

Research Article

Determination of Antibacterial Effect of *Punica granatum* Shell Extract

İpek Ada^{1*}, Fatih Candemir¹

¹Operating Room Services Programme, Health Services Vocational School, Altınbaş University, Istanbul, Turkey.

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Abstract: *Punica granatum* (Pomegranate) is used as fresh fruit or processed products such as pomegranate juice, pomegranate sour and wine, jam. Although pomegranate is produced in our country, pomegranate shell is discarded without use. In recent studies; *P. granatum* containing rich phenolic compounds is known to have antibacterial, antifungal and antioxidant activity. The aim of this study was to investigate the antibacterial effect of *P. granatum* shell extracts prepared by ethanol, methanol and distilled water mixture on bacteria isolated from degraded cheese and salami samples by well diffusion method. In this study, identification of bacteria were performed by API biochemical identification test kits. API® 20 Strep for *Listeria monocytogenes*, API® 20E for *Salmonella typhimurium* and API® Staph test kits for *Staphylococcus aureus* were used. When the results of this study are evaluated, the zone diameters were measured as 18-24 mm and it was determined that *P. granatum* shell extract has antibacterial effect against to isolated bacteria from salami and cheese samples.

Keywords: Antibacterial activity; extract; inhibition zone; pomegranate; *Punica granatum*; well diffusion

Address of Correspondence: İpek Ada - ipek.ada@kemerburgaz.edu.tr, ORCID: orcid.org/0000-0003-4787-8171

Tel: +90(212)7094528, Operating Room Services Programme, Health Services Vocational School, Altınbaş University, Kartaltepe Mahallesi, Incirli Caddesi No: 11-A, 34147 Bakırköy, İstanbul, Turkey

1. Introduction

Today, it is known that food borne infections cause an increase in poisoning. On the other hand, it has been found that synthetic preservatives used in food production have a carcinogenic effect on human health. For this reason, producers have turned to the production of food preservatives which have antibacterial properties derived from natural products and which do not adversely affect human health (Al- Zoreky, 2009).

In recent years, the numbers of studies about identification of antioxidant and antimicrobial activities of natural compounds obtained from plants or fruits and their usage rate in the preservation of food products have been increased. Pomegranate (*Punica granatum*) shell is used as antimicrobial food

supplement or drug in many countries because of its high phenolic contents. It has been determined in some experiments that the phenolic compounds obtained from pomegranate shell have antibacterial, antifungal, antioxidant, antidiabetic and anticarcinogenic effects (Vuorela et al., 2005; Wang et al., 2010).

Studies of the effect of pomegranate on human health have shown that it strengthens the immune system, balances cholesterol and blood glucose value, protects against heart diseases and has anti-carcinogenic effect. In particular, it has been determined that phenolic compounds in *P. granatum* shell have antibacterial, antifungal, antiviral and anti-helminthic activity (Fischer et al., 2011; Gundogdu et al., 2011).

The aim of this study was to investigate the antibacterial effect of *P. granatum* shell extracts prepared by ethanol, methanol and distilled water mixture on bacteria isolated from degraded cheese and salami samples by well diffusion method.

2. Materials and Methods

2.1. Preparation of Samples

The bacteria were isolated that 25 g salami and cheese samples in expiration date past. The samples were homogenized in 225 mL buffered peptone water for pre-enrichment of bacteria. For the homogenization step, all samples were placed in a sterile polyethylene bag and it was shown in Figure 1. The samples were shaken for 2 minutes in Stomacher (Stomacher 400). 1.5×10^8 CFU/mL (McFarland No: 0.5) bacterial suspensions were prepared in sterile distilled water and then a series of dilutions (10^8 , 10^7 , 10^6 , 10^5 CFU/mL) were prepared to determine the number of bacteria. 100 μ L of each dilution series were inoculated to Nutrient Agar (Oxoid). Each Petri dish was allowed to incubate at 37 °C. At the end of the period, bacterial colony counts were made.

Figure 1. Homogenization step of bacteria isolated from salami and cheese samples.



2.2. Isolation of *Salmonella typhimurium*

The homogenized samples in peptone water for the pre-enrichment stage were incubated at 37 °C for 24 hours. At the end of the time, 0.1 mL samples were inoculated into the tubes containing 10 mL of

Rappaport-Vassiliadis Broth (Merck) for selective enrichment and incubated at 42 °C for 24 hours. The samples were inoculated on to XLD (Xylose Lysine Deoxycholate) Agar medium (Merck) and incubated at 37 °C for 24 hours. *S. typhimurium* suspected bacteria colonized on XLD Agar medium (red colonies with black centers) were evaluated according to Gram staining method and oxidase activity results. Gram and oxidase negative bacterial colonies were planted on Nutrient Agar (Oxoid) medium and API® 20E (Biomériux, France) test kit was used for the identification of suspected bacteria according to the manufacturer's instructions.

2.3. Isolation of *Staphylococcus aureus*

The samples homogenized in buffered peptone water for the pre-enrichment stage were incubated at 37 °C for 24 hours. At the end of the incubation period, 10⁻³ dilution series were prepared from the samples in Buffered Peptone Water for selective enrichment and 0.1 mL samples inoculated on to Baird Parker Agar (Oxoid) medium containing 5% Egg Yolk Tellurite. The samples incubated at 37 °C for 24 hours. At the end of the period, gray-black colored colonies were evaluated as suspicious for *S. aureus*. The samples were evaluated according to Gram staining, catalase, coagulase and oxidase activity. API® Staph (Biomériux, France) test kit was used for identification of *S. aureus* according to the manufacturer's instructions.

2.4. Isolation of *Listeria monocytogenes*

The samples homogenized in peptone water for the pre-enrichment stage were incubated at 37 °C for 24 hours. At the end of the time, 0.1 mL of the samples in Buffered Peptone Water were inoculated into tubes containing 10 mL of Listeria Enrichment Broth (Merck) for selective enrichment and incubated at 37 °C for 24 hours and then plated on Palcam Agar (Merck) medium. The samples were incubated at 37 °C for 24 hours. *Listeria monocytogenes* suspected bacteria colonized on Palcam Agar medium (gray-green, black zone, round colonies) were evaluated according to Gram staining method and oxidase activity results. Gram negative, oxidase negative bacterial colonies were plated on Nutrient Agar (Oxoid) medium and API® 20Strep (Biomériux, France) test kit was used for identification of suspected bacteria according to the manufacturer's instructions.

2.5. Preparation of *Punica granatum* Shell Extract

P. granatum shells brought to the laboratory were separated into small particles by using sterile lancet and the allowed to dry at room temperature. The dried specimens were pulverized by passing 3 times through a small-diameter sieve, and then 10 g of the sample was added to 100 mL of the mixture (70% ethanol, 70% methanol, distilled water). The mixture was placed in the evaporator and allowed to stand at 30 °C an hour at magnetic stirrer. It was shown in Figure 2. At the end of the incubation period, the homogenously mixed solution was passed through a 0.22 µm pore size injector filter (Millipore) to be sterile.

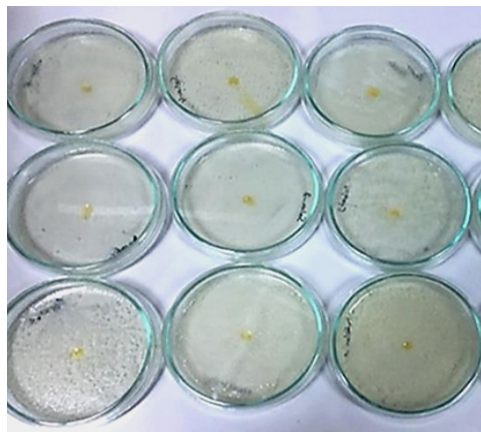
Figure 2. The evaporator used to obtain extract of *P. granatum* shell.



2.6. Evaluation of Antibacterial Activity of *Punica granatum* Extract

1.5×10^8 CFU/mL (McFarland No: 0,5) bacterial suspensions were prepared in sterile distilled water after identification of the bacteria isolated from salami and cheese samples by API biochemical test kit. 100 μ L of the samples were inoculated on Mueller Hinton Agar (Merck) medium by spreading method. All of samples were incubated at 37 °C for an hour. A well diffusion method was used to evaluate the antibacterial effect of *P. granatum* shell extract (Al-Zoreky, 2009). For this purpose, the wells were opened into Mueller Hinton Agar (Merck) medium and 100 μ L of *P. granatum* shell extract were inoculated to Mueller Hinton Agar medium. 100 μ L of sterile PBS was used as a negative control. The samples were incubated at 37 °C for 24-48 hours. All of samples were run in 3 replicates. The inhibition zone diameters at the end of the time were measured in mm and the antibacterial activity of *P. granatum* shell extract was evaluated. It was shown in Figure 3.

Figure 3. The well diffusion method used to evaluation of antibacterial activity of *P. granatum* shell extract. A 1-3: *S. aureus*; B 1-3: *S. typhimurium*; C 1-3: *L. monocytogenes*.



The antibacterial of extract of *P. granatum* shell against bacteria isolated from salami and cheese samples will be measured inhibition zone diameters (mm). Then, the results were evaluated by comparing CLSI (Clinical & Laboratory Standards Institute) (2012).

3. Results

25 g of samples isolated from salami and cheese samples were transferred to Buffered peptone water for pre-enrichment stage. Then, suspected bacteria were spread on selective media and the appearance of the colony morphology on the media was observed and then the results of colony counts were shown in Table 1 and 2.

Table 1. The results of colony counts of bacteria isolated from salami samples.

Colony counts (CFU/ mL)	Serial dilutions (10 ⁻² /10 ⁻⁵ CFU/mL)			
	10 ⁻² CFU/mL	10 ⁻³ CFU/mL	10 ⁻⁴ CFU/mL	10 ⁻⁵ CFU/mL
	1100	856	112	9

Table 2. The results of colony counts of bacteria isolated from cheese samples.

Colony counts (CFU/ mL)	Serial dilutions (10 ⁻² /10 ⁻⁵ CFU/mL)			
	10 ⁻² CFU/mL	10 ⁻³ CFU/mL	10 ⁻⁴ CFU/mL	10 ⁻⁵ CFU/mL
	1310	744	98	14

The bacterial species identification of bacteria was performed by API biochemical identification test kits.

API® 20 Strep for *L. monocytogenes*, API® 20E for *S. typhimurium* and API® Staph test kits for *S. aureus* were used. Bacteria isolated from salami and cheese specimens were cultivated on Mueller Hinton Agar (Merck) media. After biochemical identification of bacterial specimens was done, well diffusion method was used to assess the antibacterial activity of *P. granatum* shell extract. The inhibition zone diameters (mm) were shown in Table 3.

When all of results obtained were evaluated, it was determined that the extract obtained from the *P. granatum* shell had antibacterial activity against bacteria isolated from salami and cheese samples. It was shown in Figure 4.

Figure 4. The antibacterial activity of extract of *P. granatum* shell.

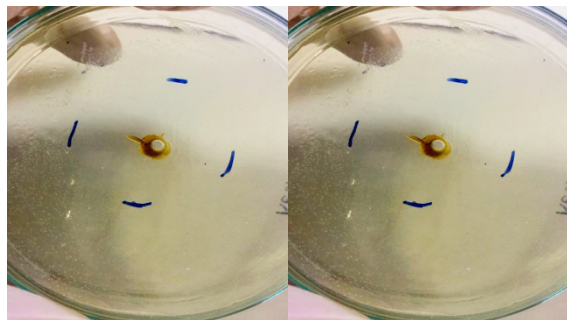


Table 3. The antibacterial effect of the extracts of *P. granatum* against bacteria isolated from salami and cheese samples.

Bacteria	Inhibition zone diameters (mm)	Average zone diameters (mm)
<i>S. aureus</i>	24 mm – 22 mm	23 mm
<i>S. typhimurium</i>	20 mm – 22 mm	21 mm
<i>L. monocytogenes</i>	18 mm – 18 mm	18 mm

4. Discussion

Because of the antimicrobial and antioxidant activity of the phenolic compounds in *Punica granatum*, the reconsidered to be effective in food preservation and as an alternative to synthetic food preservatives (Apaydin, 2008). It is thought that this product will extend the food preservation period and reduce the risk of food poisoning.

It has been determined that the extract obtained from the *P. granatum* shell (249.4 mg/L) contains much more phenolic material than the pulp extract (24.4 mg/L) (Tomas-Barberan and Espin, 2001). In the world and Turkey, pomegranate kernel sand shell is pressed in production of pomegranate juice. As a result of the pressing, there meaning portion is comprised approximately 73 percent of shell and 27 percent of kernel. The antioxidant and antimicrobial phenolic content of the shell part is higher than the other parts of the pomegranate juice; for this reason the work done in recent years has increased (Negi and Jayaprakasha, 2003; Li et al., 2006; Nuamsetti et al., 2012).

According to the studies done, the extracts obtained from pomegranate shell were detected to be effective in extending the shelf life of raw or cooked meat and poul try products (Naveena et al., 2008; Kanatt et al., 2010; Hayrapetyan et al., 2012). In our study, it was determined that the extract obtained from *P. granatum* shell was antibacterial effect against *Staphylococcus aureus*, *Salmonella typhimurium* and *Listeria monocytogenes*. According to this result, it is predicted that the shelf life of raw meat products will be extended.

In a study, when the antibacterial activity of *P. granatum* extractions grown in the Mediterranean Region was investigated, it was found to be effective against *Bacillus megaterium* DSM 32, *Pseudomonas aeruginosa* DSM 9027, *Staphylococcus aureus* Cowan 1, *Corynebacterium xerosis* UC 9165, *Escherichia coli* DM, *Enterococcus faecalis* A10 and *Micrococcus luteus* LA 2971 (Duman et al., 2009). In this study, the antibacterial activity of *P. granatum* shell extract was investigated after isolating bacteria (*S. aureus*, *S. typhimurium* and *L. monocytogenes*) from cheese and salami samples.

It was determined that *P. granatum* extract prepared with methanol had a strong antibacterial effect against *S. aureus* (32.3 mm) and *E. coli* (14.5 mm) (Shan et al., 2007). In other study, the antibacterial effect of extracts prepared from 46 medicinal plants were examined. It was determined that *P. granatum* had the highest total phenolic content compared to other plants and it was suggested that there was a direct correlation between antimicrobial activity and amount of phenolic material. In this study, it was determined that *P. granatum* extract had a strong antibacterial effect against *S. aureus* (23 mm), *S. typhimurium* (21 mm) and *L. monocytogenes* (18 mm) species isolated from cheese and salami samples.

In a study by Dahham et al. (2010), the inhibition zone diameter (25 mm) of *P. granatum* shell extract prepared with methanol against *S. aureus* and the result was found to be similar to the inhibition zone diameter (23 mm) against *S. aureus* in our study. In a study by Atya et al. (2018), the antimicrobial activity of extract of *P. granatum* at a concentration of 200 mg/ml was detected against *Pseudomonas aeruginosa* with a diameter of inhibition zone of 23.5 mm. Similarly, the diameter of inhibition zone was measured 22 mm against *S. aureus*, whereas 19.5 mm against *E. coli*, 17.5 mm against *Staphylococcus epidermidis* and 18 mm against *Staphylococcus saprophyticus*. It was detected that the antimicrobial effect of *P. granatum* shell extract against *S. aureus* (Usman, et al. 2018). In this study, the diameter of inhibition zone was measured 13.67 ± 0.47 mm against *S. aureus*. In our study, the antibacterial activity of extract of *P. granatum* shell against *S. aureus* was observed to be 23 mm.

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Conflict of Interests

Authors declare no conflict of interests

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