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ANTIMICROBIAL ACTIVITY OF RIND EXTRACTS OF *PUNICA GRANATUM* LINN.

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ABSTRACT
The present investigations evaluates the antimicrobial activity of crude extracts from the rinds of *Punica granatum* against selected gram positive, gram negative bacteria and fungal strains. In this study, we investigated the in vitro antimicrobial activity of rinds of ganesh and Kabul variety in different solvent like ethyl acetate, ethanol, methanol, water and acetone are used against the different strains. The zone of inhibition obtained was dose dependent and methanol extract of ganesh variety was found to be the most effective antibacterial agent as compared to the aqueous extract of the same variety. In case of Kabul variety the ethyl acetate extract was effective against *Bacillus*, *E.coli*, *Pseudomonas*, *Staphylococcus* and *Candida*. The Aqueous extract was also effective against *Aeromonas*, *Bacillus*, *E.coli*, *Pseudomonas*, *Staphylococcus*, *Vibrio* and *Candida*. The methanolic rind extract of *Punica granatum* ganesh variety showed high antimicrobial activity against each of the tested strains, and these values ranged from 31.75 to 1000 μg/mL and in case of Kabul variety the ethyl acetate rind extract showed activity from 62.5 to 500 μg/mL. It can be concluded that methanolic rind extract of ganesh variety has the potential to provide an effective treatment for infectious disease caused by various microorganisms.

INTRODUCTION

Nowadays researchers are increasingly turning their attention to folk medicine, looking for new discovery to develop better drugs against microbial infections caused by various pathogens (Benkeblia, 2004).The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug-resistant pathogenic strains (Bandow *et al*., 2003). It is important to discover new antimicrobial compounds with diverse chemical structures and with novel mechanisms of action for new and re-emerging infectious diseases (Rojas *et al*., 1992). The antibiotic resistance and failure of chemotherapeutics exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity (Murugaian *et al*., 2009).

According to the medicinal point of view and global environmental perspective, herb is an immeasurable wealth of nature. It plays a significant role ameliorating the disease resistant ability and combating various unfavorable metabolic activities within the living system. Plant-based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials.

The increasingly high numbers of bacteria that are developing resistance to classical antibiotics drive much of the current interest on natural antimicrobial molecules in hope that they may provide useful leads into anti-infective drugs. Several antimicrobial agents were isolated from plant including secondary metabolites such as essential oil, xanthones, benzophenones, coumarins and flavonoids (Belguith and Kthiri, 2010). These new chemical substances can also serve as templates for producing more effective drugs through semi-synthetic and total synthetic procedure. The folkloric records of many different cultures have provided information of plants with useful medicinal properties (Longtin, 2003).

The pomegranate (*Punica granatum* L.) is one of the oldest edible fruit which has a long history as a medicinal fruit and has been used extensively in the folk of many cultures (Gracious *et al*., 2001). All plant organs of the pomegranate tree have been used to ameliorate an array of common diseases (Braga *et al*., 2005). The fruit was seen by ancient Egyptians as a symbol of prosperity and ambition, and parts of the tree were used as treatment for tapeworm and other parasitic infections (Madihassan, 1984). According to ancient Chinese, the red juice was regarded as a "soul concentrate" homologous to human blood and capable of coffering on a person longevity or even immortality (Sarkhosh *et al*., 2006).

Nowadays, pomegranate is an important commercial fruit crop that is widely cultivated in parts of Asia, North Africa, the Mediterranean, and the Middle East (Sumner *et al*., 2005). In addition to the medicinal use of dried products, the fruit is consumed directly as fresh arils or juice and is used in the food industry in the manufacture of jellies, concentrates, and flavoring and coloring agents. In particular, there has been renewed global interest on the functional and nutriceutical benefits of pomegranate fruit (Dumlu and Gurkan, 2007). The phytochemicals like ellagic acid, gallic acid, punicallins, and punicalagins extracted from pomegranate revealed

antimicrobial activity when assayed against Escherichia coli, Pseudomonas aeruginosa, methicillin-resistant Staphylococcus aureus, and other harmful bacteria (Reddy *et al*., 2007). Braga *et al*. (2005) evaluated the effect of the whole pomegranate fruit methanol extract on S. aureus and subsequent enterotoxin production. They suggested that pomegranate extracts could be considered a potential antibacterial therapeutics with the additional ability to inhibit enterotoxin production. They added that the antibiotic properties of the extract are of extreme interest in light of the on-growing threat of bacterial strains developing resistance to conventional antibiotics.

Pomegranate rind is rich in polyphenols including ellagitannins , gallotannins, ellagic acids, gallic acids, catechins, anthocyanins, ferulic acids, and quercetins. These polyphenols exhibit various biological activities, such as eliminating free radicals, inhibiting oxidation and microbial growth, and decreasing the risk of cardio- and cerebrovascular diseases and some cancers. Researchers have shown that preparations containing the pomegranate rind extract can be used to prevent and/or cure atherosclerosis, diarrhea, gastric ulcer, venereal disease, and estrogen-related diseases (Reddy, *et al*., 2007). Vasconcelos *et al*., (2006) investigated the antimicrobial effect of a pomegranate phytotherapeutic gel and miconazole (Daktarin oral gel) against three standard streptococci strains, S. mutans clinically isolated and C.albicans either alone or in association. The results of the study suggested that pomegranate gel had greater efficiency in inhibiting microbial adherence than the miconazole.

The aim of the present study was to screen for the extracts of ganesh and Kabul variety of the rind of Punica granatum that could be useful for the development of new tools as antibacterial agents for the control of infectious diseases.

MATERIALS AND METHODS

Collection of plant material

The plant specimen for the proposed study was collected from local fruit market. Care was taken to select healthy fruits. It was identified as P.granatum Linn – Ganesh (yellowish red rind) and Kabul variety (reddish rind) belonging to Punicaceae family. The required fruit rind was cut and removed from the fruit. It was authenticated by Dr. P. Jayaraman, Director of National Institute of Herbal Science, Plant Anatomy Research Centre, Chennai. A voucher specimen is maintained in plant anatomy research centre, Chennai.

Preparation of the extracts

P. granatum rind was removed from the fruit and they were dried in shade and powdered mechanically. 5g of Coarse powder was weighed and extracted with 50mL of each solvent (Ethyl acetate, Ethanol, Methanol, Acetone and Water) separately and kept over night. The extract was collected after filtration using Whatman No.1 filter paper and used for phytochemical analysis. A part of extract evaporated below 40ºC was used for analysis.

Bacterial and fungal strain

Bacterial strains namely Aeromonas hydrophila (MTCC 646), Bacillus subtilis (MTCC 441), Pseudomonas aeroginosa (MTCC 1688), MRSA (ATCC 25923), Streptococcus pyogenes (MTCC 442), E coli (ATCC 25922), Vibrio fischeri (ATCC 7744), and Klebsiella pneumoniae (MTCC 432). Fungal strain namely Candida albicans (MTCC 227) were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India and American Type Culture Collection (ATCC), USA. Strains were maintained at 4ºC on nutrient agar slants. Each of the Microorganisms was freshly cultured prior to susceptibility testing by transferring them into a separate sterile test tube containing nutrient broth and incubated overnight at 37ºC. A microbial loop was used to remove a colony of each bacterium and fungus from pure culture and transfer it into nutrient broth.

Preparation of media

The growth media employed in the present study included Nutrient agar and Nutrient beef: Beef extract – 3.0 g; Peptone - 5.0 gl Agar - 15.0 g; Distilled water - 1000mL

Nutrient broth is composed of with out Agar. The medium was adjusted to pH 7.4 and sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 min.

Preparation of inoculum

Each organism was recovered for testing by sub culturing on fresh media. A loopful inoculum of each bacterium and fungus was suspended in 5mL of nutrient broth and incubated overnight at 37ºC. These overnight cultures were used for the study.

Antimicrobial activity test

The Antimicrobial susceptibility testing was done by using the Agar well diffusion method to detect the presence of anti bacterial or anti fungal activities of the samples (Jagessar *et al*., 2008). Microbial growth was determined by measuring the diameter of zone of inhibition. The diameter of inhibition zones was measured in mm and the results were recorded. Reference commercial drug used was Ciprofloxacin (200mg) purchased from Sigma Aldrich, USA. Tests were performed three times and the mean values were recorded.

Minimum inhibitory concentration (MIC) determination

The antibacterial activity of natural products was studied by employing a micro dilution method, using two different culture media: Muller-Hinton broth and Luria Bertania (LB). The inoculums were prepared as described previously. Extracts were dissolved in DMSO (10% of the final volume) and diluted with culture broth to a concentration of 2 mg/mL. Further 1:2 serial dilutions were performed by addition of culture broth to reach concentrations ranging from 2 to 0.0156 mg/mL; 100 μL of each dilution were distributed in 96-well plates, as well as a sterility control and a growth control (containing culture broth plus DMSO, without antimicrobial substance). Each test and growth control well was inoculated with 5 μL of a bacterial suspension (10^8 CFU/mL or 10^5 CFU/well). All experiments were performed in triplicate and the microdilution trays were incubated at 36ºC for 18 hr. MIC values were defined as the lowest concentration of each natural product, which completely inhibited microbial growth. The results were expressed in mg/mL (Souza *et al*., 2005).

RESULTS

The antimicrobial efficacy of *Punica granatum* rind extracts

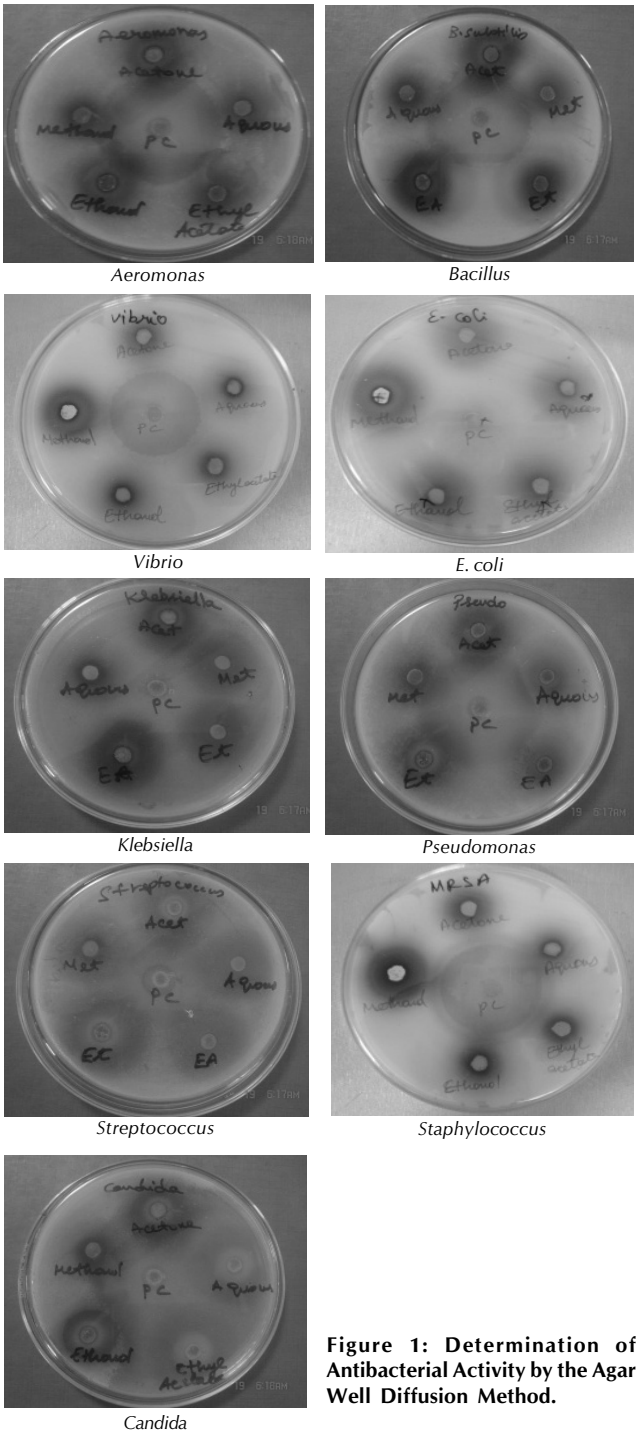

Figure 1: Determination of Antibacterial Activity by the Agar Well Diffusion Method.

against the bacterial and fungal strains was evaluated by the agar well diffusion method via determination of the surrounding zones of inhibition, as well as by evaluating the MIC using the micro dilution method (Fig. 1). Table 1 and 2 show the antimicrobial activity of *P. granatum* rind extracts of ganesh and Kabul variety as determined by the agar well diffusion method. The zone of inhibition obtained was dose dependent and methanol extract of ganesh variety was found to be the most effective antibacterial agent as compared to the aqueous extract of the same variety. The mean values of the zones of inhibition produced against the tested bacteria ranged from 14 to 22 mm, and the mean values of the zones of inhibition produced against the tested fungi was about 18 mm. In case of Kabul variety the ethyl acetate extract is effective against *Bacillus, E.coli, Pseudomonas, Staphylococcus* and *Candida*. The Aqueous extract is also effective against *Aeromonas, Bacillus, E.coli, Pseudomonas, Staphylococcus, Vibrio* and *Candida*. The mean values of the zones of inhibition produced against the tested bacteria ranged from 10 to 15 mm, and the mean values of the zones of inhibition produced against the tested fungi was about 18 mm in the case of ethyl acetate extract and 15mm in case of aqueous extract and the zone of inhibition increased in a dose-dependant manner.

Determination of MICs

The MICs of the Punica granatum rind extracts against the bacterial and fungal strains are shown in Table 3 and 4. The MICs determined using the micro dilution method confirmed the results obtained using the disc diffusion method. The methanolic extract of ganesh variety of Punica granatum showed increased antimicrobial activity against each of the tested strains, and these values ranged from 31.75 to 1000 μg/mL and in case of Kabul variety the ethyl acetate extract showed activity varying from 62.5 to 500 μg/mL for selected strains in which it shows activity.

DISCUSSION

Nowadays, a number of antibiotics have lost their effectiveness due to the development of resistant strains of bacteria, which has primarily occurred through the expression of resistance genes (Davis, 1994; Service, 1995). In addition to inducing resistance, antibiotics are sometimes associated with opposing effects such as hypersensitivity, immune suppression and allergic reactions (Ahmad *et al*., 1998). Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases (Berahou *et al*., 2007; Salomao *et al*., 2008).

Phytonutrients are plant-derived and naturally occurring compounds which possess antimicrobial activity (Srikumar *et al*., 2007). In particular the antimicrobial action of each phytonutrients is different in its intensity and specificity. In vitro antibacterial activities and antifungal activities of ethyl acetate, ethanol, methanol, aqueous and acetone extracts of rinds of ganesh and Kabul variety of Punica granatum are tabulated (Table 1 and 2). The zone of inhibition obtained was dose dependent and methanolic extract of ganesh variety was found to be the most effective antibacterial agent as compared to the aqueous extract. This may be due to the better solubility of the active components in organic solvent (Lin *et al*., 1999). These observations can be rationalized in terms of the polarity of the compounds being extracted from each solvent and, in addition to their intrinsic bioactivity, by their ability to dissolve or diffuse in the different media used in the assay. The growth media also seem to play an important role in the determination of the antibacterial activity. It was reported by Lin et al. that Muller-Hinton agar appears to be the best medium to explicate the antibacterial activity and the same was used in the present study. The methanolic extract of ganesh variety was active against Aeromonas, Bacillus subtilis,

Table 1: Antimicrobial activity of ganesh variety rind on different bacterial strains and fungal strain

S.No	Name of Organisms	Diameter of zone of inhibition*** (mm)					
		Ethyl acetateextract	Ethanolextract	Methanolextract	Aqueousextract	Acetoneextract	Ciproflaxacin
1	Aeromonas	-	-	20 ± 0.5	-	-	34 ± 0.5
2	Bacillus	10± 0.1	10± 0.1	14 ± 0.2	-	14 ± 0.1	26 ± 0.3
3	Vibrio	10±0.15	12± 0.2	18 ± 0.2	-	15 ± 0.2	32 ± 0.5
4	E.coli	-	-	-	-	-	14 ± 0.0
5	Klebsiella	-	-	18 ± 0.1	-	14 ± 0.1	24 ± 0.4
6	Pseudomonas	-	-	-	-	-	28 ± 0.4
7	Streptococcus	-	10± 0.0	20 ± 0.57	-	12 ± 0.1	24 ± 0.5
8	Staphylococcus	-	-	22 ± 0.8	-	12 ± 0.1	32 ± 0.3
9	Candida	10 ± 0.1	10± 0.1	18 ± 0.1	-	15 ± 0.1	32 ± 0.3

***Values are Mean ± SD for three replicates;'-': no zone of inhibition.

Table 2: Antimicrobial activity of kabul variety rind on different bacterial strains and fungal strain

Name of Organisms	Diameter of zone of inhibition*** (mm)					
	Ethyl acetate extract	Ethanolextract	Methanolextract	Aqueousextract	Acetoneextract	Ciproflaxacin
Aeromonas	-	-	-	10±0.0	-	22± 0.2
Bacillus	10± 0.1	-	-	10 ± 0.1	-	28 ± 0.2
Vibrio	-	-	-	12 ± 0.1	-	26 ± 0.3
E.coli	12±0.15	-	_	15 ± 0.2	-	20 ± 0.15
Klebsiella	-	-	-	-	-	18 ± 0.1
Pseudomonas	12± 0.1	-	_	15 ± 0.2	-	28 ± 0.5
Streptococcus	-	-	-	-	-	20 ± 0.3
Staphylococcus	15± 0.2	12± 0.1	12± 0.05	14± 0.15	8 ± 0.1	22 ± 0.5
Candida	18± 0.2	20± 0.1	22 ± 0.2	15±0.15	10 ± 0.1	28 ± 0.5

***Values are Mean ± SD for three replicates;'-': no zone of inhibition.

Table 3: Minimum inhibitory concentration (MIC) of methanol extract and ethanol extract of ganesh variety

Extract	MIC (μg/mL) Bacterial strains			Fungi					
	Aeromonas	Bacillus	Vibrio	E.coli	Klebsiella	MRSA	Pseudomonas	Streptococcus	Candida
Methanol	62.5	250	125	500	125	31.75	1000	62.5	125
Ethanol	1000	500	500	>1000	-	-	>1000	500	500
Ciproflaxacin(PC)	<5	2.5	1.25	2.5	>2.5	5	2.5	7.5	5

Table 4: Minimum inhibitory concentration for Ethyl acetate extract of Kabul variety

Extract	MIC (μg/mL) Bacterial strains				Fungi
	Bacillus	E.coli	Pseudo-monas	MRSA	Candida
Ethyl acetate extract	250	500	125	62.5	62.5
Ciproflaxacin (PC)	15.7	7.4	7.4	15.7	15.7

MRSA, Streptococcus pyogenes, Vibrio, and Klebsiella pneumoniae and Candida albicans whereas aqueous extract of ganesh variety was totally inactive against the studied gram positive and gram negative bacteria as well as fungi. In the present study the water was found to be least effective in extracting the active antimicrobial Component/s present in the extract of ganesh variety. The differences in the sensitivity of food associated microorganisms may be due to the differences in concentrations, methods of extraction used in each study (Kumar *et al*., 1997) and the little diffusion properties of these extracts in the agar and soil composition and water availability (Romero *et al*., 2005). In case of Kabul variety the ethyl acetate and aqueous extract are active against gram positive, gram negative bacteria and fungi. This is in agreement with previous reports that plant extracts are more active against gram-positive bacteria than gram-negative bacteria (Rabe and Staden, 1997). B. subtilis was the most susceptible gram-positive bacteria and P. aeruginosa was the most resistant gram-negative bacterial strain. The mechanisms behind the antibacterial activity are complex to understand and could be attributed to either inhibiting the cell division or to damaging the cell walls of bacteria; which should be investigated in detail in future.

The antibacterial activity of the extracts may be due to the presence of several metabolic toxins or broad-spectrum antibiotics. Several metabolites from herb species, including alkaloids, tannins and sterols, have previously been associated with antimicrobial activity (Leven *et al.,* 1979) .The site and the number of hydroxyl groups on the phenol components may increase the toxicity against the microorganisms. Cowan (1999) suggested that the antimicrobial properties of tannins might be related to their ability to inactivate microbial adhesions, enzymes, and cell envelope transport proteins, their complexity with polysaccharides, and their ability to modify the morphology of microorganisms.

In recent years the treatment for several infections has become very difficult because of multi resistant bacterial strains have increased dramatically which might reduce the therapeutic options. Present study reveals the fact that the therapeutic action of the plant may not be due to a single compound but due to synergistic action of a number of compounds. This synergistic impact could counteract the resistance of bacteria which are hard to be killed by a single antibiotic, and may hold the key to cure the health disorders due to microbial infections.

The results of present study supports the traditional usage of

plant and Punica granatum extracts which possess compounds with antibacterial properties that can be used as antibacterial agents in new drugs for the therapy of infectious diseases caused by pathogens. The most active methanolic rind extract of ganesh variety can be subjected to isolation of the therapeutic antibacterials and further work may be carried out for pharmacological evaluation.

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