Investigation of inhibition efficiency of *Punica granatum* peel extract against bacteria

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Objectives The present study aims to determine the inhibitory efficiency of *Punica granatum* peel extract and test its effectiveness against pathogenic bacteria.
Methods Acetone, ethanol, methanol, ethyl acetate and distilled water were used as solvent to obtain of crude extract of *P. granatum* peel, which tested to determine the effectiveness against five types of pathogens, such as *Pseudomonas aeruginosa, Pseudomonas oryzihabitans, Proteus vulgaris, Klebsiella pneumonia* and *Staphylococcus aureus* under a series of concentrations. It was also used to determine the most efficient concentration of solvent optimization, and then was determined minimum inhibitory concentration (MIC) of the extract more efficient.
Results The results of the current study showed that the most efficient extraction solvent is ethyl acetate showed the diameters of inhibition zone are 48.66, 51.5, 41.66, 28.0, and 40.83 mm for the types of bacteria above, respectively. The results showed that the concentration of ethyl acetate was 40% in the optimal inhibition of bacteria, amounting to diameters of inhibition zone at the concentration of 19.83, 27.33, 17.66, 15.16 and 12.33 mm for each of the bacterial species above, respectively. The results also found that MIC is 11 mg/ml of *P. aeruginosa* and 10 mg/ml of *P. varaplis* and 9 mg/ml of *P. oryzihabitans* and 3 mg/ml of *K. pneumoniae* and *S. aureus*.
Conclusion The most effective composites against pathogenic bacteria from *P. granatum* peel are using ethyl acetate solvent with the concentration of 40%.
Keywords Ethyl acetate, *Punica granatum*, antibacterial activity, MIC.

Introduction

Bacteria are microscopic single cell called microbes; They are also found in ocean soil and inside the human gut.[1] It may be beneficial or harmless (pathogenic). Pathogenic bacteria not only cause disease to human, animals and plants, but also develop more interest for researchers to study in detail.[2]

Various bacteria cause different types of diseases such as meningitis caused by *Neisseria meningitidis*; ear infection and strep throat caused by streptococcal bacteria, and toxic shock syndrome caused by *Staphylococcus aureus.*[3]

Multiple researches revealed that *Punica granatum* has pathogen-resistant. *P. granatum* is one of the oldest known fruits found in writings and artifacts of many culture and religions. It has been revered as a symbol of health, fertility and eternal life.[4] *P. granatum* yields powerful antioxidant such as flavonoids, proanthocyanidins and phenolic.[5]

Research shows that various extracts from *P. granatum* has antifungal activity against *Listeria monocytogenes, S. aureus*, *Escherichia coli and Yersinia enterocolitica.*[6] The literature also reveals that *P. granatum* has antifungal activity against dermatophytic fungi and *Trichophyton mentagrophytes.*[7]

Our study aimed to examine the antibacterial activity of extract of *P. granatum* peel against five types of pathogenic bacteria, four is gram-negative *P. aeruginosa, Pseudomonas oryzihabita, Proteus varaplis* and *Klebsiella pneumonia* and one gram-positive *S. aureus* using acetone, ethanol, methanol, ethyl acetate and water as solvents.

Materials and Methods

Microorganisms: In this study, we used five pathogenic bacteria isolated from patient in Al-Zahra Teaching Hospital in Karbala city. These bacteria are *P. aeruginosa, P. vulgaris, K. pneumonia* and *S. aureus*.

Extraction: *P. granatum* peel was grinded into powder and was uniformly divided in five beakers weighing 20 g each in a beaker. Subsequently, 100 ml of solvent (acetone, ethanol, methanol, ethyl acetate, distill water) with 70% concentration to each beaker that contain plant powder and labeled this beaker with the name of solvent and were kept in a shaking incubator over night with 150 cycle/min at 37°C.

The next day, the extracts in all five beakers were filtered using cotton and gauze. Further, the purified extracts in tubes were centrifuged at 3000 rpm for 5 min.

After centrifugation, the supernatant was transferred to glass dishes and allowed them to dry. Later, each dish was scrubbed and stored in a clean container and saved as crude extract. The total weight of the crude was also measured.[8]

Determination of antibacterial activity of the best solvent: First, Muller Hinton agar medium was prepared and sterilized. It was poured in petri dishes and allowed them to solidify. Three wells with 5 mm diameter in every dish were made by cork borer. From the pre-collected powder, 0.05 g from each container was taken and put them in five tubes. Each tube contains extract for certain type of our five solvents. 2 ml of ethanol 70% was added to each tube and shaked until the powder was dissolved. The final concentration of extract in each tube was equal to 25 mg/ml. In addition to our five tubes, a sixth tube was also added containing 2 ml for control (70% ethanol without any extract).

After activation of bacteria in nutrient broth, we cultured it in the prepared dishes. In each petri dish, 100 µl of each activation bacteria was spread by sterilized spreader, then 50 µl plant extract in each well was added. In the negative control, 50 µl ethanol 70% was added, 50 µg/ml Gentamycin was used as a positive control. After culturing, all the dishes were incubated in an incubator for 24 hrs under

37°C. In the next day, after incubation we measure the diameter of zone of inhibition was measured against bacteria by solvent.[9]

The optimal concentration of the best solvent: Depending on the results of the previous experiment, ethyl acetate extract was chosen. Five different concentrations of ethyl acetate (20, 40, 60, 80, 100%) were taken in each beaker. In each beaker, 20 g of *P. granatum* extract powder was added, and the same process of extraction method was repeated that described above and recorded the results.

In the next step, 0.05 g of dry extract in five separate tubes was taken, then added 2 ml of 70% ethanol to each tube to obtain on the final concentration 25 mg/ml. After that, 30 petri dishes with Muller Hinton agar were prepared, and followed the three tests to determine the best concentration of ethyl acetate, which has the largest inhibition zone.[9]

Minimum inhibitory concentration (MIC) of ethyl acetate: In a flask, 50 ml of nutrient broth was poured, and in the other flask, 25 ml of nutrient broth was added with 0.625 g from ethyl acetate 40% dry extract to obtain on 25 mg/ml concentration in this flask from extraction.

26 glass tubes were taken and labelled them with numbers from 0 to 25. On each tube, the extract from *P. granatum* with ethyl acetate 40% concentration which is equal to the number of the label of the tube was taken. For example: in tube number 25, 2 ml from flask 2 was added, which represents 25 mg/ml concentration. In tube number 24, 1.92 ml from flask 2 and 0.08 ml from flask 1 were added, which represents 24 mg/ml concentration, so that with other tubes, in the tube number 0 put only 2 ml from flask 1 to obtain of 0 mg/ml in this tube. The distribution between flask 1 and 2 in these tubes is according to the law C1 V1 = C2 V2.

After that, micro titter plate with 96 wells and numbered the wells from 0 to 25, twice for each type of bacteria (each type of bacteria have 52 wells twice for each number), then added 150 µl from each tube in two wells of the same number on each type of bacteria, Then added 50 µl of each type of bacteria(after dilute it with both media and take from the 3rd dilution comparison with McFarland solution) in all the 52 wells from 0 to 25 concentrations. Later, the plates were covered and incubated in an incubator for 24 hr in 37°C.

The plates were examined to see growth or no growth of bacteria to determine the minimal inhibition concentration from extract for each type of bacteria.[10]

Statistical Analysis: Statistical analysis included random complete design(RCD) with 3 replicates. 0.05 is the level of probability that was used to identify the significant differences. The significant differences between the averages were also tested using the test less significant difference (LSD) at the level of probability of 0.05.[11]

Results

The results show the percentage of materials recoverable from *P. granatum* when we use the solvents: acetone, ethanol, methanol, ethyl acetate and distilled water. The percentages are 2.45, 3, 2.5, 3.25, 2.28%, respectively (Table 1).

The higher percentage was noted by ethyl acetate 3.25% and low percentage was noted by distilled water 2.28%.

The results show that the extract of *P. granatum* with ethyl acetate give the most large inhibition zone with significance differences ($P < 0.05$) in all five type of bacteria *P. aeruginosa, P. oryzihabita, P. vulgaris, Klebsila pneumonia* and *S. aureus* (Table 2). The diameters of inhibition zone are 48.66, 51.5, 41.66, 28.0, 40.83 mm, respectively. The results were significantly different ($P < 0.05$) comparison with Gentamycin and comparison with others solvent.

The extract of *P. granatum* with (20, 40, 60, 80, 100)% of ethyl acetate was examined (Table 3). Larger inhibition zone with significant difference ($P < 0.05$) against gentamycin from hand and in other hand between ethyl acetate 40% and other concentrations was noted.

Table 1. **The percentage of materials extracted from *P. granatum* peel**

No.	Extraction solvent (100 ml)	Origin weight of plant powder (g)	Weigh of extract (g)	Percentage of extract materials (%)
1	Acetone	20	0.49	2.45
2	Ethanol	20	0.6	3
3	Methanol	20	0.5	2.5
4	Ethyl acetate	20	0.65	3.25
5	Distil Water	20	0.457	2.28

Table 2. **Inhibition zone (mm) to extract *P. granatum* peel against bacteria**

Bacteria	Extraction solvent (70%)						$LSD_{0.05}$
	Gentamycin 10 µg/ml	Acetone	Ethanol	Methanol	Ethyl acetate	Distil water	
P. aeruginosa	21.66 ± 0.88	20.5 ± 0.5	0.0 ± 0.0	27.0 ± 2.59	48.66 ± 3.21	12.83 ± 0.44	4.40
P. oryzihabitans	20.33 ± 0.88	46.66 ± 4.63	39.16 ± 0.83	39.33 ± 0.66	51.5 ± 0.76	39.16 ± 3.0	5.91
P. vulgaris	12.5 ± 0.28	33.0 ± 2.51	31.5 ± 3.25	38.83 ± 1.33	41.66 ± 1.66	23.0 ± 0.57	4.81
K. pneumonia	17.66 ± 0.33	20.66 ± 1.20	18.66 ± 0.16	19.66 ± 0.88	28.0 ± 2.46	26.0 ± 3.60	4.76
S. aureus	17 ± 0.57	39.33 ± 1.83	24.16 ± 1.83	31.0 ± 2.56	40.83 ± 4.4	31.16 ± 0.6	5.94

The numbers refer to mean ± standard error.

Table 3. **Inhibition zone (mm) of extract *P. granatum* peel against bacteria by using dilutions series of ethyl acetate**

Bacteria	Ethyl acetate ratio (%)						$LSD_{0.05}$
	Gentamycin 10 µg/ml	20%	40%	60%	80%	100%	
P. aeruginosa	21.66 ± 0.88	14.66 ± 4.1	19.83 ± 3.52	18.73 ± 1.13	4.66 ± 0.33	0.0 ± 0.0	4.80
P. oryzihabitans	20.33 ± 0.88	16.83 ± 4.1	27.33 ± 1.30	20.83 ± 1.16	13.5 ± 0.28	0.0 ± 0.0	4.68
P. vulgaris	12.5 ± 0.28	15.0 ± 0.28	18.16 ± 1.74	17.66 ± 1.09	17.66 ± 1.16	9.83 ± 0.33	2.49
K. pneumonia	17.66 ± 0.33	15.83 ± 1.09	17.16 ± 2.74	17.16 ± 1.48	15.16 ± 0.44	13.5 ± 0.28	3.45
S. aureus	17 ± 0.57	18.33 ± 0.6	21.0 ± 0.28	15.0 ± 1.52	12.33 ± 1.83	5.0 ± 0.5	2.66

The numbers refer to mean ± standard error.

Table 4. **Minimum inhibitory concentration (mm) of ethyl acetate extract *P. granatum* peel**

Extract Concentration (mg/ml)	Types of bacteria				
	P. aeruginosa	*P. oryzihabitans*	*P. vulgaris*	*K. pneumonia*	*S. aureus*
1	+	+	+	+	+
2	+	+	+	+	+
3	+	+	+	–	–
4	+	+	+	–	–
5	+	+	+	–	–
6	+	+	+	–	–
7	+	+	+	–	–
8	+	+	+	–	–
9	+	–	+	–	–
10	+	–	–	–	–
11	–	–	–	–	–
12	–	–	–	–	–
13	–	–	–	–	–
14	–	–	–	–	–
15	–	–	–	–	–
16	–	–	–	–	–
17	–	–	–	–	–
18	–	–	–	–	–
19	–	–	–	–	–
20	–	–	–	–	–
21	–	–	–	–	–
22	–	–	–	–	–
23	–	–	–	–	–
24	–	–	–	–	–
25	–	–	–	–	–

+ means growth – means not growth.

The diameter with the concentration of 40% that gives 19.83, 27.33, 17.66, 15.16, 12.33 mm against *P. aeruginosa, P. oryzihabitans, P. vulgaris, K. pneumonia* and *S. aureus,* respectively.

The less concentration of extract that caused inhibition to bacteria (Table 4). It was noted that the concentration 3 mg/ml of extraction showed inhibition activity against *K. pneumonia* and *S. aureus,* and the concentration 9 mg/ml inhibited *P. oryzihabitans*, and the concentration 10 mg/ml inhibited *P. vulgaris* and concentration 11 mg/ml inhibit *P. aeruginosa.* From this result, it can be noted that bacteria *K. pneumonia* is more sensitive than the others because it gave less MIC, and in contrast *P. aeruginosa* gave large MIC.

Discussion

Various extracts from *P. granatum* have antibacterial activity against *Listeria monocytogen, S. aureus, E. coli and Yersinia enterocytogene.*[6] In other study, *P. granatum* has antibacterial activity against the dermatophytic fungi *Trichophyton mentagrophytes.*[7]

Previous studies reveal that *P. granatum* has antibacterial activity against *S. aureus*, and its juice also has inhibitory effect to *S. epidermidis* and *K. pneumonia*.[12,13]

Methabe *et al*[14] show that methanol, ethanol, acetone and water extract obtained from *P. granatum* have antibacterial activity against *S. aureus, Salmonella typhi, E. coli, S. dysenteriae, Vibrio cholera, S. soni, Shigella boydii* and *Shigella flexneri* .

Other study[15] found that the extract from *Pnica granatum* also showed antibacterial activity against *E. coli, Enterobacter cloacae, P. fluorescens, Proteus vulgaris, Alcaligenes faecalis, Serratia marcescens, Enterobacter aerogenes, S. aureus, Micrococcus luteus, Bacillus cereus, Bacillus subtilis, Micrococcus roseus, Mycobacterium phlei, Bacillus coagulans, Micrococcus rodochrus and Mycobacterium smegmatis* with inhibition zone 11–31 mm.

Ahmed and Beg[16] show that alcohol extract of *P. granatum* have antibacterial activity against *E. coli, S. aureus* and *Shigella dysenteriae.* Also Al-Zoreky[6] started to notice that the water extract of *P. granatum* have no antibacterial activity against *S. aureus and E. coli* but it was found that methanol extract had high inhibitory effect.

In one study reported that *E. coli* is more resistant to all *P. granatum* extract than other Gram -ve bacteria.[17]

In our study, the difference between previous studies may be in use of different solvents or type of bacteria and also the composition of plant help us to gain more inhibitory effect that contain flavonoids, proanthocyanidins and phenolic.

Conclusion

From the results, we concluded that the extract of *P. granatum* peel by ethyl acetate 40% has high antibacterial activity against the pathogenic bacteria that isolated from patients.

Recommendations

Other study on *P. granatum* peel is proposed to purify the material, which owns the effectiveness of anti-bacterial pathogenesis. ■

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