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Original Article

Portrayal of *Punica granatum* L. peel extract through High Performance Liquid Chromatography and antimicrobial activity evaluation

Retrato do extrato de casca de *Punica granatum* L. por meio de cromatografia líquida de alto desempenho e avaliação da atividade antimicrobiana

M.S. Mahmood[a], A. Ashraf[b], S. Ali[a], A.B. Siddique[c], F. Asad[b], R.Z. Abbas[d], F. Siddique[e], A. Aslam[f], R. Aslam[a] and A. Rafique[b]*

[a]Institute of Microbiology, University of Agriculture, Institute of Microbiology, Faisalabad, Pakistan
[b]Department of Zoology, Government College University, Faisalabad, Pakistan
[c]Department of Microbiology, GC University, Faisalabad, Pakistan
[d]Department of Parasitology, University of Agriculture, Faisalabad, Pakistan
[e]Department of Microbiology, Cholistan University of Veterinary and Animal Sciences, Bahawalpur, Pakistan
[f]Department of Microbiology, The Islamia University of Bahawalpur, Bahawalpur, Pakistan

Abstract

Increasing trend in antimicrobial resistance and failure of chemically synthesized antibiotics lead to discover alternative methods for the treatment of bacterial infections. Various medicinal plants are in use traditionally and their active compounds can be further applied for treatment of bacterial diseases. This study was designed to determine the antibacterial activity of *Punica granatum* (*P. granatum* L.) (pomegranate) peel extract against Enterobacteriaceae [*Escherichia coli* (*E. coli*), *Salmonella Typhimurium* (*S. Typhimurium*) and *Shigella Dysenteriae* (*S. Dysenteriae*)] and gram-positive bacterium [*Staphylococcus aureus* (*Staph aureus*)]. Methanolic extract of *P. granatum* L. peel was prepared by Soxhlet apparatus method. Total flavonoid and phenolic contents from the extract were determined by High Performance Liquid Chromatography (HPLC). The antibacterial activity of *P. granatum* L. peel extract was evaluated through agar well diffusion method. HPLC showed the range of phenolics (gallic acid, caffeic acid, benzoic acid, cinnamic acid) and flavonoid compounds. The chemical structures of flavonoid and phenolics found in the methanolic extract of *P. granatum* L. peel have been reported for the first time. The methanolic peel extract (50 ul) of yellow *P. granatum* L. showed 26, 10, 10 and 9mm zones of inhibition (ZOI) against *S. aureus*, *S. Typhimurium*, *S. Dysenteriae* and *E. coli*, respectively. The methanolic extract of red *P. granatum* L. (100 ul) showed 27, 8, 12 and 15 mm ZOI against *Staph. aureus*, *S. Typhimurium*, *S. Dysenteriae* and *E. coli*, respectively. Highest ZOI was observed against *Staph. aureus*. Many of the bacteria studied in the present work may cause serious gastrointestinal infections, which can lead to hemorrhagic diarrhea in children. These infections can be life-threatening to young children and the elderly. There is an incentive to find alternative control measures, such as plant and herbal extracts, especially in lesser-developed countries where traditional antibiotics may not be readily available.

Keywords: *P. granatum* L., antimicrobial activity, Enterobacteriaceae, *Staphylococcus aureus*, HPLC.

Resumo

A tendência crescente na resistência antimicrobiana e na falha dos antibióticos sintetizados quimicamente leva à descoberta de métodos alternativos para o tratamento de infecções bacterianas. Várias plantas medicinais estão em uso tradicionalmente e seus compostos ativos podem ser posteriormente aplicados para o tratamento de doenças bacterianas. Este estudo foi desenhado para determinar a atividade antibacteriana do extrato de casca de *Punica granatum* (*P. granatum* L.) (romã) contra Enterobacteriaceae [*Escherichia coli* (*E. coli*), *Salmonella Typhimurium* (*S. Typhimurium*) e *Shigella Dysenteriae* (*S. Dysenteriae*)] e bactéria gram-positiva [*Staphylococcus aureus* (*Staph aureus*)]. O extrato metanólico da casca de *P. granatum* L. foi preparado pelo método do aparelho de Soxhlet. O conteúdo total de flavonoides e fenólicos do extrato foi determinado por cromatografia líquida de alta eficiência (HPLC). A atividade antibacteriana do extrato da casca de *P. granatum* L. foi avaliada através do método de difusão em ágar. HPLC mostrou a gama de compostos fenólicos (ácido gálico, ácido cafeico, ácido benzoico, ácido cinâmico) e flavonoides. As estruturas químicas de flavonoides e fenólicos encontradas no extrato metanólico da casca de *P. granatum* L. foram relatadas pela primeira vez. O extrato metanólico da casca (50 ul) de *P. granatum* L. amarelo

*e-mail: azharrafique96@gmail.com
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apresentou zonas de inibição (ZOI) de 26, 10, 10 e 9mm contra *S. aureus*, *S. Typhimurium*, *S. Dysenteriae* e *E. coli*, respectivamente. O extrato metanólico de *P. granatum* L. vermelho (100 ul) apresentou 27, 8, 12 e 15 mm IOI contra *Staph. aureus*, *S. Typhimurium*, *S. Dysenteriae* e *E. coli*, respectivamente. O ZOI mais alto foi observado contra *Staph. aureus*. Muitas das bactérias estudadas no presente trabalho podem causar infecções gastrointestinais graves, que podem levar à diarreia hemorrágica em crianças. Essas infecções podem ser fatais para crianças pequenas e idosos. Há um incentivo para encontrar medidas de controle alternativas, como extratos de plantas e ervas, especialmente em países menos desenvolvidos, onde os antibióticos tradicionais podem não estar prontamente disponíveis.

Palavras-chave: *P. granatum* L., atividade antimicrobiana, Enterobacteriaceae, *Staphylococcus aureus*, HPLC.

1. Introduction

Excessive use of antibiotics is the leading cause of resistance in pathogenic bacteria mainly in *Staph. aureus*, *E. coli O157*, *Salmonella* and *Pseudomonas*. In recent years, the identification of food containing nutrients and biologically active components has been gaining immense attention. The active components in plants (phytochemicals) are helpful components against pathogenic microorganisms in replacement of antibiotics (Devi et al., 2011; Ahmad et al., 2020).

Due to bacterial resistance against antibiotics, herbal medicines can be used for the treatment of many diseases. Medicinal plants are natural products for the treatment of diseases without any adverse side effects (Moghaddam et al., 2013; Javed et al., 2021; Majeed et al., 2021; Rafay et al., 2021).

Different plant extracts have been reported for their antimicrobial activities including some of the important appended here *i.e*., *Aframomum melegueta* (Doherty et al., 2011), aqueous and hydroalcoholic extracts of *Acanthospermum australe* (Loefl.) Kuntze against diarrhea-inducing bacteria (Mallmann et al., 2018), antibacterial and antiproliferative activities of the fresh leaf essential oil of *Psidium guajava* L. (Silva et al., 2019) and antimicrobial activity of *Campomanesia aurea* against three strains of *Listeria monocytogenes* (Pacheco et al., 2020).

Punica granatum (*P. granatum L.*) locally named as pomegranate is a plant fruit that belongs to the *punicaceae* (family). It is an important horticultural fruit crop. Polyphenols, important minerals and saccharides are present in edible part of fruit. Various types of phytochemicals are present in the *P. granatum L.* fruit, tree and seed. The polyphenols are the major class of phytochemicals. Polyphenols have multiple hydroxyl groups on the phenolic rings. Polyphenols have hydrolysable tannins, condensed tannins and flavonoids. The hydrolysable tannin includes ellagitannins and gallotannins (Elalamy et al., 2020). The condensed tannins include pro-anthocyanidins. Flavonols, anthocyanin and flavanols are included in the flavonoids (Abbas et al., 2020).

The components of *P. granatum L.* plant includes juice, leaves, seed oil, peel, rind (pericarp), barks, roots and flowers. The constituents of *P. granatum L.* include ascorbic acid, anthocyanin, amino acids, glucose, caffeic acid, gallic acid, catechin, ellagic acid, minerals and iron. *P. granatum L.* leaves contain tannins (punicafolin and the punicalin) and flavones glycosides (include apgenin and the luteolin). The *P. granatum L.* seed oil has ellagic acid, sterols, fatty acids and punicic acid. The components of *P. granatum L.* peel and rind include gallic acid, phenolic punicalagins, rutin, flavonols (flavonones, flavones and anthocyanidins), quercetin, and fatty acids (catechin). *P. granatum L.* barks and roots have piperidine alkaloids and Ellagitannins (punicalagin and punicalin). Gallic acid, triterpenoid, ursolic acid, and other unidentified components are included in flower part of the *P. granatum L.* plant. According to chemical composition (per 100 grams), *P. granatum L.* has 1.6% protein, 14.5% carbohydrates, 0.7% minerals, 0.1% fat, 5.1% fiber and 78% moisture. The iron concentration of *P. granatum L.* is 0.3 mg. The concentration of calcium in *P. granatum L.* is 10 mg and concentration of phosphorus is 70 mg. Vitamin C (16.0 mg) is present in *P. granatum L.* plant. Vitamin B is present in trace amount. The caloric value of *P. granatum L.* is in 65 mg (Dahham et al., 2010).

All parts of the *P. granatum L.* plant including trunk skin, seed, root and flower are used in medicine. *P. granatum L.* flowers are used in the treatment of diarrhea. *P. granatum L.* has bioactive components that can be used for the treatment of the dental diseases, cardiovascular diseases, cancer, diarrhea , skin infections, diabetes mellitus, hemorrhoids and other bacterial and fungal infections (Hajifattahi et al., 2016).

P. granatum L. peel extract has polyphenols that inhibit the oxidation process, microbial growth, eliminate free radicals and decrease the risk of cancer and cardiovascular diseases. *P. granatum L.* peel extract is also rich in vitamin C. It is useful as the raw material for pharmaceutical industry because of its antimicrobial properties. Gallic acid, punicallins and ellagic acid extracted from the *P. granatum L.* revealed good inhibitory effect against pathogenic microorganisms (Opara et al., 2009). Pomegranate has exhibited bactericidal activity against food and waterborne pathogenic bacteria including *Salmonella* Typhi (*S.* Typhi) (Perez and Anesini, 1994; Rani and Khullar, 2004), *Vibrio cholerae* (Guevara et al., 1994; Mathabe et al., 2006), *Yersinia enterocolitica*, *Shigella* spp. (Alanis et al., 2005; Mathabe et al., 2006), and *Listeria monocytogenes* (*L. monocytogenes*) (Lucas and Were, 2009; Shan et al., 2011). Typhoid fever (causal agent, *S.* Typhi) is a life-threatening enteric infection that can be transmitted by consuming food or drinking water contaminated with feces from an infected person. It is more common in less industrialized countries. Extracts of pomegranate fruit pericarp were tested by agar well diffusion and found to be highly active when compared to a reference concentration-response curve for ampicillin. In one study, ethanolic pomegranate extracts exhibited greater antibacterial activity than the antibiotic chloramphenicol, but lower activity than trimethoprim (Alanis et al., 2005; Zhang et al., 2020). *Shigella sonnei* showed the highest susceptibility to the extracts.

This study includes the phytochemical analysis of *P. granatum L.* through HPLC and antibacterial effect against

range of gram negative and gram-positive bacteria. The active compounds, present in *P. granatum L.* and their antibacterial activity was evaluated through HPLC and agar well diffusion method, respectively.

2. Materials and Methods

2.1. Plant sample

The fresh *P. granatum L.* plant fruits were purchased from the local market of Faisalabad, Punjab, Pakistan. The fruit was confirmed and verified from a botanist following the Bessey classification system. These collected fruit peels were dried and stored safely for further proceedings.

2.2. Test organisms

The test organisms were obtained from the culture bank of the Institute of Microbiology, University of Agriculture, Faisalabad Pakistan. The confirmed isolates of three-gram negative bacteria belong to the Enterobacteriaceae *E. coli, S. Typhimurium* and *S. Dysenteriae* and a gram positive bacteria *Staph. aureus* in a final culture strength of 0.5 (1500×10^7 cells /ml) were used. MacFarland standard inoculum was prepared in nutrient broth.

2.3. Preparation of the P. granatum L. extract

The 10 grams of dried peels of *P. granatum L.* were crushed in the electric blender machine to make fine powder. Methanolic extract of *P. granatum L.* peel was prepared through Soxhlet apparatus method in 500ml of methanol solvent by following the already reported method (Lin et al., 2021). *P. granatum L.* powder was poured in the thimbles and plugged with cotton. The thimble and solvent (methanol) was placed in the Soxhlet apparatus. The extract was prepared after 8 hours of cyclic procedure of the apparatus. The cycles were repeated in the apparatus continuously until the solvent changed to colorless from colored. The extract was removed from apparatus and dried in the rotary evaporator. The extract was stored in the refrigerator at 4°C in the Falcon tubes.

2.4. Phytochemical analysis

High Performance Liquid Chromatography (HPLC) was performed for the phytochemical analysis of yellow and red pomegranate ethanolic extracts in Shim-Pack CLC-ODS (C-18) column, 25cm×4.6mm, 5µm in the mobile phase of Gradient: A(H2O:AA-94:6. pH=2.27), B(ACN 100%), flow rate was 1ml/min. The detection was done through UV-Vis Detector 280 nm SPD-10Av. Reverse Phase Gradient HPLC made by Shimadzu, Japan was used for analysis. The pump used in this system was LC-10AT. The diluted extracted solution is injected into the HPLC system in a specific volume of 10 µm (Yasmin et al., 2020).

2.5. Antibacterial assay

The antibacterial activity of *P. granatum L.*) peel extract was determined against three gram negative bacteria (*E. coli, S. Typhimurium* and *S. Dysenteriae*) and a gram positive bacteria *Staph. aureus* by following the agar well diffusion method used by Dahiya and Purkayastha, 2012 with little modifications. 50µl and 100µl of yellow and red pomegranate ethanolic extracts were tested with the positive (Ampicillin and piperacillin tazobactam) and negative control. Zones were measured after incubation at 37°C for 24 hours.

2.6. Minimum Inhibitory Concentration (MIC)

The MIC of *P. granatum L.* peel extract was determined by broth dilution method. Total 2ml volume contains nutrient broth, inoculum standardized through 0.5 MacFarland standard (1500×10^7 cells /ml). Each tube was incubated at 37°C for 24 hours and results were analyzed.

3. Results

3.1. Antibacterial activity

Chemical structures of flavonoid and phenolics found in the methanolic extract of *Punica granatum L.* peel is shown in Figure 1. *P. granatum L.* peel extracts (red and yellow) showed significant antibacterial activity against *Staph. aureus, S. Typhimurium, S. Dysenteriae* and *E. coli*. The extract produced greatest zones of inhibition against *Staph. aureus* as compared to bacteria including even positive control. The zones of inhibition (ZOI) of both red and yellow *P. granatum* L. extracts against different bacteria have been shown in Table 1 and Figure 2.

3.2. Phytochemical analysis of P. granatum L. peel methanol extract

The HPLC chromatogram identified various phenolics and flavonoid compounds in methanolic extract of *P. granatum L.* peel. The most important phenolic compounds identified through HPLC in both red and yellow pomegranate are given in Table 2.

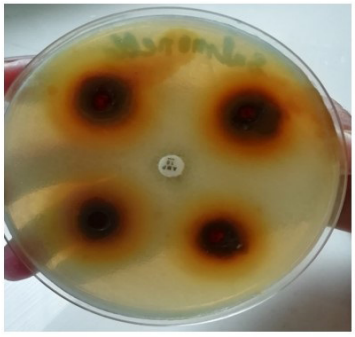
3.3. Minimum Inhibitory Concentration (MIC)

The MIC values of red and yellow pomegranate extract against *Staph. aureus* were 40 µl/ 2ml and 80 µl/2ml or 20 µl/ml and 40 µl/ml, respectively.

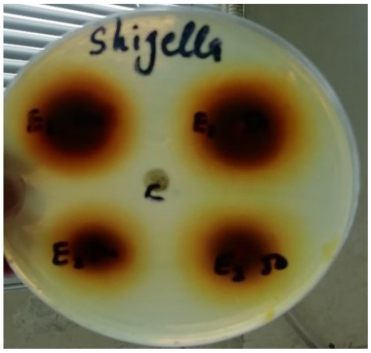
4. Discussion

Red and yellow *P. grantum L.* are already reported and showed comprehensively good results against different microbes. ZOI against *E. coli* and *Staph. aureus* determined by Khan and Hanee, 2011 were 25.5 and 22.5 mm, respectively. In current study, a significant difference was observed against *Staph. aureus*, between methanolic extract of both red and yellow *P. granatum L.* showed upto 30 mm ZOI in triplicates. It was more than positive control. So, it can be stated that methanolic extract of *P. granatum L.* peel can be effective against infections that are caused by *Staph. aureus* and it was more effective against gram positive bacteria than gram negative. In the study carried by Jaisinghani et al., 2018. The inhibition zone was greater against *S. Typhimurium* than *Staph. aureus* and *E. coli*. The

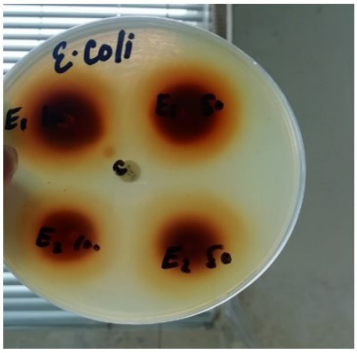
Zones of Inhibitions of *Staph. aureus*



Zones of Inhibition of *S. Typhimurium*



Zones of Inhibition of *S. Dysenteriae*



Zones of Inhibition of *E. coli*

Figure 1. Chemical structures of flavonoid and phenolics found in the methanolic extract of *Punica granatum L.* peel.

Table 1. ZOI of bacterial strains against different concentrations of P. grantum L. peel methanolic extracts.

Bacterial cultures	Control discs	Yellow Extract		Red Extract	
		50µl	100µl	50µl	100µl
Staph. aureus	13mm	26mm	28mm	25mm	27mm
S. Typhimurium	24mm	10mm	15mm	9mm	8mm
S. Dysenteriae	-	10mm	11mm	11mm	12mm
E. coli	-	9mm	13mm	12mm	15mm

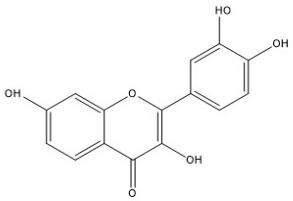
Table 2. Concentration of different flavonoid and phenolics compounds in *P. granatum L.* methanolic extract determined through HPLC.

Components	Red pomegranate	Yellow pomegranate
Flavonoid		
Quercetin	114.26 ppm	33.91 ppm
Phenols		
Gallic acid	25.31 ppm	178.95 ppm
Caffeic acid	10.79 ppm	34.27 ppm
Benzoic acid	42.15 ppm	99.94 ppm
P-coumaric acid	2.94 ppm	5.95 ppm
M-coumaric acid	-	4.23 ppm
Cinnamic acid	3.29 ppm	12.47 ppm
Ferulic acid	12.74 ppm	-

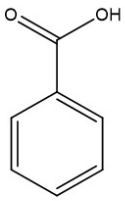
zones of inhibition against *S. Typhimurium* were around 28mm while against *Staph. aureus* and *E. coli* were 26 and 23mm, respectively. In current study, the highest zones of inhibition were against *Staph. aureus*.

Antibacterial activity of *P. granatum L.* peel extract in ethanol solvent against *S. typhimurium* showed minimum-inhibitory concentration (MIC) ranged from 62.5 to 1000x03BCgmL−1. The zones of inhibitions were 11, 14 and 15mm against *S. Typhimurium*. All the three strains showed inhibition zones (Choi et al., 2011). In the current study, the average of zone of inhibition is 16 and 11 mm. There is much similarity in the diameter of inhibition zones against *S. Typhimurium*.

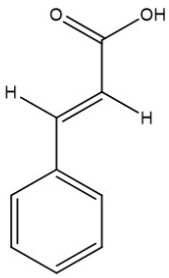
Chebaibi and Filali (2013) reported that antimicrobial activity of *P. granatum L.* is due to the presence of active components. They isolated the components (phenols, tannins, flavonoids and steroids) but they did not explain the quantities of the active components. We isolated the



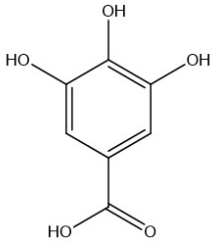
A. Quercetin structure



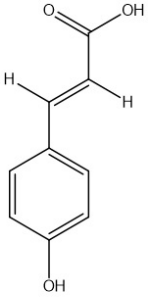
D. Benzoic acid



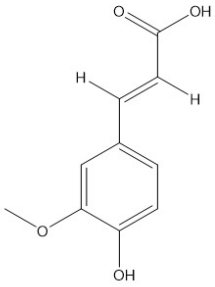
G. Cinnamic acid



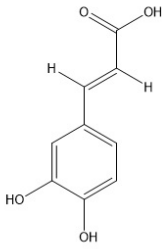
B. Gallic acid



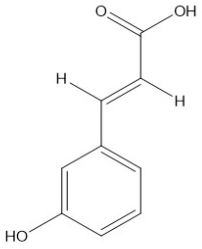
E. P-Coumaric acid



H. Ferulic acid



C. Caffeic acid



F. M-Coumaric acid

Figure 2. Zones of inhibition of flavonoid and phenolics found in the methanolic extract of *P. granatum* L. peel against different public health bacteria.

same active components and with their quantity in ppm (parts per million).

P. granatum L. extract has no antibacterial effects against gram negative bacteria (Kanatt et al., 2010). They reported that there were no effects of *P. granatum L.* extract on *E. coli* and *S. Typhimurium*. In the gram-positive bacteria, *P. granatum L.* extract showed good antimicrobial activity even at the concentration of 0.01%. In the current study, the *P. granatum L.* extract shows good antimicrobial activity against both the gram positive and gram-negative bacteria.

P. granatum L. is good antimicrobial agent against gram positive and gram-negative bacteria (Opara et al., 2009). There is much similarity in our results of anti-microbial activity against the pathogenic bacteria (gram positive and gram negative).

Pomegranate has exhibited bactericidal activity against food and waterborne pathogenic bacteria including *Salmonella* Typhi (*S*. Typhi) (Perez and Anesini, 1994; Rani and Khullar, 2004), *Vibrio cholerae* (Guevara et al., 1994; Mathabe et al., 2006), *Yersinia enterocolitica*, *Shigella* spp. (Alanis et al., 2005; Mathabe et al., 2006), and *Listeria monocytogenes* (*L. monocytogenes*) (Lucas and Were, 2009; Shan et al., 2011). Typhoid fever (causal agent, *S*. Typhi) is a life-threatening enteric infection that can be transmitted by consuming food or drinking water contaminated with feces from an infected person

particularly pediatric infections. The use of pomegranate extracts as therapeutic intervention is more common in less industrialized countries. Extracts of pomegranate fruit and peel are also tested by agar well diffusion and found to be highly active when compared to a reference concentration-response curve for ampicillin. In one study, ethanolic pomegranate extracts exhibited greater antibacterial activity than the antibiotic chloramphenicol, but lower activity than trimethoprim (Alanis et al., 2005).

5. Conclusion

From the study undertaken, it can be concluded that methanol extract of *P. granatum L.* peel has flavonoid and phenolic contents and showed antimicrobial activity particularly against *Staph. aureus*. Pomegranate extracts may be used therapeutically against public health bacteria and infections causing pediatric infections *e.g.*, diarrhea and dysentery along with several other diseases.

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