



Original Research Article

In vitro anti-microbial activity and phytochemical analysis of selected fruit wastes

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A B S T R A C T

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disc diffusion method.

The medicinal plants are reservoir of potential phytochemical compounds that could be useful as an alternative to antibiotics and synthetic chemicals which causes serious side effect. In the present study, *Punica granatum* peel and *Vitis vinifera* seeds, the waste material of the fruits were extracted with ethanol as solvent. The ethanolic extract of both the fruit materials were analyzed for its antimicrobial potential and tested for presence of phytochemicals. Qualitative phytochemicals analysis showed presence of potent bioactive constituents such as Polyphenols, Terpenoids, Flavonoids, tannins, alkaloids, cardiac glycosides, carbohydrates and phytosterols were present in the selected extracts. The two selected extracts were evaluated for *in vitro* antimicrobial activity against bacterial strains *Staphylococcus aureus*, *Bacillus circulans*, *Klebsiella pneumonia*, *Vibrio vulnificus* and *Salmonella typhi*. The fungal strains selected for the studies were *Candida albicans*, *Cryptococcus neoformans* and *Candida tropicalis*. The antimicrobial activity was determined using agar plate well diffusion method and minimum inhibitory concentration was also evaluated. Both the extracts showed significant antimicrobial activity against the tested organisms. However, *P. granatum* peel extracts showed higher antimicrobial potential than *V. vinifera* seed extract. The minimum inhibitory concentration study was done using micro dilution technique and *Staphylococcus aureus* growth was completely inhibited at lesser concentration by both the extracts selected for this study.

Introduction

Medicinal plants play an important role in the human health care and are one of the oldest forms of health care known to mankind. In India, over 2600 plant species has been considered useful in the traditional system of medicines like

Ayurveda, Unani and Siddha (Khandelwal, 1999). A country like India is much suited for development of drugs from plant origin. Many naturally occurring compounds found in dietary and medicinal plants and fruits have been

shown to possess antimicrobial activities (Kouassi and Shelef, 1998; Larson *et al.*, 1996). Antimicrobials of plant origin have enormous therapeutic potential and have been used from ancient times. They have been shown to be good alternatives to synthetic chemical antimicrobial agents and antibiotics because of the serious side effects (Pawar and Nabar, 2010; Olila *et al.*, 2001). The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug-resistant pathogenic strains (Bandow *et al.*, 2003). Several antimicrobial agents were isolated from plants including secondary metabolites such as essential oil, xanthenes, benzophenones, coumarins and flavonoids (Belguith and Kthiri, 2010)

Pomegranate tree, *Punica granatum* L., is a native shrub of Asia and Mediterranean Europe that has a rich history of traditional use in medicine. For centuries, the barks, leaves, flowers, fruits, and seeds of this plant have been used to ameliorate diseases (Jayaprakasha *et al.*, 2006). It is widely reported that pomegranate exhibits antiviral, antioxidant, anticancer, and antiproliferative activities (Faria *et al.*, 2007; Adhami and Mukhtar, 2006). Pomegranate peels are exploited in traditional medicine because of their strong astringency, making them a popular remedy throughout the world. In the form of an aqueous decoction (i.e., boiling the hulls in water for 10-40 minutes), it was used for dysentery and diarrhea, and also for stomatitis it can be drunk, used as a mouthwash, douche or enema (Lansky and Shubert, 2004).

Grapes (*Vitis Vinifera*), is cultivated today in all temperature regions of the world (Gruenwald *et al.*, 2004). The grape seed extract has been reported to possess a broad spectrum of pharmacological and therapeutic effects such as antioxidative, anti-inflammatory and antimicrobial

activities, as well as having cardioprotective, hepatoprotective, and neuroprotective effects. The seeds of the grape are used in herbal medicine and as a dietary supplement (Shenoy *et al.*, 2007). GSE is considered as a powerful antioxidant nutritive supplement that prevents premature ageing and diseases (Chedea *et al.*, 2010).

Materials and methods

Collection of plant material

The plant specimens for the proposed study were collected from Koyambedu market, Chennai, India of same cultivar. The fruits *Punica granatum* belonging to Lythraceae family and *Vitis vinifera* belonging to Vitaceae family were selected for this study. Care was taken to select healthy fruits. The *Punica granatum* peel and *Vitis vinifera* seeds were removed, washed and used for the studies. The plant materials (*P.granatum* - PRAC/2009/360 and *V. vinifera*-PRAC/2009/361) selected for the studies were authenticated by Dr. P. Jayaraman, Director of National Institute of Herbal Science, Plant Anatomy Research Centre, Chennai.

Preparation of the extracts

P.granatum peel and *V.vinifera* seeds were removed from the fruits and washed thoroughly using sterilized water. The selected plant materials were shade dried and pulverized to fine powder in a mechanical grinder individually. 100g of Course powder were weighed and extracted with 1 Litre of Ethanol as solvent. These extract were collected after filtration using Whatman No.1 filter paper. The solvent were concentrated under reduced pressure in a rotary evaporator until solvents are completely evaporated

from the extract. This obtained alcoholic extracts were used for further studies.

Phytochemical analysis

Qualitative phytochemical analysis was performed to find out the presence of potent phytochemicals using the procedures of (Kokate *et al.*, 1995; Harborne, 1998; Trease and Evans, 1989; Sofowora, 1993).

Determination of antimicrobial activity by disc diffusion assay

Bacterial and fungal strain

Bacterial strains *Staphylococcus aureus* (MTCC 1144), *Bacillus circulans* (MTCC 7635), *Klebsiella pneumonia* (MTCC 3040) and *Vibrio vulnificus* (ATCC 33817) were selected along with fungal strain *Candida albicans* (MTCC 227) and *Candida tropicalis* (MTCC 184). All the above selected strains were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India and American Type Culture Collection (ATCC), USA. Fungal strains *Cryptococcus neoformans*, and bacterial strain *Salmonella typhi* were clinical isolates obtained from Saveetha Medical College, Thandalam, Tamilnadu, India. Strains were maintained at 4°C 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 37°C. 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 inoculums

The organisms selected for studies were

recovered and sub cultured on fresh media. A loopful inoculum of each bacterium and fungus was suspended in 5ml of nutrient broth and incubated overnight at 37°C. These overnight cultures were used for the study. Composition of nutrient broth were Beef extract -3.0 g, Peptone -5.0 g, Distilled water-1000 ml. The medium was adjusted to pH 7.4 and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min.

The disc diffusion method (Bauer AW *et al.*, 1966) was used to screen the antimicrobial activity. *In-vitro* antibacterial assay was done using Muller Hinton Agar (MHA, pH 7.3 ± 0.1) for anti-bacterial and Potato dextrose agar for anti-fungal activity. The Muller Hinton Agar and Potato dextrose agar were prepared and autoclaved at 121°C for 20min. The plates were prepared by pouring 15ml of molten media into sterile petri plates.

The plates were allowed to solidify for 10 minutes. 0.1% standardized inoculums suspension was swabbed uniformly, and the inoculums were allowed to dry for 5 minutes. Sterile HiMedia paper disc (6mm) were soaked in 20µl of the extract with different concentration diluted in 25% DMSO and dried at 37°C overnight. The loaded disc was placed on the surface of medium, the compound was allowed to diffuse for 5 minutes, and the plates were kept for incubation at 37°C for 24hrs.

Antibiotic disc containing Chloramphenicol (30µg) was used as controls. A disc loaded with 25% DMSO alone served as the negative control. The antibacterial activity was evaluated by measuring the diameter of the inhibition zone formed around the discs. In antifungal activity determination the loaded disks were placed in the surface of

medium and the plates were kept for incubation at 28^oc for 72 hours. Antibiotic disc containing Amphotericin B was used as controls. The antifungal activity was evaluated by measuring the diameter of the inhibition zone formed around the discs. These studies were performed in triplicate.

Minimum inhibitory concentration (MIC) determination

The antimicrobial 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 in above. 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 0.01575 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 (10⁸ CFU/ml or 10⁵ CFU/well). All experiments were performed in triplicate and the micro dilution trays were incubated at 36°C for 18 hours. MIC values were defined as the lowest concentration of each natural product, which completely inhibited microbial growth. The results were expressed in milligrams per milliliters (Souza *et al.*, 2005).

Results and discussion

Qualitative phytochemical analysis

The result of qualitative phytochemical analysis of the selected fruit extracts revealed the presence of various potent phytochemical such as flavonoids, tannins, alkaloids, cardiac glycosides, carbohydrates, terpenoids, chalcones, phlobatannins and phytosterols were present in both the extract whereas saponins was present only in *V.vinifera* seed extract and steroids in *P.granatum peel* extract (Table 1). Flavonoids and tannins were abundant in both the selected extract.

The antimicrobial efficacy of *Punica granatum peel* and *Vitis vinifera* seeds against the bacterial and fungal strains was evaluated by determination of the zones of inhibition. The results of the antibacterial activity of both the extracts were presented in Table 2,3. Both the extracts showed bactericidal activity against the selected Gram negative and Gram positive bacteria. Chloramphenicol was used as a positive control. The zone of inhibition showed by *P.granatum peel* extract against test bacteria ranged from 7-16 mm and seed extract produced zone of inhibition at a range of 7-15 mm. Both the selected extracts showed higher antibacterial activity against *Staphylococcus aureus* (Tables 2, 3). The two extracts selected for this study showed antifungal activity against all the three fungal strains. Both the extract showed maximum zone of inhibition against *Candida tropicalis*. (Table 4)

Table.1 Qualitative analysis *Punica granatum* Peel and *Vitis vinifera* seed extract Antimicrobial activity by Agar well diffusion method

Phytochemicals	<i>Punica granatum</i> Peel	<i>Vitis vinifera</i> seeds
Flavonoids	++	++
Anthraquinones	-	-
Saponins	-	+
Tannins	++	++
Aminoacids	+	+
Carbohydrates	++	+
Phlobatannins	+	+
Chalcones	+	+
Alkaloids	+	++
Steroids	+	-
Terpenoids	+	+
Phytosterol	++	+
Cardiac Glycosides	+	+

Table.2 Antibacterial activity of *Punica granatum* peel extract against selected bacteria

S.No	Microorganism	Zone of Inhibition (mm)					
		1000 µg	500 µg	250 µg	100 µg	DMSO	Chloramphenicol 30µg
1.	<i>Staphylococcus aureus</i>	16	14	13	12	-	26
2.	<i>Bacillus circulans</i>	13	12	10	8	-	21
3.	<i>Salmonella typhi</i>	11	8	7	-	-	15
4.	<i>Klebsiella pneumonia</i>	13	12	11	9	-	20
5.	<i>V.vulnificus</i>	12	10	8	7	-	20

Table. 3 Antibacterial activities of *Vitis vinifera* seed extract against selected bacteria

S.No	Microorganism	Zone of Inhibition (mm)					
		1000 µg	500 µg	250 µg	100 µg	DMSO	Chloramphenicol 30µg
1.	<i>Staphylococcus aureus</i>	15	13	12	11	-	27
2.	<i>Bacillus circulans</i>	12	10	8	8	-	20
3.	<i>Salmonella typhi</i>	8	7	7	-	-	15
4.	<i>Klebsiella pneumonia</i>	13	11	11	10	-	21
5.	<i>V.vulnificus</i>	11	10	8	8	-	17

Table. 4 Antifungal activity of fruit extracts against selected strains

S.No	Microorganism	<i>Candida albicans</i>			<i>Cryptococcus neoformans</i>			<i>Candida tropicalis</i>		
		Zone of Inhibition (mm)								
		Day 1	Day2	Day3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
1.	<i>Punica granatum</i> Peel	7	8	11	7	8	10	10	12	15
2.	<i>Vitis vinifera</i> seeds	6	8	9	7	7	8	-	7	10
3.	Amphotericin B	14	15	16	13	15	17	15	18	20

The alcoholic extract of *Punica granatum* peel and *Vitis vinifera* seeds showed antimicrobial activity against each of the tested strains at different concentration. The values of *Punica granatum* peel extract ranged from 62.50 to 1000 µg/ mL and *Vitis vinifera* seed extract showed activity varying from 125 to >1000µg/mL against the selected strains. The MICs determined by micro dilution method confirmed the results obtained using the disc diffusion method. The MIC results showed both *Punica granatum* peel and *Vitis vinifera* extracts showed growth inhibition of *Staphylococcus aureus* at lowest concentration (Tables 5, 6).

Phytonutrients and secondary metabolites naturally occurring from plants possess antimicrobial activity (Srikumar *et al.*, 2007). The antibacterial activity of the extracts may be due to the presence of several metabolic toxins. Several metabolites from plant materials, including alkaloids, tannins and sterols possess 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. Tannins are water soluble polyphenols known as tannic acid acts as antimicrobial agents. Presence of tannins is to prevent the development of microorganism by precipitating microbial proteins (Pranay Jain and GulhinaNafis, 2011). 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 (Cowan, 1999). Sterol molecules Gamma.-Sitosterol is present in both the selected extracts whereas

Stigmasterol is present only in *V.vinifera* seed extract (Ashok kumar and Vijayalakshmi, 2011). Sterol molecules shows antibacterial activity and their mode of action may be due to surface interaction of it with the bacterial cell wall and membrane ultimately leading to pore formation and degradation of the bacterial components (Devjani Chakraborty and Barkha Shah, 2011). *P.granatum* peel and *V.vinifera* seed extracts contains 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl- a flavonoid fraction (Ashok kumar and Vijayalakshmi, 2011) which possess anti-microbial, anti-oxidant and anti-inflammatory activities (Teoh *et al.*, 2011; Kumar and Maneemegalai 2008; Kumar *et al.*, 2010) which may play a role in anti-microbial activity of the extract against the selected microbial strains.

Most of the antibiotics used nowadays have lost their effectiveness due to the development of resistant strains of microbes, which is primarily due to the expression of resistance genes (Davis, 1994; Service, 1995). The antibiotics are sometimes associated with serious side effects such as hypersensitivity, immune suppression and allergic reactions (Ahmad *et al.*, 1998). Therefore, more interest is shown to develop alternative antimicrobial drugs for the treatment of infectious diseases without side effects. (Berahou *et al.*, 2007; Salomao *et al.*, 2008). The results of present study support the traditional usage of plant materials *P.granatum* and *V.vinifera* extracts which possess compounds with antibacterial and antifungal potential that can be used as anti-microbial agents as new drugs for the therapy of infectious diseases caused by pathogens.

Table.5 Minimum Inhibitory Concentration of *Punica granatum* peel extract against selected microorganisms

S.No	Microorganism	Minimum Inhibitory Concentration ($\mu\text{g/ml}$)							
		>1000	1000	500	250	125	61.50	31.25	15.175
1.	<i>Staphylococcus aureus</i>	-	-	-	-	-	-	+	+
2.	<i>Bacillus circulans</i>	-	-	-	+	+	+	+	+
3.	<i>Salmonella typhi</i>	-	-	+	+	+	+	+	+
4.	<i>Klebsiella pneumonia</i>	-	-	-	-	+	+	+	+
5.	<i>V.vulnificus</i>	-	-	+	+	+	+	+	+
6.	<i>Candida albicans</i>	-	-	-	+	+	+	+	+
7.	<i>C.neoformans</i>	-	-	+	+	+	+	+	+
8.	<i>Candida tropicalis</i>	-	-	-	-	+	+	+	+

Table.6 Minimum Inhibitory Concentration of *Vitis vinifera* seed extract against selected microorganisms

S.No	Microorganism	Minimum Inhibitory Concentration ($\mu\text{g/ml}$)							
		>1000	1000	500	250	125	61.50	31.25	15.175
1.	<i>Staphylococcus aureus</i>	-	-	-	-	-	+	+	+
2.	<i>Bacillus circulans</i>	-	-	-	+	+	+	+	+
3.	<i>Salmonella typhi</i>	-	+	+	+	+	+	+	+
4.	<i>Klebsiella pneumonia</i>	-	-	-	+	+	+	+	+
5.	<i>V.vulnificus</i>	-	-	+	+	+	+	+	+
6.	<i>Candida albicans</i>	-	-	+	+	+	+	+	+
7.	<i>C. neoformans</i>	-	-	+	+	+	+	+	+
8.	<i>Candida tropicalis</i>	-	-	-	+	+	+	+	+

+ = growth of organism, - = inhibition of growth

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