

Antibacterial effects of Iranian native sour and sweet pomegranate (*Punica granatum*) peel extracts against various pathogenic bacteria

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(Received 19 Oct 2011; revised version 17 Jun 2012; accepted 24 Jul 2012)

Summary

Nowadays, uncontrolled and frequent use of antibiotics may cause emergence of microbial resistance among pathogenic agents. Therefore, the use of new synthetic and natural antimicrobial compounds is inevitable. One source of natural compounds in this respect comes from plants. The purpose of this study was to examine the antibacterial effects of peel extracts from sour and sweet pomegranate. Methanolic extracts of sour and sweet pomegranate peels and aqueous solutions of tetracycline and chloramphenicol were prepared. Antibiogram tests using disk diffusion technique and serial dilution method were performed against ten pathogenic bacteria isolated from animals, and relative minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were also determined for the above compounds. The greatest zone of inhibition induced by the action of pomegranate peel extracts was obtained for *Staphylococcus aureus* (about 25 mm) and the smallest zone of inhibition was obtained for *Pasteurella multocida* (about 9 mm). In addition, the lowest MIC and MBC values of pomegranate peel extract were obtained for *Staphylococcus aureus* (7.8 and 62.5 mg/ml, respectively). Results of serial dilution tests indicate that bactericidal effect of sour pomegranate peel extract was more than that for sweet pomegranate peel extract; and sweet pomegranate peel extract exerts a bacteriostatic action against bacteria. The antibacterial effect was greater against Gram-positive bacteria compared to that for the Gram-negative bacteria. Effects of these extracts were considerably lower than those for tetracycline and chloramphenicol. In conclusion, methanolic extracts of pomegranate peels exhibit relatively good bacteriostatic and bactericidal effects.

Key words: Pomegranate peel, Antibacterial effects, Antibiogram, Tetracycline, Chloramphenicol

Introduction

Development in pharmaceutical sciences has made the production and formation of synthetic drugs possible and they are widely used in the treatment of diseases in humans and animals, among which antimicrobial agents are included. Incidence of toxic side effects, development of resistance, and sensitivity of individuals are several reasons for the need to substitute these synthetic drugs with new ones, particularly compounds with natural origin (Pakdaman and Mostafavi, 1979). Medicinal plants usually contain several compounds that can vary with climate and growing region

(Mojtabae and Semsar, 1968). Iran is very rich in the rate and diversity of vegetation in the Middle East (Khosravi Haghghi, 1986) and so far many studies have been conducted to reveal the medicinal properties of some of these plants (Shokri *et al.*, 2011; Taati *et al.*, 2011). Knowing the composition of these plants, one can alter their chemical structures in order to produce semisynthetic compounds to improve their efficacy against microorganisms. Use of pomegranate (*Punica granatum*) has been reported in traditional medicine (Mirheydar, 1993; Mirjalili, 2002). In addition to seeds, that are the edible part of pomegranate fruit, medicinal properties of various parts of the

plant have also been studied (Mirheydar, 1993; Mirjalili, 2002). For instance, the effects of different parts of pomegranate against intestinal helminthes (Naqvi *et al.*, 1991; Lansky *et al.*, 2000), Giardia (Ponce-Macotella, 1994), pathogenic intestinal bacteria such as microorganism causing typhoid (Pérez and Anesini, 1994) and cholera (Guivara, 1994), *Bacillus subtilis* and *Escherichia coli* (De *et al.*, 1999), *Staphylococcus aureus* (Braga *et al.*, 2005a), and more recently against viruses such as Herpes (Zhang, 1995), Polio and HIV (Stewart, 1995; Neurath *et al.*, 2004), antifungal effects on *Penicillium* species (Azzouz and Bullerman, 1982), *Candida albicans* (Nair and Chanda, 2005; Vasconcelos *et al.*, 2006), *Saccharomyces* (De *et al.*, 1999) and anti-tumor effects (Mavlyanov *et al.*, 1997) have been reported. The antioxidant and antimutagenic effect of pomegranate extract has been investigated, too (Negi *et al.*, 2003). The use of pomegranate gel for controlling attachment of different organisms in oral cavity, such as *Streptococcus mutans*, *Streptococcus mitis* and *Candida albicans* (in comparison with miconazole) has also been reported (Vasconcelos *et al.*, 2006).

In the present study, antibacterial effects of Iranian native sour and sweet pomegranate were examined and their efficacy was compared with two synthetic antibiotics, chloramphenicol and tetracycline, using disc diffusion and serial dilution methods.

Materials and Methods

Preparation of methanolic extract of Iranian native sour and sweet pomegranate peels

Pomegranates were collected from Shiraz city retail stores and were divided into two groups of sour and sweet pomegranates on the basis of physical characteristics (taste and color). Peels were first prepared and dried for a week at room temperature in dark. Then the dried peels were separately ground to obtain uniform powders. Each powder (500 g) was dissolved in 1800 ml methanol and was left for 96 h in a dark environment; the decoctions

were filtered using filter paper (Millipore, AP2512450). Each solution was evaporated by vacuum evaporator (Heidolph, LABOROTA 4000-eficieat), the semisolid products were lyophilized by a Vaco 5 freeze dryer (ZIRBUS Technology, D-37539 Bad Grund) and the dried powders were kept in dark capped bottles (Moattar and Samsam Shareat, 1985).

Making 1/100 dilution of McFarland 0.5 bacterial culture

Each Gram-positive bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus cereus*, *Clostridium perfringens*, *Listeria monocytogenes*) and Gram-negative bacteria (*Escherichia coli* O₁₅₇:H₇, *Pasteurella multocida*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Yersinia enterocolitica*) isolated from different species of animals in Shiraz Veterinary School were first identified and then cultured in the sterile tryptic soy broth medium (TSB) (Difco Co.) for 3-5 h and the opacity of the medium was adjusted with that of McFarland 0.5 tube (Quinn *et al.*, 1994). Finally, 1/100 dilution of the culture was prepared to get an approximate colony count of 10⁶ CFU per milliliter.

Susceptibility testing

A) Disc diffusion method

The tests were done based on Kirby-Bauer standard method (Bauer *et al.*, 1966). In summary, 20 µl of various concentrations (50, 100, 200 and 400 mg/ml) of pomegranate peel extract was added to each sterile standard disc. So, each disc contained 1, 2, 4 and 8 mg sour or sweet pomegranate peel extract. Commercial standard discs (Padtanteb Iran) containing 30 µg antibiotics (tetracycline and chloramphenicol) were also used for comparison. Then 100 µl of each 1/100 dilution of McFarland 0.5 bacterial culture was added on Mueller Hinton agar (Difco Co.) plate and was uniformly spread on the surface. The discs (for each concentration of each extract and antibiotic, in triplicate) were planted as well and then plates were kept in an incubator (37°C) for 24 h. Diameters of the bacterial growth inhibition zones were exactly measured using a cullis vernier.

It should be mentioned that except for *Clostridium perfringens* (incubated at anaerobic conditions using anaerobic jar containing gas pack Anaerocult A, Merck) all other bacteria were incubated in aerobic conditions.

B) Serial dilution method

The tests were done to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (Lorian, 1996; Andrews, 2001). For this purpose, stock solutions of pomegranate peel extracts (500 mg/ml) were prepared using dimethyl sulfoxide as the solvent. Tetracycline (2 mg/ml) and chloramphenicol (2.5 mg/ml) were dissolved in sterile distilled water. A volume of 0.5 ml from each stock solution and 0.5 ml of TSB were added to the first tube and serially diluted in the next 10 to 15 tubes containing 0.5 ml of TSB medium. Then, 0.5 ml of 1/100 dilution of McFarland 0.5 bacterial culture was added to each tube. Seven test tubes were considered as controls for culture medium, solvent, testing bacteria, pomegranate peel extracts and testing antibiotics (the tests were done in triplicates). Concentration in the tube showing no bacterial growth and turbidity after 24 h incubation at 37°C was considered as MIC. Aliquots of 100 µl from each transparent tube showing no turbidity were separately cultured on Mueller Hinton agar plates. After 24 h of incubation at 37°C, the concentration of antibacterial agent in the tube that showed no bacterial growth was recorded as MBC (Lorian, 1996).

Statistical analysis

Data are brought as mean \pm SEM and the values of MICs, MBCs and zone of inhibition for sour and sweet pomegranate peel extract, tetracycline and chloramphenicol were compared using analysis of variance (One way ANOVA) followed by LSD multiple comparison test. For comparison of these values between Gram-positive and Gram-negative bacteria, independent sample t-test was used. Statistical analysis was carried out using SPSS 16.0 computer software. Differences were considered significant when p-value was less than 0.05.

Results

Table 1 summarizes the inhibition zone of various bacteria by the pomegranate peel extracts and antibiotics. Comparison of means of inhibition zones indicates that with discs containing 8 mg sour or sweet pomegranate peel extract, *Staphylococcus aureus* shows the most sensitivity (25.7 ± 0.3 and 25.3 ± 0.3 mm, respectively). The lowest sensitivity was illustrated by *Pasteurella multocida* with inhibition zones of 9.3 ± 0.9 and 9.7 ± 0.9 mm, respectively. Corresponding results against *Staphylococcus aureus* (25.7 ± 0.3 and 25.3 ± 0.3 mm) and *Pseudomonas aeruginosa* (15.7 ± 0.3 and 15.7 ± 0.3 mm) have shown greater zones of inhibition ($P < 0.05$) compared to those obtained for tetracycline and/or chloramphenicol (Table 1), but the efficacy of tetracycline and chloramphenicol was more than those ($P < 0.001$) for sour and sweet pomegranate peel extracts as the quantities of each of the above antibiotics added to each disc (30 µg) was considerably less than peel extracts (1 to 8 mg).

Table 2 shows the results obtained from serial dilution tests. The lowest MICs of sour and sweet pomegranate peel extracts were 15.6 and 7.8 mg/ml, respectively. In addition, the lowest MBCs of sour and sweet pomegranate peel extracts were found to be 62.5 and 62.5 mg/ml, respectively. In most of the studied bacteria (7 out of 10 bacteria), MICs for sweet pomegranate peel extract were generally lower than those for sour pomegranate peel extract ($P < 0.05$). This is in contrast to the MBC value (7 out of 10 bacteria) as it was more for sweet pomegranate peel extract compared to that for sour pomegranate peel extract ($P < 0.05$) (Table 2).

Mean inhibition zones and MIC values indicate that pomegranate peel extracts (sour and sweet) exert a more powerful effect on Gram-positive than Gram-negative bacteria ($P < 0.05$), but the difference in MBC values is not significant (Tables 1 and 2).

Discussion

Comparison between means of growth inhibition zones and the MIC and MBC

Table 1: Comparison of the inhibitory zone diameters (mm)¹ for pomegranate peel extracts and tested antibiotics against various bacteria isolated from animals

Bacteria	Sour pomegranate peel extract (mg/disc)				Sweet pomegranate peel extract (mg/disc)				Tetracycline (mg/disc)	Chloramphenicol (mg/disc)
	1	2	4	8	1	2	4	8	0.03	0.03
<i>S. aureus</i>	15.3±0.3	18.3±0.3	20.0±0.0	25.7±0.3	16.0±0.0	18.3±0.3	19.7±0.3	25.3±0.3	23.7±0.3	22.3±0.3
<i>E. faecalis</i>	7.3±0.3	11.0±0.0	14.7±0.3	17.7±0.3	7.0±0.0	9.7±0.3	13.3±0.3	15.3±0.3	27.7±0.3	21.7±0.3
<i>B. cereus</i>	14.3±0.3	14.7±0.3	17.3±0.3	20.7±0.3	14.3±0.3	15.0±0.0	17.0±0.6	19.0±0.0	24.7±0.3	25.0±0.6
<i>C. perfringens</i>	13.7±0.3	16.7±0.3	18.7±0.3	23.3±0.3	13.0±0.6	16.7±0.3	18.3±0.3	23.0±0.6	22.0±0.6	27.0±0.6
<i>L. monocytogenes</i>	9.3±0.3	9.7±0.3	12.0±0.0	17.7±0.3	8.7±0.3	9.0±0.0	11.0±0.0	16.0±0.0	29.7±0.3	26.3±0.3
<i>E. coli O₁₅₇:H₇</i>	7.0±0.0	7.7±0.3	9.3±0.3	10.3±0.3	6.7±0.3	7.3±0.3	8.7±0.3	10.3±0.3	22.7±0.3	23.0±0.6
<i>P. multocida</i>	6.7±0.3	7.7±0.7	8.7±0.3	9.3±0.9	7.0±0.6	7.3±0.3	7.7±0.3	9.7±0.9	22.7±0.3	28.0±0.6
<i>P. aeruginosa</i>	9.7±0.3	11.7±0.3	13.0±0.0	15.7±0.3	9.3±0.3	11.3±0.3	12.3±0.3	15.7±0.3	13.3±0.3	12.3±0.3
<i>S. typhimurium</i>	6.0±0.0	6.7±0.3	8.0±0.6	12.0±0.6	6.0±0.0	6.3±0.3	7.7±0.3	12.0±0.0	24.7±0.3	20.0±0.0
<i>Y. enterocolitica</i>	11.7±0.3	12.7±0.3	14.3±0.3	17.7±0.3	9.3±0.3	11.0±0.0	13.3±0.3	16.3±0.3	29.3±0.3	27.7±0.3

¹Mean ± SEM (n=3)

Table 2: Comparison of MICs and MBCs¹ of pomegranate peel extracts and tested antibiotics against various bacteria isolated from animals

Bacteria	MIC				MBC			
	Sour pomegranate peel extract (mg/ml)	Sweet pomegranate peel extract (mg/ml)	Tetracycline (µg/ml)	Chloramphenicol (µg/ml)	Sour pomegranate peel extract (mg/ml)	Sweet pomegranate peel extract (mg/ml)	Tetracycline (µg/ml)	Chloramphenicol (µg/ml)
<i>S. aureus</i>	15.6±0.0	7.8±0.0	0.2±0.0	1.2±0.0	62.5±0.0	125±0.0	125±0.0	>1250±0.0
<i>E. faecalis</i>	31.3±0.0	15.6±0.0	0.2±0.0	2.4±0.0	125±0.0	250±0.0	31.3±0.0	78.1±0.0
<i>B. cereus</i>	31.3±0.0	15.6±0.0	1.3±0.3	1.6±0.4	208.3±41.7	>250±0.0	250±0.0	1041.7±208.3
<i>C. perfringens</i>	31.3±0.0	15.6±0.0	2.0±0.0	1.2±0.0	62.5±0.0	83.3±20.8	416.7±83.3	78.1±0.0
<i>L. monocytogenes</i>	31.3±0.0	26.0±5.2	0.1±0.03	2.4±0.0	125±0.0	250±0.0	250±0.0	1250±0.0
<i>E. coli O₁₅₇: H₇</i>	83.3±20.8	31.3±0.0	1.3±0.3	4.9±0.0	125±0.0	250±0.0	250±0.0	>1250±0.0
<i>P. multocida</i>	83.3±20.8	>62.5±0.0	1.0±0.0	2.4±0.0	125±0.0	166.7±41.7	125±0.0	78.1±0.0
<i>P. aeruginosa</i>	>62.5±0.0	31.3±0.0	13.0±2.6	65.1±13	>62.5±0.0	125±0.0	1000±0.0	>1250±0.0
<i>S. typhimurium</i>	83.3±20.8	31.3±0.0	2.0±0.0	4.1±0.8	125±0.0	250±0.0	125±0.0	312.5±0.0
<i>Y. enterocolitica</i>	31.3±0.0	26.0±5.2	0.5±0.0	2.4±0.0	62.5±0.0	83.3±20.8	125±0.0	312.5±0.0

¹Mean ± SEM (n=3)

values show that *Staphylococcus aureus* and *Pasteurella multocida* have, respectively, the highest and the lowest sensitivities to the methanolic extracts of sour and sweet pomegranate peels. As the MIC value for sweet pomegranate peel extract and MBC value for sour pomegranate peel were found to be relatively low ($P < 0.05$), it can be concluded that methanolic extract of sour pomegranate peel may exert a bactericidal effect, but the methanolic extract of sweet pomegranate peel may have a bacteriostatic effect. These differences may be due to the variations between the amount of antibacterial substances (such as tannins and phenolic substances) in the sour and sweet pomegranate peels. Studies on extracts of various parts of pomegranate fruit did not focus on the type of pomegranate as sour or sweet. However, according to a study searching for bacteriostatic and bactericidal effects of tannins against various bacteria, the MIC values for pomegranate peel were determined to be similar compared to that for tannin solution in the Beckman study (2007). The polyphenol content and the antibacterial effects of these polyphenolic compounds have been reported by several studies in recent years (Haslam, 1996; Machado *et al.*, 2002; Naz *et al.*, 2007). The fact that tannin and polyphenolic agents are the most abundant compounds in pomegranate fruit extract is also reported by Haslam (1996). Effects of tannins on bacterial metabolism are identified by their effect on bacterial membrane, because tannins can pass through cell walls, which contain polysaccharides and proteins and bind to their surface, preventing their normal activity (Scalbert, 1991; Chung *et al.*, 1993; Vasconcelos *et al.*, 2006). Machado *et al.* (2002) isolated punicalagin ellagitannin in ethylacetate extract of pomegranate fruit using chromatographic techniques, and specified that these agents are effective against methicillin resistant *Staphylococcus aureus* strains. Naz *et al.* (2007) isolated various compounds from methanolic extract of pomegranate fruit and showed that pomegranate's phenolic compounds, especially gallic acid, exert certain antibacterial effects against *Corynebacterium*, *Staphylococcus*, *Streptococcus*, *Bacillus subtilis*, *Shigella*, *Salmonella*,

Escherichia and *Vibrio* species.

Results of this study revealed a stronger effect of pomegranate peel extract on Gram-positive bacteria compared to that against Gram-negative bacteria ($P < 0.05$). Due to differences in cell wall structure in these bacteria, the results are not unexpected. Comparison of the results of disc diffusion tests and MIC and MBC values for the tested extracts with those found for tetracycline and chloramphenicol suggests a higher efficacy of these two antibiotics against the studied bacteria ($P < 0.001$). Lower efficacy for the peel extracts may be related to the impurities, as an extract comprises many different compounds, of which only a few may have antibacterial activity. Nascimento *et al.* (2000) showed that pomegranate extract inhibited *Pseudomonas aeruginosa* growth and has a synergistic effect against bacteria resistant to the known antibiotics. Synergistic effects of methanolic pomegranate extract and chloramphenicol, gentamicin, ampicillin, tetracycline and oxacillin against *Staphylococcus aureus* are also reported by Braga *et al.* (2005b).

In the present study, the effects of methanolic pomegranate peel extract were investigated. The use of various solvents for extraction may change the degrees of antibacterial effect of the extracts as the active antimicrobial substances may have different solubilities in various solvents. Negi and Jayaprakasha (2003) determined that acetonitrile pomegranate peel extract is the most effective extract against the studied bacteria compared to the methanolic or water extracts. Sirirak *et al.* (2005) and Voravuthikunchai *et al.* (2006) also reported a greater antibacterial effect of ethanolic pomegranate extract compared to the values found in the present study. The variation in antibacterial spectrum can also be related to the differences in the amount of antibacterial substances (such as tannins and phenolic substances) in various parts of plant. Minor differences in laboratory techniques and the strain of specified bacterial species used in the experiments run by researchers may also be involved in the variation of the reported results (Lowburg and Ayliffe, 1974).

In conclusion, methanolic extracts of pomegranate peels exhibit relatively good

bacteriostatic and bactericidal effects and could potentially be a good substitute for the synthetic antibiotic against pathogenic resistant bacteria. But further research is required to identify and isolate the active compounds present in the pomegranate's peel to replace the synthetic additives with these natural plant-based products and also to confirm these effects *in vivo*.

Acknowledgements

We thank J. Jalae and A. Shahed for technical help. This work was financially supported by Shiraz University and Natural Antimicrobial Center of Excellence (NACE) which is greatly appreciated.

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