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Bactericidal activity and phytochemical screening of Moroccan pomegranate (*Punica granatum* Linn.) peel aqueous extracts

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***Punica granatum* L. is an important medicinal plant in Morocco. Several researches were focused on antimicrobial activity of different pomegranate extracts, only few were on traditional preparations. To optimize and enhance the traditional use of this plant, aqueous extracts (decoction, infusion, maceration) of pomegranate fruit peels were examined for its antibacterial activity against some strains of human pathogenic bacteria. The sensitivity test performed with commonly used antibiotic test discs showed that majority of the bacteria tested are multiresistant to antibiotics. In spite of this, the plant extract was found to be more or equally effective as compared to standard antibiotics. Also, the decoction has a higher bactericidal activity with inhibition zones ranging from 12.3 to 30.3 mm, and minimum inhibitory concentration ranging from 0.048 to 3.12 mg/ml. The results provide a scientific basis for the centuries-old usage of aqueous extracts of this medicinal plant.**

Key words: Antibiotic resistance, aqueous extracts, bactericidal activity, *Punica granatum* L.

INTRODUCTION

Recently, a number of antibiotics have lost their effectiveness due to the development of resistant strains of bacteria, which has primarily occurred through the expression of resistance genes (Davis, 1994; Service, 1995). In addition to inducing resistance, antibiotics are sometimes associated with opposing effects such as hypersensitivity, immune-suppression and allergic reactions (Ahmad et al., 1998). Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases (Berahou et al., 2007; Salomao et al., 2008).

As part of our effort to reach this aim, we evaluated the antibacterial activity of some plants used in Moroccan traditional medicine. The pomegranate (*Punica granatum* L.) belongs to the Punicaceae family. It is a shrub native to Asia and has been cultivated since ancient times throughout the Mediterranean region of Africa and parts of Europe. In Morocco, it is valued for its delicious edible fruit (Bellakhdar, 2006; Oukablie, 2004). The fruit is consumed directly as fresh or juice and used in the food industry in the manufacture of jellies and syrups (grenadine). Fruit pericarp (peel, high in tannins) has been used in Moroccan traditional medicine as astringent, diuretic, natural dye, and it is also recommended for the treatment of diarrhoea, dysentery, gastric ulcer, bleeding disorders, gum inflammation, and for the treatment of some infections (Bellakhdar, 2006; Hmamouchi, 1999).

Although many reports on the antimicrobial activity of pomegranate exist in the literature, few of them evaluated the traditional preparations of extracts. This study describes the antibacterial efficacy of traditional preparations of pomegranate peel extracts.

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MATERIALS AND METHODS

Plant

Fresh fruits of *P. granatum*, collected near Fès city (Morocco) in October 2007, were used for extract preparation. The fruits were manually peeled and collected peels were then rinsed with distilled water. The peels were air dried under ambient conditions and grounded by mechanical mill.

Preparation of extracts

Decoction was prepared by boiling finely ground peels in water (10% w/v) (100°C) for 20 min. Infusion was prepared by adding boiling water to the herb material and left it standing for 20 min. After cooling, both preparations were vacuum filtered (through Whatman no.1 filter paper) using a Büchner funnel, and filtrate was concentrated to dryness in Rotavapor rotary-evaporation unit (Büchi Labortechnik; Flawil, Switzerland) at 40°C, and was weighed.

The residue obtained after drying was dissolved in distilled water for a final concentration of 200 mg/ml. Maceration: 20 g of finely ground plant material was macerated in 100 ml of distilled water for 24 h at room temperature. The extract was filtered as previously indicated and was stored. In all cases, the extracts were stored in a refrigerator at 4°C until use.

Test microorganisms

Eleven Gram-positive (two strain of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*) and Gram-negative (*Escherichia coli, Klebsiella pneumoniae, Serratia liquefaciens, Citrobacter freundii, Proteus mirabilis, Acinetobacter baumannii, Pseudomonas aeruginosa*) bacterial strains were used in this study. They were human pathogenic bacteria recovered from clinical isolates in Meknès regional hospital. All isolates were identified by BD Phoenix^{TM}100 Automated Microbiology System (Becton Dickinson Diagnostic Systems [BD], Pont de Claix, France).

Sensitivity test

Antibiograms were carried out by disc diffusion method using commonly used antibiotics (Bauer et al., 1966; FSM, 2007). Antibiotic sensitivity was tested in Mueller-Hinton agar plates (Oxoid). The media surface was inoculated with bacteria from a broth culture. Antibiotic impregnated discs (Oxoid) were placed on the solid medium and the plates were then incubated at 37°C for 24 h. The clear inhibition zones diameters formed around the discs were measured and interpreted in accordance to the manufacturer's instruction. The different bacteria were then ranked according to their sensitivity to the antibiotic being tested (sensitive, intermediate or resistant).

Determination of antibacterial activity

Disc diffusion method

The sensitivity of different bacterial strains to aqueous plant extract was measured in terms of zone of inhibition using a modified agar diffusion method (Bauer et al., 1966). Bacterial strains grown on nutrient agar at 37°C for 18 h were suspended in saline solution (0.9% NaCl) and adjusted to a turbidity of 0.5 MacFarland standards. The suspension was used to inoculate Petri plates. 6-mm-diameter sterile paper discs (Antibiotic test discs, Fioroni ref. 905) were impregnated with 50 µl of the extract (10 mg/disc), and were placed on the inoculated agar. Negative controls were done using paper discs loaded with 50 µl of sterile distilled water.

The plates were incubated at 37°C for 24 h. Antibacterial activities were evaluated by measuring the inhibition zone diameters. The experiments were conducted simultaneously in triplicate and the mean diameter of the inhibition zones was calculated.

Determination of minimum inhibitory concentration (MIC)

MIC values were determined for microorganisms that were found to be sensitive to the most active extract revealed by the previous screening test (disc diffusion assay).

A serial micro-dilution technique using 96-well micro-plates, as described by Eloff (1998) was used to obtain MIC values of the plant extract. Thiazolyl blue reagent (0.2 mg/ml) was used to indicate the presence of uninhibited bacterial growth (a blue colour) or inhibition (colourless) of bacterial growth in each well. MIC values are recorded as the lowest concentration of the extract that completely inhibited bacterial growth, giving a clear well.

Determination of minimum bactericidal concentration (MBC)

Referring to the results of the MIC assay, the wells showing complete absence of bacterial growth were identified and 5 µl of each well were transferred to Mueller Hinton agar plates and incubated at 37°C for 24 h. The complete absence of growth was considered as the minimum bactericidal concentration.

Phytochemical screening

Phytochemical screening was carried out on the powdered plant material for the presence of bioactive components such as tannins, phenols, alkaloids, cardiac glycosides, saponins, steroids and terpenes, flavonoids and mucilages according to the methods described by Raman (2006) and each of the tests was qualitatively expressed as negative (-) or positive (+).

RESULTS AND DISCUSSION

Sensitivity test

The antibiotic sensitivity profile of the bacterial strains tested is listed in Table 1. All bacteria tested (except 1 strain of *S. aureus*) were resistant to several antibiotics indicating the appearance of multiple drug-resistance phenotypes of the bacteria tested. Consequently, we could not use these antibiotics as therapeutic agents for treating diseases related with these bacteria.

Antibacterial activity

Antibacterial activity of aqueous extracts of *P. granatum* fruit peels were evaluated by measuring the diameters of zones of growth inhibition on bacterial strains and the results are presented as shown in Table 2.

The results showed that all the extracts did not have any antibacterial activity against the *Enterobacteriaceae*

Table 1. Antibiotic sensitivity profile of bacteria against commercially available antibiotics.

Name of the antibiotic	Inhibition zone diameter (mm)											
	MSSA	***MRSA***	***S. epidermidis***	***E. faecalis***	***A. baumannii***	***P. aeruginosa***	***K. pneumonia***	***E. coli***	***C. freundii***	***S. liquefaciens***	***P. mirabilis***	***S. typhi***
Ampicillin (10 µg)	NT	NT	NT	16 (I)	NT	NT	0 (R)	0 (R)	0 (R)	0 (R)	9 (R)	NT
Amoxycillin/clavulanic acid (20 µg)	NT	NT	NT	NT	NT	NT	10 (R)	9 (R)	9 (R)	8 (R)	9 (R)	17 (I)
Gentamicin (15 µg)	23 (S)	15 (R)	9 (R)	NT	0 (R)	0 (R)	0 (R)	13 (R)	10 (R)	14 (R)	16 (I)	17 (I)
Oxacillin (5 µg)	30 (S)	12 (R)	16 (R)	NT	NT	NT	NT	NT	NT	NT	NT	NT
Ciprofloxacin (5 µg)	27 (S)	22 (S)	0 (R)	16 (I)	NT	NT	20 (R)	22 (I)	16 (R)	18 (R)	25 (S)	22 (I)
Amikacin (30 µg)	NT	NT	NT	NT	18 (S)	0 (R)	11 (R)	13 (R)	11 (R)	14 (R)	16 (I)	16 (I)
Ceftazidim (30 µg)	NT	NT	NT	NT	0 (R)	12 (R)	NT	NT	NT	NT	NT	21 (S)
Piperacillin (75 µg)	NT	NT	NT	NT	0 (R)	0 (R)	NT	NT	NT	NT	NT	NT
Imipenem (10 µg)	NT	NT	NT	NT	21 (R)	10 (R)	NT	NT	NT	NT	NT	NT
Erythromycin (15 µg)	23 (S)	22 (R)	10 (R)	0 (R)	NT	NT	NT	NT	NT	NT	NT	NT
Vancomycin (30 µg)	19 (S)	16 (R)	15 (R)	15 (R)	NT	NT	NT	NT	NT	NT	NT	NT

MSSA: *methicillin-sensitive Staphylococcus aureus*; MRSA: *methicillin-resistant Staphylococcus aureus.* R: Resistant; S: sensitive; I: Intermediate; NT: Not Tested.

tested (*E. coli, K. pneumoniae, C. freundii, S. liquefaciens* and *P. mirabilis*). To some extent, these results were similar to those of previous studies. Indeed, ethanolic extract of pomegranate pericarp was inactive against the following *Enterobacteriaceae*: *K. pneumoniae, E. coli, Proteus* species*, Enterobacter aerogenes, Salmonella choleraesuis* and *Shigella* species (Nascimento et al., 2000); the water extract was also not inhibitory to *E. coli* (Negi and Jayaprakasha, 2003; Opara et al., 2009), *P. mirabilis* (McCarrell et al., 2008) and *K. pneumonia* (Prashanth et al., 2001). In contrast, Prashanth et al. (2001) and Altuner (2011) stated, respectively, that water extract of *P. granatum* fruit rind showed antibacterial activity against *E. coli, Proteus vulgaris* and *Salmonella typhi*, and that ethanol and solvent cocktail extract of *P. granatum* peel induces antibacterial activity against *E. coli* and *S. enterica*.

These differences in the antibacterial activity of pomegranate peel extracts among studies could be partially explained by variations in extraction methods, freshness of fruits, variations in the season and region of growth, strains sensitivity and antimicrobial procedures adopted in tests (Opara et al., 2009; Al-Zoreky, 2009).

On the other hand, all other bacterial strains tested (methicillin-sensitive *S. aureus* (*MSSA*)*,* methicillin-resistant *Staphylococcus aureus* (*MRSA*)*, S. epidermidis, E. faecalis, A. baumannii and P. aeruginosa*) were sensitive to the pomegranate peel aqueous extracts though to varying degrees. *MSSA, MRSA* and *S. epidermidis* were the most susceptible bacteria to the plant extract, followed by *E. faecalis* and *P. aeruginosa*; *A. baumannii* was only sensitive to decoction and infusion. These results are in accordance with those obtained by Opara et al. (2009), Reddy et al. (2007) and Nascimento et al. (2000). Their findings showed inhibitory effect of *P. granatum* extracts against *S. aureus* and *P. aeruginosa*. Other studies, demonstrated the high antibacterial activity of pomegranate extracts or phytochemicals against different strains of *MSSA* and *MRSA* (Machado et al., 2003; Machado et al., 2002; Meléndez and Capriles, 2006; Sadeghian et al., 2011).

Our results also show that the highest zone of growth inhibition was shown by decoction against *MSSA* (30.3 mm), *S. epidermidis* (22.3 mm), *MRSA* (22.0 mm), *A. baumannii* (16.0 mm), *E. faecalis* (14.2 mm), and *P. aeruginosa* (12.3 mm). For infusion, the measured zone diameter was *MSSA* (20.7 mm), *S. epidermidis* (16.3 mm), *MRSA* (12.7 mm), *E. faecalis* (12.7 mm), *P. aeruginosa* (11.7 mm), and *A. baumannii* (11.0 mm). Maceration give inhibitory zone of 17.7 mm for *MSSA*, 14 mm for *S. epidermidis*, 11.3 mm for *MRSA*, 10 mm for *E. faecalis*, and 8.3 mm for *P. aeruginosa*, while *A. baumannii* was not sensitive to this extract.

In this study, we also note as the temperature was increased, the antibacterial activity also

Table 2. Inhibition zone diameter (mm)[a] of aqueous extracts of *P. granatum* fruit peels against human pathogenic bacteria.

Bacteria	Decoction	Infusion	Maceration
MSSA	30.3 ± 0.33	20.7 ± 0.33	17.7 ± 067
MRSA	22.0 ± 0.58	12.7± 0.33	11.3 ± 0.33
S. epidermidis	22.3 ± 0.33	16.3 ± 0.33	14.0 ± 0.58
E. faecalis	14.2 ± 0.17	12.7 ± 0.67	10.0 ± 0.00
P. aeruginosa	12.3 ± 0.33	11.7 ± 0.33	8.3 ± 0.33
A. baumanii	16.0 ± 0.58	11.0 ± 0.58	-
E. coli	-[b]	-	-
K. pneumoniae	-	-	-
C. freundii	-	-	-
S. liquefaciens	-	-	-
P. mirabilis	-	-	-

[a]Inhibition zone diameter expressed in Mean ± Standard error; [b]No inhibition.

Table 3. CMI and CMB of decoction of pomegranate fruit peels.

Bacteria	MIC (mg/ml)	MBC (mg/ml)
MSSA	0.048	0.048
MRSA	0.097	0.097
S. epidermidis	0.097	0.097
E. faecalis	3.12	6.25
P. aeruginosa	1.56	1.56
A. baumannii	1.56	1.56
E. coli	ND[c]	ND
K. pneumoniae	ND	ND
C. freundii	ND	ND
S. liquefaciens	ND	ND
P. mirabilis	ND	ND
S. typhi	ND	ND

ND: Not done.

increased (Decoction > Infusion > Maceration), similar to the data presented by El-Mahmood (2009) which showed that the antibacterial activity of *Euphorbia hirta* is enhanced at elevated temperatures. The traditional practitioners usually boil the plants before dispensing out to patients. The results obtained in this study support the methods used by the traditional healers.

The MIC values obtained in our experiments (Table 3) with decoction of pomegranate peel (the most active extract) were very interesting. First, they confirmed the results obtained using the disc diffusion method. Then, the MICs against *S. aureus* and *P. aeruginosa* were much lower than those reported in number of studies. Indeed, MIC was 70 mg/ml against *P. aeruginosa* (Nascimento et al., 2000) and ranging from 0.25 to 25 mg/ml against *S. aureus* strains (Prashanth et al., 2001; Machado et al., 2003; Al-Zoreky, 2009).

Table 4. Phytochemical screening of pomegranate peels

Assay	Result
Phenols	+
Tannins	+
Flavonoids	+
Alkaloids (Dragendroff reaction)	-
Saponins	+
Steroides and terpenes	+
Cardiac glycosides	+
Mucilages	+

+: Present ; -: Absent.

From the MIC values (0.048 to 3.12 mg/ml) obtained in the present study, it can be concluded that decoction of pomegranate peel could be a good source of bioactive components with antimicrobial potency.

Also, the MIC and MBC results demonstrated that aqueous pomegranate extract has a bactericidal effect even on multi-drug resistant bacteria. In fact, when the MBC value is close to MIC (1≤MBC/MIC≤2), the antibiotic (or extract) is bactericide (Éberlin, 1994). This the first study describing the bactericidal activity of pomegranate peel decoction against *S. epidermidis*, *E. faecalis*, *P. aeruginosa* and *A. baumannii*.

Although, several studies showed weak or not effect of *P. granatum* water extract (Al-Zoreky, 2009; Sadeghian et al., 2011), the surprising point in our study is the bactericidal effect of *P. granatum* peel water extract (specially decoction) which was more than those obtain with solvent extracts and antibiotics (Prashanth et al., 2001; Machado et al., 2003; Meléndez and Capriles, 2006; Altuner, 2011; Sadeghian et al., 2011), particularly on *P. aeruginosa* and *A. baumannii* which are resistant to different antibiotics.

Phytochemical screening

This bactericidal activity of *P. granatum* peel water extract on Gram-positive and Gram-negative bacteria is attributed to its contents of bioactive components. Indeed, as shown in Table 4, the phytochemical screening revealed the presence of tannins, phenolics, cardiac glycosides, saponins, and flavonoids. These compounds have potentially significant application against human pathogens. Several authors have linked the presence of these bioactive compounds to the antimicrobial properties of crude plant extracts (Nascimento et al., 2000; Reddy et al., 2007; Machado et al., 2003). This activity of pomegranate aqueous extracts, suggest that the active principles are soluble in water. In fact, pomegranate peels contain very important amount of phenolics including water soluble tannins which are well known to possess antimicrobial properties (Reddy et al., 2007;

Machado et al., 2003; Machado et al., 2002; Al-Zoreky, 2009; Shan et al., 2007; Cowan, 1999). Other authors related such antimicrobial activity of pomegranate to its high content of total flavonols, phenolics, anthocyanins and organic acids (Duman et al., 2009). While Opara et al. (2009), associated this activity with the presence of vitamin C in pomegranate peel, they reported that the best activity against *S. aureus* and *P. aeruginosa* were found in fruit peel fractions, particularly from Oman, which coincide with the highest levels of vitamin C detected in these samples.

Conclusion

Conclusively, our results show that aqueous extracts of pomegranate peel have great potential as antibacterial compounds against several microorganisms. Thus, they can be exceedingly effective in the treatment of infectious diseases caused by resistant bacteria such as *MRSA, P. aeruginosa* and *A. baumannii*, which cause nosocomial infections.

This study thus validates, in systematic way, the use of traditional preparations (especially decoction) of *P. granatum* used for many centuries, in Morocco, to treat various ailments. Further isolation and purification of our extracts are in progress to determine the active components responsible for their activity; also, clinical trials will be required to confirm its antibacterial action *in vivo*.

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