used in the study
Bacterial strains used in the study were the isolates obtained from clinical samples at Kasturba hospital,Manipal and standard strains from National Institute of Cholera and Enteric Diseases (NICED) Kolkata, India

All the bacterial cultures, viz. *Vibrio cholerae* 01[El Tor];*Vibrio parahemolyticus*, species of *Shigella*, *Salmonella*, Enteropathogenic *Escherichia.coli*(EPEC), Enterotoxigenic *Escherichia. Coli*(ETEC) ,Enteroaggregative *Escherichia.coli* (EAEC) , *Aeromonas hydrophila*, and few *Candida* spp were used in the study. The reference strains of bacteria were maintained on nutrient agar slants (16), sub cultured regularly (every 30 days) and stored at 4°C .

Inoculum preparation:
A loopful of isolated colonies was inoculated into 4 ml peptone water and incubated at 37°C for 4 hours. The turbidity of actively growing bacterial suspension was adjusted to match the turbidity standard of 0.5 McFarland units prepared by mixing 0.5 ml of 1.75% (w/v) barium chloride dihydrate with 99.5 ml 1% (v/v) sulphuric acid. This turbidity was equivalent to approximately 1–2 × 10^8 colony-forming units per millilitre (cfu/ml). This 4-h grown suspension was used for further testing[17,18].

Determination of antibacterial activity:
Antibacterial activity was tested on Muller-Hinton Agar(MHA) plates by employing Punch well method. Varying concentrations of the pomegranate rind extracts were prepared (alcoholic and aqueous) by dissolving in Dimethyl Sulphoxide (DMSO) [20] to obtain a final concentration of 1%, 3%, 5%, 7% and 9%. against the test organisms. The test inoculums were then swabbed uniformly onto the MHA plates and wells of diameter 6mm were punched out in each plate. 50µl of each of these extracts were pipetted out into these wells, the plates incubated upright at 37°C overnight.. The sensitivity of different bacterial strains to aqueous and alcoholic extracts of the peel of *Punica granatum* was calculated by measuring the diameter (in millimeters) of inhibition zone. Readings were taken at the end of 24hrs and 48hrs. Bacteria showing a clear zone of inhibition > 8 mm were considered to be sensitive**..** Experiments were performed in triplicates for each combination of extract and the bacterial strain .

Sensitivity of bacteria to standard antibiotics
The sensitivity pattern of the reference strains of bacteria were compared with the five commonly employed antibiotics, viz. Ampicillin, Cefixime, Chloramphenicol, Co-trimoxazole, Gentamicin

RESULTS AND DISCUSSION

The results of the present study were encouraging as ethanolic peel extract showed significant antibacterial activity against many enteric pathogens tested in comparison with the aqueous

extract. The results of this study strengthens the claims of the available published data on this useful drug **.** Alcoholic extracts of *Punica granatum* appeared to be the most effective with the zone of inhibition sizes ranging from 15 to 30 mm. The different bacterial cultures responded to standard antibiotics and aqueous/alcoholic extract in a variable manner, resulting in zones of inhibition of 9–38 mm. Statistical analysis revealed that aqueous /alcoholic extracts of Punica granatum were better/equally effective to that of standerd antibiotics used in the experiment against some of the bacterial strains . Some enteric pathogens like *ETEC, EAEC, Shigella flexneri* were more susceptible to alcoholic extracts than to cefixime, chloramphenicol or co-trimoxazole. *Shigella ,Salmonella* species and *E. coli* were all inhibited to a considerable extent by the peel extracts of *Punica granatum.* Among the E.coli strains , significant activity was observed in ETEC by use of 7% and 9% of ethanolic extract. The results did not vary much in proportion when compared to all 3 strains of E.coli with the use of aqueous extract .Same was with that of Shigella strains too. Considerable significant activity of ethanolic extract was observed on *Aeromonas hydrophila*, *Shigella flexneri* (Fig 2 and 3) and *Candida albicans* and that of aqueous extract on *Aeromonas hydrophila* and *Shigella flexneri*.

Ethanolic extract had shown considerable activity against *Shigella flexneri* even with the use of 1%(Fig.1). Our study also revealed that as the increase in concentration of the extract increased the inhibitory zone size in at least 9 out of the 12 strains tested.

Antibacterial zone produced by aqueous and ethanol extracts of pomegranate peel against few medically important enteric pathogens

	Test strains	Extracts	Concentration Of Extracts (%)				
			1%	3%	5%	7%	9%
1.	*Vibrio cholerae*	Aq	10	12	15	16	16
		Et	11	14	16	18	18
2.	*Vibrio parahemolyticus*	Aq	12	14	17	17	17
		Et	10	12	12	15	15
3.	*Aeromonas hydrophila*	Aq	14	17	19	19	19
		Et	16	19	20	22	23
4.	*ETEC*	Aq	10	12	14	15	15
		Et	15	15	16	19	21
5.	*EPEC*	Aq	8	10	12	12	12
		Et	12	12	12	15	17
6.	*EAEC*	Aq	10	11	14	15	15
		Et	12	14	16	19	19
7.	*Salmonella typhimurium*	Aq	10	11	12	14	14
		Et	11	13	14	20	20
8.	*Shigella flexneri*	Aq	16	18	21	21	22
		Et	22	26	27	30	30
9.	*Shigella dysentriae*	Aq	9	11	11	13	14
		Et	10	12	13	13	14
10.	*Shigella sonnei*	Aq	10	11	11	12	12
		Et	12	13	13	16	16
11.	*Shigella boydii*	Aq	9	10	14	17	17
		Et	14	14	16	16	17
12.	*Candida albicans*	Aq	9	9	12	12	14
		Et	16	19	21	25	28

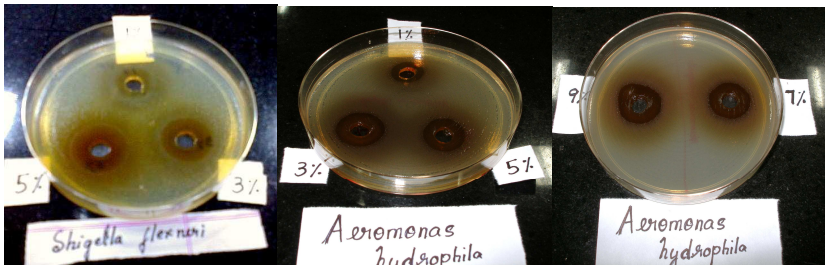

Fig 1 **Fig 2** **Fig 3**

CONCLUSION

Traditional medicinal practice has been known for centuries in many parts of the world for the treatment of various human ailments [21]. The use of antibiotics has revolutionized the treatment of various enteric bacterial infections. However, their indiscriminate use has led to an alarming increase in antibiotic resistance among microorganisms, thus necessitating the need for development of novel antimicrobials. Recent years have witnessed a renewed interest in exploring natural resources for developing such compounds [22,24]. Medicinal plants are relied upon by 80% of the world's population, and in India, the use of plants as therapeutic agents remains an important component of the traditional medicinal system. A number of plants have been documented for their biological and antimicrobial properties[23].

The aqueous extracts of pomegranate peel have shown to have antidiarrhoeal activity as tested in animal models[25]. Most of the above-mentioned microorganisms are developing resistance to commonly employed antibiotics and are a common cause of many enteric infections[26]. Thus the antimicrobial activity of the medicinal plant reported in the present study is noteworthy considering the havoc created by these enteric pathogens.

In conclusion, peel of the *Punica granatum* exhibited significant antibacterial activity. The peel of this plant have been in use for many years as decoctions or infusions prepared in water to treat various other ailments. This study thus provides a scientific basis for the use of these aqueous /alcoholic plant extracts as home-made remedies and their possible application in treating gastrointestinal disorders. Further studies may lead to their use as safe alternatives to synthetic antimicrobial drugs.

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