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Original Article

Inhibitory effects of ethanolic extract of two Iranian pomegranates peel cultivars on *Staphylococcus aureus* and *Salmonella typhimurium*

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Abstract

In last decades, the antibiotic resistance is considered one of the essential problems. Therefore, uses of waste agricultural products such as pomegranate peel have drawn attention to be used as an effective preservative in food industry. Ethanolic extract of pomegranate peels (*Naderi* and *Mallas*) were prepared. Then, the antibacterial effects on two foodborne pathogens (*Staphylococcus aureus* and *Salmonella typhimurium*) in meat broth and TSB media at two temperatures levels (4 °C and 15 °C) during storage were investigated. The Minimal Inhibitory Concentration (MIC) values of *Naderi* and *Mallas* cultivar for *Staphylococcus aureus* and *Salmonella typhimurium* were measured 15.62 and 62.5 mg/ml, and 19.5 and 64.5 mg/ml respectively. The Minimum Bactericidal Concentration (MBC) of *Naderi* and *Mallas* cultivar were evaluated 125 and 130 mg/ml for both bacteria. All concentration of *Mallas* and *Naderi* Pomegranate Peel Extract (PPE) in meat broth at 4 °C and 15°C inhibited *Staphylococcus aureus* growth. It was reported that PPE was less effective in decreasing the *S. typhimurium* growth compared to the *S. aureus*. *Naderi* cultivar showed better effects on bacterial inhibition in compared to *Mallas* cultivar. According to achieved results, it could be suggested to use the ethanolic extract of pomegranate peel as a useful preservative against foodborne bacteria in the food processing industry.

Keywords: Pomegranates peel, *Naderi* cultivar, *Mallas* cultivar, *Staphylococcus aureus*, *Salmonella typhimurium*

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Introduction

The Infectious disease is known as the one of the major causes of death in the world. During last decades, by increasing concerns about antibiotic resistance the uses of natural and conventional products as a preventive agent for bacterial disease have gained much attention. Therefore, many studies

on antimicrobial activities of herbal medicines have been reported (Amirmohammadi et al., 2014; Asadi-Samani et al., 2014; Bahmani et al., 2014). The presence of antibacterial compounds in plants could have preventive role in the growth of pathogens. Pomegranate (*Punica granatum L.*) is the oldest functional fruits cultivated in Iran and neighboring countries (Gullon et al., 2016). Pomegranate peel is a waste part of the juice industry which compromise about 50% of the total weight of pomegranate fruit (Fawole et al., 2012). The pomegranate peel is rich in bioactive compounds such as ellagic tannins, flavonols, anthocyanins, catechin, procyanidins, ellagic acid and gallic acid (Fawole et al., 2012; Gullon et al., 2016). These constitute differs from one cultivar to another as well as climate changes, and place of growth (Fawole et al., 2012). It was shown that all parts of pomegranate fruit are useful for the treatment of common diseases (Ross et al., 2001). Presence of tannins and other biochemical compounds such as phenolic compounds in pomegranate fruit introduced this fruit as an antibacterial, antiviral, antioxidant, and anti-inflammatory bioactive agent (Fawole et al., 2012; Malviya et al., 2014). Although the pomegranate peel is considered to be effective enough to be used without any enrichments. It was reported that phenolics compounds in pomegranate peel are involved in bacterial cell lysis by precipitation of membrane proteins and inhibition of enzymatic activities (such as glycosyltransferases) (Ismail et al., 2012).

Due to various medical effects of pomegranate and its different parts, it could be helpful to investigate the antibacterial effects of its extracts. However, there is a need to examine the inhibitory effects of different varieties of pomegranates peel extract cultivated in Iran against foodborne pathogens. In current study *S. aureus* and *S. typhimurium* were chosen because of their outbreaks and emergence of drug resistance strains (Gullon et al., 2016). By knowing the efficacy of antibacterial activity of pomegranate peel extract (PPE), the use of its extract in food industry as natural food additives could be proven. Therefore, the objective of the present study was the investigation of antibacterial effects of pomegranate peel extract of two cultivar (*Naderi* and *Mallas* cultivated in Iran) against *Staphylococcus aureus* and *Salmonella typhimurium* in red meat extract and TSB at two temperatures (4 °C and 15 °C). Also, the differences in antibacterial activities of two pomegranate cultivars (*Naderi* and *Mallas*) were studied.

Material and Methods

Pomegranate fruit and preparation of extract

Two cultivars of *Naderi* and *Mallas* pomegranate fruits were purchased from local market of Iran. The fresh fruits were cleaned, peeled manually, dried in an incubator (30 - 40 °C), and milled. One gram of powdered pomegranate peel was dissolved in 10 ml aqueous ethanol (80%) and placed in a shaker for 24 hours at room temperature. Then extracts were filtered and dried in oven at 37 °C. The crude extract was stored at 4 °C until use (Derakhshan et al., 2018).

Methods of red meat extraction

Fresh beef were purchased from the market and transferred immediately to the laboratory under suitable condition. The meat broth was prepared by mixing the ground meat and homogenizing in distilled water at a ratio of 1 to 10 by stomacher. In order to coagulate the proteins, homogenized samples were cooked and the resulting broth was passed through filter and, then sterilized by autoclave at 121 °C for 15min.

Microorganisms

The *Staphylococcus aureus* (PTCC 1113) and *Salmonella typhimurium* (RTCC 1735) were obtained from the Persian Type Culture Collection (PTCC) and the Razi Vaccine and Serum Research Institute (RTCC), Tehran, Iran, respectively. The bacterial strains were cultured on Tryptic Soy Broth (TSB, Merck, Germany) at 37 °C for 20 min and 0.5 Mc Farland dilutions were prepared (Abdollahzadeh et al., 2011).

Determination of antibacterial activity

Separate tubes containing 1 ml of red meat extract and 1 ml of TSB were prepared for each bacterium. The amount of 10 ml of bacterial suspension was added to each test tube to reach the concentration of 10^7 colony forming units (CFU). Thereafter, pomegranate extract was added to reach the dilutions of 0.5%, 1%, 2.5%, and 5%. One tube of meat extract and one tube of TSB without pomegranate addition were considered as controls. All tested groups were incubated at 4°C and 15°C temperatures. The bacterial count was done at 0, 1, 3, 6, 24 and 48 hours after incubation. Then, the samples were cultured on

Tryptic Soy Agar (TSA, Merck, Germany) and incubated at 37 °C for 24 hours (Boniadian et al., 2014).

Minimum inhibition concentration (MIC) and Minimum bactericidal concentration (MBC) assays

The MIC of the pomegranate extract was determined using macro dilution technique wherein the extract was diluted serially in a series of ten test tubes containing TSB (0.5 ml) and all tubes were loaded with 0.5 ml of each bacterium (10^{6} CFU/ml) and then incubated at 37 °C for 24 h. The first tube with transparent appearance was considered as a MIC. All analysis was done in triplicate (Boniadian et al., 2014). The transparent tubes were cultured on Muller Hinton agar plates and after 24 h incubation (37 °C) and the tubes with no bacterial growth recorded as MBC (Naziri et al., 2012).

Statistical analysis

Statistical analyses of the results were carried out using SPSS and the bacterial count was analyzed by a one way ANOVA and LSD comparison test.

Results and Discussion

Minimum Inhibition Concentration and Minimum Bactericidal Concentration

The pomegranate extracts minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) are shown in table 1. It was shown that two cultivar of PPE had antibacterial activity against both bacteria. According to Al-Zoreky (2009) research MIC of *S. aureus*, was evaluated 2 mg/ml which was estimated lower than results of current study. The MIC of *S. aureus* in active pomegranate extract was reported 40 and 90 µg/ml by Duman et al. (2009). In Hayouni et al. (2011) study *S. typhimurium* was considered the second most sensitive microorganism to methanolic extract of pomegranate peel with the MIC of 0.25 mg/ml, which is not in agreement with present study. Fawole et al. (2012) showed the MIC value of Methanolic extract of pomegranate ranges from 0.2 to 0.78 mg/ml. By comparing results of present study with these reports, the MIC values achieved in current research are considerably higher than previous studies. The MIC and MBC values of 50 and 60 mg/ml against *Salmonella sp* and *S. aureus* were reported by Gullon et al. (2016). The MBC of present research was evaluated 125 and 130 mg/ml for *Naderi* and *Mallas* PPE respectively which was not in line with Gullon et al. (2016) report. It was shown that the variation in results achieved by different researchers is due to differences among pomegranate cultivars, extraction methods, strain sensitivity, and antibacterial procedures (Gullon et al., 2016). According to our result MIC value of two investigated cultivars were varied, which is due to inter-genetic cultivar variability, geo-environmental spatial variation, and chemical heterogeneity (Fawole et al., 2012).

Table-1. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) (mg/mL) of pomegranate ethanolic extract

Pomegranate cultivar	Bacterium	MIC	MBC
Naderi	*Staphylococcus aureus*	15.62	125
	Salmonella typhimurium	62.5	125
Mallas	*Staphylococcus aureus*	19.5	130
	Salmonella typhimurium	64.75	130

Antibacterial effect of PPE in meat broth

In different studies, various degrees of bacterial growth inhibition were reported by using PPE. Because of the importance of *S. aureus* and *S. typhimurium* in food poisoning of Iran, their sensitivity to PPE in meat broth and TSB media at 4 °C and 15 °C were investigated.

Results of bacterial growth in different concentration of PPE at 4 °C and 15 °C were shown in Table 2 to 5. It was shown the *S. aureus* inhibition by *Naderi* PPE in all concentration at 4 °C and 1%, 2.5% and 5% concentrations at 15 °C. Otherwise, all concentrations of *Mallas* PPE were significantly effective in reduction of *S. aureus* in meat broth at both temperatures.

The reduction in initial population of *S. typhimurium* in meat broth samples treated by *Naderi* PPE at 4 °C were recorded at 0.5% and 1% concentrations and 2.5% and 5% concentrations in 6 and 3 hours of storage, respectively. Results of current study manifested that by increasing in the concentration of *Naderi* PPE, the antibacterial activity at 15 °C and 4 °C was increased.

It could be concluded that the sensitivity of *S. aureus* to *Naderi* PPE was more pronounced at 15°C compared to 4°C. The achieved result indicated the better function of *Naderi* PPE in inhibiting S. *aureus*

growth than *Mallas* one. This could be attributed to antibacterial compounds in pomegranates peel such as phenols, flavonoids and tannins (Al-Zoreky, 2009) and lower pH of *Naderi* PPE. The inhibitory effect of *Mallas* PPE on *S. typhimurium* in meat broth was obtained at 2.5% and 5% concentrations at 4 °C by the end of storage time, which was significantly reduced by 1.5 logs at 5% concentration. On the other hand, the reduction in *S. typhimurium* growth at 0.5% and 1% levels was only shown until the first hour of storage. The bacteriostatic activity of *Mallas* PPE against *S. typhimurium* at 15 °C was reported in 1, 3 and 6 hours of storage. It could be suggested that 2.5% and 5% PPE inhibit *S. typhimurium* at 4 °C better than 15 °C.

PPE was less effective in inhibiting the *S. typhimurium* compared to the *S. aureus*. The existence of outer phospholipidic membrane in Gram negative bacteria had been resulted in higher resistance to different treatments (Djenane et al., 2011). Moreover, hydrophobic constituents in extracts and essential oils increase the ion permeability and leakage of intracellular component of Gram positive bacteria (Djenane et al., 2011). Ahmad and Beg (2001) reported antibacterial activity of alcohol extracts of pomegranate against *S. aureus*, *Escherichia coli* and *Shigella dysenteriae*. Opara et al. (2009) determined the antimicrobial activity against *S. aureus* in all studied varieties of pomegranates. Overall, the current findings approved the results achieved by Negi and Jayaprakasha (2003) which was investigated the ethyl acetate, acetone, methanol extract of pomegranate peel. They indicated a high inhibition activity on both Gram-positive and Gram-negative bacteria such as *S.aureus*, *Pseudomonas aeruginosa*, and *B. aurous*, although *E.coli* had been resistance to extract (Negi and Jayaprakasha, 2003). Al-Zoreky (2009) reported inhibition effect of methanolic extract of pomegranate peel against *S. aureus, Listeria monocytogenes*, *E. Coli* and *Yersinia enterocolitica*. Hayrapetyan et al. (2012) studied the effect of pomegranate peel extract in meat pate and it was shown that *S. aureus* had the highest sensitivity among *Bacillus cereus, E. Coli, B. subtilis and L. monocytogenes*.

Table-2. Bacterial count (log, CFU/ml) in different concentrations of Naderi PPE in meat broth at 4 °C and 15 °C

Bacteria	Temperature	Time (h)	Naderi PPE concentrations (Mean ± SD)				
			control	0.5%	1%	2.5%	5%
Staphylococcus aureus	4°C	0	6.3	6.3	6.3	6.3	6.3
		48	6.01±0.31	5.02±0.37	4.57±0.51	4.64±0.67	3.84±0.59
Salmonella typhimurium		0	6.3	6.3	6.3	6.3	6.3
		48	6.88±0.31	6.55±0.37	6.50±0.51	6.54±0.67	6.30±0.59
Staphylococcus aureus	15 °C	0	6.3	6.3	6.3	6.3	6.3
		48	6.32±0.08	5.90±0.40	4.91±0.89	4.32±0.88	3.26±1.09
Salmonella typhimurium		0	6.3	6.3	6.3	6.3	6.3
		48	8.81±1.14	7.38±0.82	7.84±0.78	7.23±0.65	6.32±0.41

Table-3. Bacterial count (log, CFU/ml) in different concentration of Naderi PPE in TSB at 4 °C and 15 °C

Bacteria	Temperature	Time (h)	Naderi PPE concentrations (Mean ± SD)				
			control	0.5%	1%	2.5%	5%
Staphylococcus aureus	4°C	0	6.3	6.3	6.3	6.3	6.3
		48	6.02±0.64	5.38±0.68	4.76±0.69	4.66±0.75	3.70±1.03
Salmonella typhimurium		0	6.3	6.3	6.3	6.3	6.3
		48	8.792	7.491	7.064	4.984	4.132
Staphylococcus aureus	15 °C	0	6.3	6.3	6.3	6.3	6.3
		48	6.98±0.67	5.97±0.43	5.82±0.82	5.08±0.23	3±1.03
Salmonella typhimurium		0	6.3	6.3	6.3	6.3	6.3
		48	6.94±1.13	6.52±0.56	6.31±0.80	6.51±0.47	6.54±0.80

Table-4. Bacterial count (log, CFU/ml) in different concentration of Mallas PPE in meat broth at 4 °C and 15 °C

Bacteria	Temperature	Time (h)	Mallas PPE concentrations (Mean ± SD)				
			control	0.5%	1%	2.5%	5%
Staphylococcus aureus	4°C	0	6.3	6.3	6.3	6.3	6.3
		48	6.49±0.13	5.90±0.43	5.82±0.38	4.94±0.80	4.23±1.06
Salmonella typhimurium		0	6.3	6.3	6.3	6.3	6.3
		48	6.88±0.24	7.45±0.58	6.90±0.51	5.90±0.46	4.86±0.70
Staphylococcus aureus	15 °C	0	6.3	6.3	6.3	6.3	6.3
		48	7.24±0.39	5.54±0.34	4.58±0.62	4.53±0.79	4.12±1.14
Salmonella typhimurium		0	6.3	6.3	6.3	6.3	6.3
		48	6.91±0.53	7.34±.0.76	7.10±0.43	7.51±0.89	7.60±0.58

Table-5. Bacterial count (log, CFU/ml) in different concentration of Mallas PPE in TSB at 4 °C and 15 °C

Bacteria	Temperature	Time (h)	Mallas PPE concentrations (Mean ± SD)				
			control	0.5%	1%	2.5%	5%
Staphylococcus aureus	4°C	0	6.3	6.3	6.3	6.3	6.3
		48	6.02±0.13	5.91±0.36	5.83±0.34	4.93±0.68	4.51±0.76
Salmonella typhimurium		0	6.3	6.3	6.3	6.3	6.3
		48	6.94±0.30	6.60±0.18	6.34±0.22	6.54±0.23	5.60±0.52
Staphylococcus aureus	15 °C	0	6.3	6.3	6.3	6.3	6.3
		48	7.34±0.65	5.51±0.71	4.34±0.78	3.82±0.88	2.84±0.99
Salmonella typhimurium		0	6.3	6.3	6.3	6.3	6.3
		48	6.81±0.83	7.54±0.45	7.65±0.66	7.30±0.81	8.46±0.68

Antibacterial effect of PPE in TSB media

According to results, *S. aureus* reduction was reported in samples treated with *Naderi* PPE at 4 °C in all concentrations and at 15 °C in 2.5% and 5% concentrations. However, initial populations of *S. aureus* were decreased in all TSB samples treated by *Mallas* PPE ($p<0.05$) at both 4 °C and 15 °C.

Naderi PPE with 2.5% and 5% concentrations reduced the bacterial growth during 48 hours of storage. The reduction in growth of *S. typhimurium* in TSB media at 4 °C was reported in 5% *Mallas* and 2.5% and 5% *Naderi* PPE, respectively. These bacteriostatic effects were shown more powerful at higher concentrations. During the storage at 15 °C, the inhibition of *S. typhimurium* in TSB media treated by *Mallas* PPE was shown at the first three hours of storage. In overall, *Naderi* PPE had better function in reduction of bacterial load. This can attributed to the lower pH of *Naderi* PPE (pH: 3) than pH of *Mallas* PPE (pH: 3.5). The wide difference in bacterial inhibition activity may be related to pomegranate varieties and agro climatology differences (Hayrapetyan et al., 2012; Opara et al., 2009). It was seen that PPE was less effective in inhibiting the *S. typhimurium* compared to the *S. aureus*. Results of current study are compatible with those of Panichayupakaranant who reported antibacterial activity of pomegranate rind extract against *S. aureus* and resistance of *S. typhimurium* (Panichayupakaranant et al., 2010). The antibacterial compounds in pomegranate peel which cause destruction in cell wall, cytoplasmic membrane and membrane proteins have essential role in bacterial death (Ibrahium, 2010). The inhibitory effect of PPE on *S.typhimurium* was more pronounced at lower temperatures (4°C) than higher one (15°C). The inhibition effect of ethanolic extract was shown at lower temperature for *S. aureus* than for *S. typhimurium.* These differences might be due to differences in cell membrane permeability of bacteria at various temperatures (Wu et al., 2016).

It was shown that PPE in meat broth were more effective than in TSB media which could be attributed to low pH of meat broth. Djenane et al. (2011) infested the importance of meat pH on bacterial activity of essential oil, which probably could be referred to extracts. According to their results, by lowering the pH, the hydrophobicity of

essential oil will be increased and their solubility in lipid phase of bacterial membrane facilitated. The current results are in agreement with Durairaj who reported that by increasing the pH value, the antibacterial effects decreased (Durairaj et al., 2009). Natural antibacterial agents such as ellagitannins, punicalagin, ellagic acid and gallic acid and also, the ability of phenolic compounds to precipitation of membrane proteins and inhibition of enzymes such as glycosyl transferase, make pomegranate peel as a powerful antibacterial preservative.

Conclusion

Result of current study, clearly confirmed the effectiveness of pomegranate peel on inhibition of bacterial activity. It was reported that two cultivar of PPE had antibacterial activity against *S. aureus* and *S. typhimurium*. It was shown that PPE in meat broth were more effective than in TSB media which could be attributed to low pH of meat broth. In general, it could be concluded the Gram positive bacteria was more sensitive to ethanolic extract of pomegranate peel than Gram negative one. Also, the PPE was less effective in inhibiting the *S. typhimurium* compared to the *S. aureus*, which could be due to their outer lipopolysaccharide (LPS) membranes. Also, the inhibitory effect of PPE on *S. typhimurium* was more pronounced at lower temperatures (4°C) than higher one (15°C). According to achieved results, the antibacterial activity of pomegranate and its use in traditional medicine are proved.

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Contribution of Authors

Tadi M: Conceived idea, designed research methodology, collected data and wrote manuscript
Boroujeni HM: Helped in data collection, analysis and manuscript write up
Rafieian-Kopaei M: Helped in data collection, analysis and manuscript write up
Sadrabad EK: Supervised research, data analysis and final approval of article