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Punica granatum L. Kabuklarinin Etanol Ekstraktlarının Sitotoksik ve Antibakteriyel Aktivitelerinin Değerlendirilmesi

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ÖZET: Çalışmada, nar kabuğu ekstraktlarının antibakteriyel aktivitesi ve güvenilirliğini kanıtlamak amacıyla insan dermal fibroblast primer hücreleri üzerindeki sitotoksisitesinin araştırılması amaçlanmıştır. Nar kabuklarının etanol özütlerinin antibakteriyel etkinliği *Acinetobacter baumannii*, *Escherichia coli*, *Staphylococcus aureus* MRSA ATCC 67101, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Burkholderia cepacia*, *Bacillus cereus*, *Citrobacter freundii* ve *Cedecea neteri*'ye karşı disk difüzyon ve mikrodilüsyon yöntemleriyle test edildi. Aynı zamanda, ekstraktın insan dermal fibroblast hücreleri üzerindeki etkileri de WST-8 deneyi ile belirlendi. Çalışmanın sonuçlarına göre; nar kabuklarının etanol özütü, *A. baumannii*, *E. coli*, *S. aureus* MRSA ATCC 67101, *P. aeruginosa*, *E. faecalis*, *S. epidermidis*, *B. cepacia*, *B. cereus*, *C. freundii* ve *C. neteri*'ye karşı 100 ila 500 µg mL^{-1} arasında değişen oranlarda MİK değerleri ile potansiyel olarak etkiliydi. Çalışmada ekstraktın *K. pneumoniae*, *S. aureus* and *S. pneumoniae*'ye karşı inhibisyon aktivitesi gözlenmedi. Aynı zamanda *Punica granatum* L. kabuğunun etanol özütü, dermal fibroblast hücrelerine karşı da herhangi bir sitotoksisiteye sahip değildi. Çalışmanın sonuçlarına göre, ekstraktın antibakteriyel özelliğe sahip olduğu ve toksik etkisi olmaması nedeniyle de birçok endüstriyel üründe doğal koruyucu bir bileşen olarak kullanılabileceği önerilmektedir.

Anahtar Kelimeler: *Punica granatum* L., nar kabuğu, antibakteriyel aktivite, sitotoksisite, WST-8 test

The Evaluation of Cytotoxic and Antibacterial Activity of the Ethanol Extract of *Punica granatum* L. Peels

ABSTRACT: In this study, it was aimed to investigate the antibacterial activity of pomegranate peel's extract and the cytotoxicity on the human dermal fibroblast primary cells to rationalize the safe usage of this extract. The antibacterial efficiency of ethanol extract of pomegranate peels was evaluated against *Acinetobacter baumannii*, *Escherichia coli*, *Staphylococcus aureus* MRSA ATCC 67101, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Burkholderia cepacia*, *Bacillus cereus*, *Citrobacter freundii* and *Cedecea neteri* by disc diffusion and microdilution assays. At the same time, the effects of this extract on the human dermal fibroblast primary cells were determined by WST-8 assay. The ethanol extract of pomegranate peels was potentially effective with different efficiency against *A. baumannii*, *E. coli*, *S. aureus* MRSA ATCC 67101, *P. aeruginosa*, *E. faecalis*, *S. epidermidis*, *B. cepacia*, *B. cereus*, *C. freundii*, and *C. neteri* at MIC's ranged from 100 to 500 µg mL^{-1}. However, it exhibits no inhibition activity against *K. pneumoniae*, *S. aureus* and *S. pneumoniae*. The ethanol extract of *Punica granatum* L. peel exhibited no cytotoxic activity against the normal human dermal fibroblast primary cells. According to results it may be suggested that this extract possess antibacterial properties and the safety, and therefore, it can be used as a natural preservative ingredient in many industrial products.

Keywords: *Punica granatum* L., pomegranate peels, antibacterial activity, cytotoxicity, WST-8 assay

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INTRODUCTION

Pomegranate (*Punica granatum* L.), one of an oldest and common fruit trees, belongs to the family Punicaceae. The pomegranate has been naturally grown in a wide region from Iran to India. However, it has been cultivated all over the world, especially over the Mediterranean Region for many years (Divyashree and Kunnaiah, 2014). The fruits of pomegranates have been consecrated to be a symbol of life, femininity, immortality, permanence and erudition since ancient times (Lamar et al., 2008, Yahya et al., 2018). The fruits of pomegranate attract a great deal of attention in pharmacology, food, new drug development due to its multiple bioactive properties such as antioxidant, antibacterial, hypolipidemic, antiviral, anti-diabetic, anti-neoplastic, anti-diarrhea and helminthic (Rios and Recio, 2005; Lamar et al., 2008; Akhtar et al., 2015). Besides, pomegranate has been used for the production of many food products such as oil, jelly, jam, dietary supplements and as beverages such as juice and wine (Kaur et al., 2018). The ripe and deep red fruits of the plant are about five inches wide with leathery peel and the peels are generally wasted (Divyashree and Kunnaiah, 2014). The fruit peels are the major problem for the pollution monitoring agencies and the fruit processing industries in terms of environmental health. Therefore, the researchers have given increasing attention to produce useful products from waste peels (Manthey and Grohmann, 2001). Many researchers have reported that peels of pomegranate have nutritional value and bioactive compounds as much as its edible parts (Akhtar et al., 2015; Kaur et al., 2018; Yahya et al., 2018). It can be used in pharmacology such as an antimicrobial agent (Akhtar et al., 2015). However, unnecessarily and extremely use of the antibiotic drugs has increased the pathogenic bacteria resistant against all antibiotic treatments and therefore the acquired resistance has become threat for both human and animal (Bbosa and Mwebaza, 2013). Extremely use of antibiotics has also adverse effects on microorganisms and their efficiency in ecosystem since they have a significant role in the decomposition of organic wastes. In this context, natural antimicrobial agents might be an alternative treatment. For example, the pomegranate peel extract (PPE) is an alternative antibacterial agent to antibiotics and synthetic antibacterial substances. The PPE exhibited antimicrobial effect due to its diverse phytochemicals. Its phytochemicals are described as punicalin, punicalagin, pedunculagin, granatin, methyl gallate, gallic acid, ellagic acid, corilagin, casuarinin, catechin, cyanidin, epicatechin, epigallocatechin 3-gallate, kaempferol, luteolin, naringin, quercetin, etc. (Middha et al., 2013).

In this study, it was aimed to investigate the antibacterial activity of pomegranate peel's extract and the cytotoxicity on the human dermal fibroblast primary cells to rationalize the safe usage of this extract.

MATERIALS AND METHODS

Extraction of *P. granatum L.* Peels

The fresh fruits of pomegranate used in the study were obtained from Antalya and delivered to the laboratory in fruit season (December, 2018). The washed fruits were peeled. The peels were air-dried in the shade for a week and then were grounded into powder by using a grinder. Fifty grams of the grinded peels was extracted in 500 mL ethanol by Soxhlet extraction technique for 10 h. The extract was filtered and the ethanol was completely evaporated at 40 ºC in a rotary evaporator. Then, the extract was weighed and dissolved with 96% ethanol to prepare in different concentrations (100, 200, 300, 400, 500, 600 µg mL^{-1}) with 96% ethanol and stored at +4 ºC for the further studies (Wang et al., 2006).

Bacterial Strains

The bacterial strains used in this study were given in Table 1. They were supplied from Erzurum Technical University, Molecular Biology and Genetics Laboratory. The bacteria were cultured

aerobically on Mueller Hinton Agar (MHA, Merck 1.05437) and in Mueller Hinton Broth (MHA, Merck 1.10293) at 37 ºC for 24 h.

Antibacterial Studies

In this study, different concentrations of PPE prepared with ethanol (96%) were evaluated against 13 bacteria. Disc diffusion assay and microdilution assay were performed to determine the antibacterial potential of PPE.

Disc diffusion assay

One hundred µL of microbial culture (10^8 cfu mL^{-1}) was surface-inoculated on MHA with a sterile swab. The discs (6 mm in diameter) were individually saturated with 10 µL of the prepared concentrations of PPE and placed on the same medium. Absolute ethanol was used as negative control (10 µL/disc). Ofloxacin (10 µg/disc), Netilmycin (30 µg/disc) and Cefsulodin (30 µg/disc) were used as positive controls (Table 1). After the application of the test materials and antibiotics, the petri dishes were incubated at 37 °C for 24-48 h and antibacterial potentials of test materials were evaluated by measuring the inhibition zones around the discs. Each experiment was performed in triplicate (Gormez et al., 2015).

Micro-well dilution assay

The minimum inhibitory concentrations (MIC) values of the extracts against bacterial strains were determined by micro-well dilution method. In this purpose, the final concentrations of the extracts were ranged from 600 to 100 µg mL^{-1} and prepared by using 96% ethanol. The bacteria were adjusted to 0.5 McFarland standard turbidity (Jorgensen *et al.* 1999). The 96-well plates were prepared by dispensing 95 µL of MHB and 5 µL of the inoculum of the tested bacteria into each well. Then, one hundred µL of each prepared concentrations of the final extracts were individually also added into the wells. The negative control was prepared by dispensing 195 µL MHB and 5 µL of the bacterial inoculate. The plate was covered with a sterile plate sealer and incubated at 37 °C for 24-48 h. Bacterial growth was determined by measuring the absorbance at 600 nm with a microplate reader (EL×800 universal microplate reader). The MIC was defined as the lowest concentration of the compounds to inhibit the growth of bacteria. Each experiment was performed in triplicate (Gormez et al., 2015).

Cell Line and Culture Conditions

The human dermal fibroblast primary cells (PCS-201-012) were obtained from American Type Culture Collection (ATCC). Dermal fibroblast primary cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 µg mL^{-1} streptomycin, 100 U mL^{-1} penicillin, and 7,5 mM L-glutamine. This cell line was incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 48 h.

Cell Viability Analysis

The cultured cells were sub-cultured 2 to 3 times per week when they reached 80-90% confluence. Experiments were performed in 10 groups of cells as follow: Group I: control group, from Group Π to Group X were pre-treated with different doses of *P. granatum* extracts (12,5, 25, 50, 100, 200, 300, 400, 500 and 600 µg mL^{-1}). The extracts were tested for *in vitro* cytotoxicity, using "Cell Viability Detection Kit-8 (CVDK-8, Ecotech Biotechnology, Turkey)" which based on WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5 (2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] quantification on the human dermal fibroblast primary cells (Ishiyama *et al.* 1997). Healthy fibroblast cells were incubated in the 96-well plates with $5x10^3$ cells in each well in 100 µL of media and grown overnight. The cells were then incubated with determined concentrations of PPE (12.5, 25, 50, 100, 200, 300, 400,

500 and 600 µg mL^{-1}) for 48 h at 37 °C under 5% CO_2. After the incubation process, 10% of WST-8 solution was added to each well in aseptic and dark conditions and the cultures were incubated at 37 °C in 5% CO_2 for 3-4 h. The absorbance of 96-well plate was measured at 450 nm wavelength by spectrophotometer (BioTek, EPOCH). The control group was used to determinate only the absorbance of cells and growth medium.

Statistical Analysis

All experiments were performed in triplicate. All analyses were performed with GraphPad Prism software and the results presented as mean ± standard deviation.

RESULTS AND DISCUSSION

The antibacterial effect of PPE was tested against microorganisms including seven strains of Gram-negative bacteria (*A. baumannii*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *B. cepacia*, *C. freundii*, *C. neteri*) and six strains of Gram-positive bacteria (*E. faecalis*, *B. cereus*, *S. aureus*, *S. epidermidis*, *S. aureus* MRSA, *S. pneumoniae*) by using disc diffusion method. The antibacterial results and MIC values of PPE were given in Table 1. According to the obtained results, PPE did not show antibacterial effect against *K. pneumoniae*, *S. aureus* and *S. pneumonia*. These results were significantly different from the previous studies performed against several other bacteria (Abdollahzadeh et al., 2011; Fawole et al., 2012). In these mentioned literatures, the researchers have extracted the peel of pomegranate in different solvent (methanol) and applied to pathogenic microorganisms at different (higher) concentrations. Thus, they have obtained different results than the current study. It has also been reported that ethanol extract of *P. granatum* peel have shown antimicrobial activity against many microorganisms by different researchers (Voravuthikunchai et al., 2004; Choi et al., 2011; Khan and Hanee, 2011; Anibal et al., 2013).

Table 1. Antibacterial activity of the ethanol extracts of *Punica granatum* L.

Bacteria	Disc Diffusion Test*						MIC**	Negative Control	Standard antibiotic discs***		
	Concentrations (µg mL^{-1})										
	600	500	400	300	200	100		Ethanol	OFX	NET	CFS
Acinetobacter baumannii	7.5	7	-	-	-	-	500	-	9	8	8
Bacillus cereus	8.5	8.5	8	7.5	-	-	300	-	11	8.5	8
Burkholderia cepacian	9.5	8.5	8	8	7.8	-	200	-	10	10	9.5
Cedecea neteri	9	8	7.5	7.5	7.0	-	200	-	10.5	10	9
Citrobacter freundii	10	9.5	9	9	8.5	8	100	-	11	11	10
Escherichia coli	8	7.8	7.5	7.4	7	-	200	-	9.8	9.8	7.8
Enterococcus faecalis	11	10	9.5	9	8.5	8.5	100	-	11	10.5	9.5
Klebsiella pneumoniae	-	-	-	-	-	-	-	-	9	8	8
Pseudomonas aeruginosa	10	9	8	7.5	7.0	-	200	-	10	10	9
Staphylococcus aureus	-	-	-	-	-	-	-	-	10	10	9
Staphylococcus aureus MRSA ATCC 67101	9	8	7.8	7.5	-	-	300	-	11	10	8
Staphylococcus epidermidis	8.5	8	-	-			500	-	13	12	12
Streptococcus pneumoniae	-	-	-	-	-	-	-	-	10	10	8

*Diameter of inhibitory zone [mm] for different concentrations, **MIC: Minimum inhibitory concentrations, ***OFX: ofloxacin (10 µg/disc), NET-30: Netilmycin (30µg/disc), CFS: Cefsulodin (30µg/disc) were used as positive reference standard antibiotic discs (Oxoid).

In our present study, PPE was exhibited static activity against *B. cepacia*, *C. neteri* and *S. aureus* MRSA ATCC 67101. In the literature, no research has been found in the antibacterial activity of PPE against *B. cepacia* and *C. neteri*. However, Gould et al. (2009), Abdollahzadeh et al. (2011) and Bakkiyaraj et al. (2013) reported the antibacterial activity of PPE against *S. aureus* MRSA, *C. albicans*,

and *S. aureus*, respectively. Gould et al. (2009) applied PPE with cupric sulphate. It is thought that the difference in result may be due to cupric sulphate application against MRSA. The other microorganisms used in this study were sensitive to PPE. It was determined that PPE had an antibacterial effect with the concentration of 600 μg mL^{-1} against *E. faecalis*, *C. freundii* and *P. aeruginosa* giving inhibition zones of 11, 10, and 10 mm, respectively. The MIC values of PPE for *E. faecalis*, *C. freundii* and *P. aeruginosa* were determined by 100, 100, 200 μg mL^{-1}, respectively. According to the previous studies, pomegranate extracts were more effective against Gram-positive bacteria because of the structural differences between the cell walls (Wang et al., 2010; Rosas-Burgos et al., 2017). However, there was not any significant differences found in this study between Gram-positive and Gram-negative bacteria in terms of antibacterial results. It was determined that different *S. aureus* strains were sensitive against the extract at different levels in this study. The highest antibacterial activity of PPE was determined against *E. faecalis*. On the other hand, the inhibition effect of the extract was lower than the standard antibiotic discs. There have been many studies showings that the antibacterial activity of plant compounds can be effective as much as the antibiotics (Gormez et al., 2015). These results can be explained by the used solutions for dissolution of the plant ingredients and the concentrations of the extract. If the concentrations of the extract were increased, the results could be found more effective.

The compounds with lower antibacterial activity still have an important role in the development of antibacterial drugs that can be used particularly for the treatment of some infectious diseases in children and of non-severe infections (Efe, 2019). Besides, the plant extracts and their effective ingredients can be used as alternatives to chemical additives.

The obtained results for antimicrobial studies are in accordance with previous studies of PPEs (Al-Zoreky, 2009; Duman et al., 2009; Abdollahzadeh et al., 2011; Fawole et al., 2012). According to the literature, it has been reported that the antibacterial efficacy of pomegranate peel extracts and constituents were differ in relative abundance of phenolic and flavonoid constituents (Dey et al., 2012). Their contents have been described mainly as polyphenolic compounds such as flavonoids, anthocyanins, phenolic acids, and tannins (ellagitannin, punicalin, gallic acid, punicalagin, ellagic acid, gallotannins, etc.) (Singh et al., 2018). Polyphenols have characteristic aromatic rings having hydroxyl groups. They behave as antimicrobial agents by forming complexes with proteins of bacteria cell walls thus, lysis the cells (Akhtar et al., 2015; Singh et al., 2016). Also, they interact with the sulfhydryl groups of the soluble and extracellular microbial proteins and inhibit their activities (Dey et al., 2012). Besides the hydroxyl groups of polyphenols induce delocalization of electrons and behave as protons exchangers and pH gradient around the cell membrane decrease. In the end, ATP transport system and membrane functions for the nutrient uptake are damaged and microbial cell death occurs (Pisoschi et al., 2017).

The cytotoxic effect of PPE on the normal human dermal fibroblast primary cell line was evaluated through WST-8. The cells were exposed to PPEs with the concentrations ranging from 12.5 to 600 μg mL^{-1} and the results showed that there were dose-dependent increases in proliferation as compared to untreated control groups. The negative control exhibited no cytotoxic activity against the normal human dermal fibroblast primary cell line. As seen in Fig.1, the cell viability was consistently going up based on the increasing concentration of the extract (12.5, 25, 50, 100, 200, 300, 400, 500 and 600 μg mL^{-1}). There were 3, 3.2, 2.6, 4.1, 4.8, 5.5, 5.5, 7.9- and 10-fold increase of cell proliferation when the cells were exposed to a specified dose of the test material, respectively (Fig. 1). According to the previous studies, it has been known that pomegranate shows very different properties such as anti-proliferative, anti-inflammatory, antioxidant, anti-angiogenic, anti-metastatic and anti-invasive and also induces apoptosis in cancer cell lines (Ismail et al., 2012; Orgil et al., 2014; Zhou et al., 2015; Khwairakpam et al., 2018). It also affects different signalling pathways such as PI3K/AKT/mTOR, NF-κB, and Wnt, and

down-regulates the expression of genes that are responsible in cancer development, such as pro-inflammatory cytokines, VEGF, MMPs, cyclins, c-met, Cdks, and antiapoptotic genes (Khwairakpam et al., 2018). In addition, the pomegranate has been demonstrated to apply antitumor effects on different cancer cells such as human prostate cell line, lung cancer cell line etc. in many studies (Sánchez-Lamar et al., 2008; Annu et al., 2018; Sineh et al., 2018). According to another study pomegranate phenolic compounds were applied to the human keratinocyte cell line (HaCaT). A standard commercial pomegranate extract (Pomella®), ellagic acid, punicalagin, and urolith A containing phenolic compounds applied on HaCaT cell line showed protective effects against oxidative stress caused by hydrogen peroxide (H_2O_2). The pomegranate extract, punicalagin, and ellagic acid reduced the production of H_2O_2-induced ROS in HaCaT cell line by 1.03-, 1.37-, and 2.67-fold, respectively. At the same time, these extracts increased the cell viability of H_2O_2-stimulated HaCaT cells by 89.9, 94.9, and 90.0%, respectively (Malik et al., 2005). In this study, PPE was applied to the normal cell line instead of cancer cell lines differently from the literature in order to assure the safety of the plant extract. It is known that PPE contains phenolic compounds, antioxidants, phytochemicals and volatile aroma compounds. It is thought to increase the viability of the normal cells due to their components it contains.

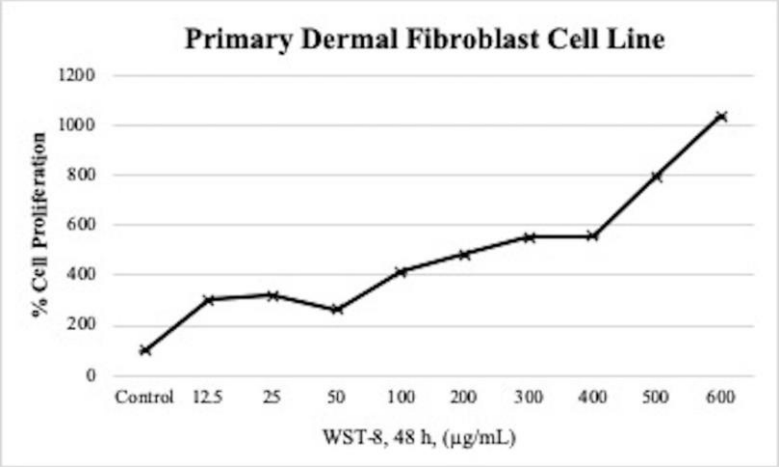

Figure 1. Cell viability of primary dermal fibroblast cell line treated with *P. granatum* peel extract. Cells were treated with extracts of plant from 12,5 to 600 µg mL^{-1} concentration for 48 h. Cytotoxicity was determined using WST-8. Data expressed as mean ± standard deviation.

CONCLUSION

In this study, it has been showed that the extract of pomegranate peels was active against *E. coli*, *E. faecalis*, *B. cepacia*, *C. freundii*, *C. neteri*, *P. aeruginosa* and *S. aureus* MRSA ATCC 67101. Among these bacteria *E. faecalis* and *C. freundii* were found to be more sensitive. At the same time, it was demonstrated that the increasing concentrations of extract of pomegranate peels has increased the normal cell proliferation. As a result, it is possible to say that it can be use an alternative antibacterial agent due to the antibacterial activity and non-cytotoxic potential of the pomegranate peels extract.

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Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

The authors declare that they have contributed equally to the article.

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