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EVALUATION OF ANTIMICROBIAL ACTIVITY OF *PUNICA GRANATUM* PEEL EXTRACTS USING DIFFERENT SOLVENT SYSTEM

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ABSTRACT

Punica granatum are utilized by local people as the part of their meal. Peoples supposed to consume *Punica granatum* seed and their peel was thrown as waste. Present study was designed to evaluate the antibacterial activity of *Punica granatum* peel against human pathogens. The extract was prepared using water, ethanol, methanol, acetic acid and petroleum ether. Antimicrobial activity was tested against four gram positive bacteria and six gram negative bacteria. Evaluations were based on the zone of inhibition using Agar well diffusion assay. The inhibitory activity was found to be dose dependent. The maximum antimicrobial activity was reported at 10mg/ml dosage tested. Results showed that *Punica granatum* showed highly significant antimicrobial activity against both the classes of bacteria. Aqueous, ethanol and methanol extracts were found to be more active towards the microorganisms tested than acetic acid and petroleum ether extracts. However with respect to pathogens the effectiveness will vary depending on the nature of extraction as well as on concentration of extract being administered. *Salmonella typhi* and *Proteus vulgaris* was reported to have significant susceptibility against most of the extract. This study represents that aqueous, ethanol and methanol extracts of waste material (peel) of *Punica granatum* may be utilizes as a potential source of antimicrobial agents. Phytochemical analysis of *P. granatum* peel show presence of alkaloids, flavanoids, steroids, tannin, cardiac glycosides and terpenoids using different solvent system suggest extractions of bioactive compounds are solvent dependent.

Key words: *Punica granatum*, Dadam, Antimicrobial agents, Phytochemicals, Solvent, Extraction.

INTRODUCTION

Punica granatum is also well known by different local name like dalim, anar, pomegranate. It belongs to the family of Punicaeae [1]. *Punica granatum* are widely available in Mediterranean basin and Southern Asia in warm environment [2]. The chief production of pomegranates is carried out at Alicante and Murcia provinces of India [2]. Different part of pomegranate like bark, leaves, immature fruits, and fruit rind have some medicinal importance [3]. Various investigations were carried out to determine antioxidant, anticarcinogenic, and anti-inflammatory properties of pomegranate constituents [3-7]. Various studies focuses on treatment and prevention

of cancer, cardiovascular disease, diabetes, dental conditions, erectile dysfunction, bacterial infections and antibiotic resistance, and ultraviolet radiation-induced skin damage, infant brain ischemia, male infertility, Alzheimer's disease, arthritis, and obesity using various extract from plant [6-8]. This study was devised to demonstrate the antibacterial activity of Pomegranate peel extract against pathogenic strain and to explore the natural source of drugs in comparison with traditional chemosynthetic drug to control the activity of pathogens. Various extractions from different part of *Punica granatum* was reported to have antimicrobial activity against *Escherichia coli* O157:H7,

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Bacillus cereus, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Shigella sonnie*, *Staphylococcus aureus* and many other species. [6, 8]. This study includes extraction of antimicrobial active compounds from peel of *Punica granatum* using different solvent system to demonstrate effectiveness of different extracts as antimicrobial agents.

MATERIALS AND METHODS

Collection of Plant Material

Fresh peel of *Punica granatum* were purchased from local market of Surat, Gujarat, India during January 2013 and the specimen samples was identified by the expert at Department of Biotechnology, Bhagwan Mahavir College of M. Sc. Biotechnology, Surat. Collected materials was washed in running tap water, rinsed properly in distilled water and then subjected to drying at room temperature for about 5 days in open air. These air dried material was grind into powdered and stored under refrigeration until their further utilization.

Preparation of Plant Extracts

Aqueous extraction was prepared as well as soxhlet extraction was carried out using different solvent such as methanol, ethanol, acetic acid and petroleum ether. The filtrates were evaporated to get concentrated residue. This residue treated as experimental drug for the present study. The extract was stored at 4°C until assay was completed.

Test organisms

Four strains of Gram-positive bacteria - *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, *Bacillus megaterium* and six strains of Gram negative bacteria - *Escherichia coli*, *Salmonella typhi*, *Salmonella paratyphi A*, *Salmonella paratyphi B*, *Proteus vulgaris* and *Pseudomonas aeruginosa* were used for antibacterial activity. All strains were obtained from Microbiology Diagnostic Centre, Surat, Gujarat, India. All bacterial cultures were grown and maintained on nutrient agar plates and were stored at 4°C. The bacterial cultures were periodically sub-cultured.

Preliminary Antimicrobial Activity Screening

The screening of different solvent extraction of peel of *Punica granatum* was carried out using agar well diffusion method. The test organisms were grown on nutrient agar plates and subculture prior to start the screening. The subculture plate containing test organism was kept in incubator at 37°C for 24 hours. The inoculums for each test microorganism were prepared which have approximately 10⁵ CFU/ml. The plant extracts were dissolved in DMSO for conducting antimicrobial activity. Antibiotics such as Tetracycline (1mg/ml) were used as positive controls, while DMSO was used as negative controls. The plates for antimicrobial activity were

incubated at 37°C for 24hrs. After 24 hours the plates was examined for zone of inhibition (Table 1).

Phytochemical screening

Phytochemical analysis of the extract was carried out using various procedures describe by various authors [10-13]. Phytochemical screening was performed to detect the presence of several phytochemicals like Alkaloids, Flavonoids, Steroids, Saponins, Cardiac glycoside, Tannins, Terpenoids and free Amino acid (Table 2).

Test for Alkaloids [10]

Dragendroff's reagent test was conducted for detection of alkaloids. 0.5 g of peel extract was dissolved in 5 ml of 1% HCl and the mixture was kept for 2 minutes in water bath. 1 ml of filtrate is treated with dragendroff's reagent. Turbidity or precipitation was indication for presence of alkaloids.

Test for Tannins [11]

For detection of tannin, the method describes by E. Y. Qnais et al, 2007 was utilized with certain modification. About 0.5 g of peel extract was dissolved in 10 ml of boiling water. The solution was filtered and to filtrate few ml of 6% FeCl₃ was added. Development of deep green colour shows presence of Tannin.

Test for Flavanoids [12]

Yellow precipitation was observed when the test solution was treated with 10% lead acetate solution indicates presence of Flavanoids.

Test for Saponin [12]

The test solution was mixed with water in the test tube and shaken properly. Foaming arises suggest presence of saponin.

Test for Steroids and Triterpenoids [12,13]

The Salkowaski method was used as describe by Agarwal et al (2011) with certain modifications. About 0.5 g of extract was dissolved in 3 ml of chloroform and filtered. Concentrated H₂SO₄ was added to the filtrate to form a lower layer. Reddish brown color develop was considered as positive results for the presence of steroids ring.

Test for Cardiac glycoside [12]

About 0.5 g of the extract was dissolved in 2ml of glacial acetic acid containing 1 drop of 1% FeCl₃. This was under laid with conc. H₂SO₄. A brown ring obtained at the interphase indicates the presence of deoxy-sugar. A violet ring appeared below the ring while in the acetic acid layer a greenish ring appeared just above ring and gradually spread throughout this layer.

Test for Free Amino Acids:[12]

Ninhydrin Test was utilizing to detect free amino acid. Peel extract solution boiled with 0.2% Ninhydrin solution. Purple colour formation indicates positive result.

Dose dependent Antimicrobial Activity Test

The dose dependent studies were conducted for different solvent extraction of peel of *Punica granatum* on microorganisms showing positive screening for antimicrobial activity. Different doses of 1 mg/ml, 2 mg/ml,

5 mg/ml and 10 mg/ml was tested for each peel extract for their antimicrobial activity. The dose dependent antimicrobial activity was carried out using agar well diffusion method (Table 4). The plates for antimicrobial activity were incubated at 37°C for 24hrs. After 24 hours the plates was examined for zone of inhibition and compared with the Tetracycline control (Table 3). The zone was measured in mm. All the tests were conducted in triplicate.

RESULTS**Table 1. Preliminary Screening for Antimicrobial Activity of *Punica granatum* Peel**

Pathogenic Organisms	Zone of Inhibition				
	Aqueous Extracts	Methanolic Extracts	Ethanollic Extracts	Acetic Acid Extracts	Petroleum Ether Extracts
<i>Escherichia coli</i>	-	-	+	-	+
<i>Bacillus subtilis</i>	+	+	+	-	-
<i>Bacillus megaterium</i>	+	+	+	+	+
<i>Bacillus cereus</i>	+	+	+	-	+
<i>Salmonella typhi</i>	+	+	+	-	+
<i>Salmonella paratyphi A</i>	+	+	+	-	+
<i>Salmonella paratyphi B</i>	-	-	-	-	-
<i>Proteus vulgaris</i>	+	+	+	+	+
<i>Pseudomonas aeruginosa</i>	-	-	+	-	+
<i>Staphylococcus aureus</i>	+	+	+	-	-

+ indicates = Zone of Inhibition, - indicates = No Zone of Inhibition

Table 2. Phytochemical Analysis of *Punica granatum* Extracts

Phytochemical Parameters	Solvent systems				
	Aqueous	Methanol	Ethanol	Acetic Acid	Petroleum Ether
Steroids	+	+	-	-	-
Alkaloids	-	-	-	+	-
Flavonoids	+	+	+	-	-
Saponins	-	-	-	-	-
Tannins	-	+	-	+	-
Cardiac glycosides	+	+	-	-	-
Terpenoids	+	-	-	-	-
Amino acids	-	-	-	-	-

+ indicates = Positive results, - indicates = Negative results.

Table 3. Antimicrobial Activity of Tetracycline Control

Pathogenic Organisms	Zone of Inhibition (m. m)
<i>Escherichia coli</i>	11
<i>Bacillus subtilis</i>	14
<i>Bacillus megaterium</i>	10
<i>Bacillus cereus</i>	10
<i>Salmonella typhi</i>	6
<i>Salmonella paratyphi A</i>	12
<i>Salmonella paratyphi B</i>	10
<i>Proteus vulgaris</i>	5
<i>Pseudomonas aeruginosa</i>	7
<i>Staphylococcus aureus</i>	21

Table 4. Dose Dependent Antimicrobial Activity of *Punica granatum* Extracts

Bacterial strains	Doses of Peel Extract of <i>Punica granatum</i>																			
	Aqueous				Methanol				Ethanol				Acetic acid				Petroleum ether			
	1 mg/ml	2 mg/ml	5 mg/ml	10 mg/ml	1 mg/ml	2 mg/ml	5 mg/ml	10 mg/ml	1 mg/ml	2 mg/ml	5 mg/ml	10 mg/ml	1 mg/ml	2 mg/ml	5 mg/ml	10 mg/ml	1 mg/ml	2 mg/ml	5 mg/ml	10 mg/ml
<i>Escherichia coli</i>	-	-	-	-	-	-	-	-	4	6	11	12	-	-	-	-	-	1	2	6
<i>Salmonella typhi</i>	3	5	10	12	1	2	5	11	4	4	5	10	-	-	-	-	1	3	5	6
<i>Salmonella paratyphi A</i>	-	-	-	-	1	2	3	12	6	5	3	11	-	-	-	-	1	2	5	6
<i>Salmonella paratyphi B</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Proteus vulgaris</i>	6	11	12	13	1	2	5	12	5	7	9	11	1	2	4	6	2	4	6	7
<i>Pseudomonas aeruginosa</i>	-	-	-	-	-	-	-	-	9	10	11	12	-	-	-	-	1	2	3	5
<i>Bacillus cereus</i>	-	-	-	-	2	7	8	11	2	3	6	12	-	-	-	-	1	2	4	8
<i>Bacillus subtilis</i>	1	4	5	8	1	2	5	12	3	2	4	9	-	-	-	-	-	-	-	-
<i>Bacillus megaterium</i>	2	2	4	9	1	2	9	12	4	5	5	9	2	3	4	8	-	1	2	5
<i>Staphylococcus aureus</i>	4	8	10	11	1	2	10	10	1	1	2	10	-	-	-	-	-	-	-	-

DISCUSSION

The peel of *Punica granatum* shows various therapeutic applications as Antibacterial, antifungal, antioxidant, antitumor, antiviral, antimalarial and antimutagenic effects as reported by different authors [3-6]. Much work has been carried out to demonstrate ethno-medicinal value of various plants in India because traditional natural products are widely used to cure certain diseases. The present investigated focuses on peel extract using various solvent systems to show the antimicrobial activity of each extract. Antimicrobial activities were measured with respect to pathogenic microorganism's reveals that each peel extracts from *Punica granatum* show significant antimicrobial activity as reported by different researchers [11, 14, 15].

During screening for antimicrobials, *Punica granatum* aqueous and methanolic extract was shown to be effective against *B. subtilis*, *B. megaterium*, *B. cereus*, *S. typhi*, *S. paratyphi A*, *P. vulgaris* and *S. aureus*. However, unlike aqueous and methanolic extract, ethanolic extract also show significant antimicrobial activity against *P. aeruginosa*. Acetic acid extract show antimicrobial activity against *B. megaterium* and *P. vulgaris*, whereas petroleum ether shows antimicrobial activity against *E. coli*, *B. megaterium*, *B. cereus*, *S. typhi*, *S. paratyphi A*, *P. vulgaris* and *P. aeruginosa*.

The dose dependent study using aqueous extract show significant antimicrobial activity against *S. typhi* and *P. vulgaris* at the concentration of 10mg/ml. The dose dependent study using methanolic extract show antimicrobial activity against *S. typhi*, *P. vulgaris*, *B. cereus*, *B. megaterium*, *S. paratyphi A* at extract concentration of 10mg/ml. The ethanolic extract shown to be effective against *B. cereus*, *P. vulgaris*, *S. typhi*, *E. coli* and *P. aeruginosa* at extract concentration of 10mg/ml. *Punica granatum* peel Acetic acid extract shows that zone of inhibition with *P. vulgaris*, *B. megaterium*, but dose

dependent study show significant antimicrobial activity against only *P. vulgaris* at 10 mg/ml extract concentration. *Punica granatum* peel Petroleum ether extract shows that zone of inhibition with *E. coli*, *B. megaterium*, *B. cereus*, *S. typhi*, *S. paratyphi A*, *P. vulgaris* and *P. aeruginosa* but dose dependent study show significant antimicrobial activity against *S. typhi* and *P. vulgaris* only at 10 mg/ml extract concentration. Tetracycline was used as positive control and DMSO as negative control for each tested extract.

The phytochemical screening of *Punica granatum* peels show presence of flavanoids, steroids, cardiac glycosides and terpenoids in aqueous extract. The ethanolic extract show presence of flavanoids, steroids, cardiac glycosides and Tannin. The Methanolic shows presence of only flavanoids whereas acetic acid extracts show presence of only alkaloids. The petroleum ether extraction show absent of above tested phytochemicals. Hence the extractions of bioactive compounds responsible for antimicrobial activity are solvent dependent.

In the present study, the antibacterial activity of *Punica granatum* peel extracts towards clinically significant microbes are reported and it was observed that the active constituents in the plant material was extracted in polar as well as non-polar solvent system. However, antimicrobial activity was demonstrated effectively in polar solvent as compared to non-polar one. The study shows the pharmacological importance of peel of *Punica granatum*, thereby exploring bioactive phytochemicals from waste material (peel) showing antimicrobial activity and thus substantiates traditional medicinal use. The separation and further activity mediated approach was emphasize to conduct in future to demonstrate active phytochemicals to be utilize as lead compounds for antimicrobials. Thus, the study provides a strong direction for proper investigation of various plants to explore molecules having antimicrobial properties against human pathogens using waste sources.

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