Revista de Ciências Farmacêuticas Básica e Aplicada
Journal of Basic and Applied Pharmaceutical Sciences

Rev Ciênc Farm Básica Apl., 2015;36(2):219-225
ISSN 1808-4532

Antibacterial activity of crude ethanolic and fractionated extracts of *Punica granatum* Linn. fruit peels

Éverton da Silva Santos[1]; Jaqueline Hoscheid[2,*]; Patricia Terron Ghezzi da Mata[1].

[1]Pontifícia Universidade Católica do Paraná, Escola de Saúde e Biociências, 85902-532 Toledo - PR, Brasil
[2]Universidade Estadual de Maringá, Departamento Farmácia, 87020-900 Maringá – PR, Brasil

ABSTRACT

Currently it is clear the need to develop new antimicrobial seeking to solve problems such as antibiotic resistance, in this context medicinal plants has been using a prominent place, and knowledge of popular medicine shows itself to be a promising search tool. Peel of Punica granatum fruits are popularly used for the treatment of diarrhea, eye and upper airway inflammation, and in the external treatment of infectious sores. Thus, this study had the objective to evaluate the *in vitro* inhibitory effect of the crude ethanol extract of peels of *P. granatum*, three organic fractions and also fractions obtained by column chromatography, on reference microorganisms (*Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium* and *Pseudomonas aeruginosa*) by disk diffusion method. The obtained results evidenced that the ethyl acetate and aqueous fractions facing *S. aureus* and *E. coli* showed significant antimicrobial activity, close to the antimicrobial gentamicin and penicillin, respectively. In its turn the crude ethanolic extract of *P. granatum* and aqueous fraction showed inhibitory effect similar to the antimicrobial tetracycline facing *P. aeruginosa*. It was observed an increase in the inhibition of the microorganisms with increasing extract volume (from 10 to 30 µL), being *S. aureus* and *P. aeruginosa* the most susceptible microorganisms. Differences in activity between the extracts and fractions can be partly explained by qualitative and quantitative variations in the secondary metabolites present in the extracts and fractions.

Keywords: Pomegranade. Medicinal plants. Antimicrobial action.

Autor correspondente: Jaqueline Hoscheid. Phone number: +55(44) 30115966. Fax number:+55 (44) 3263623. Complete postal address: Universidade Estadual de Maringá, Centro de Ciências da Saúde, Departamento de Farmácia, Avenida Colombo, 5790 block K80. Zona Sete, 87020-900- Maringá - PR, Brasil.

INTRODUCTION

Microbial resistance to drugs is a serious problem that has been worsening along the years, affecting developed as well as developing countries. Clear examples of resistance are observed by *S. aureus* and *P. aeruginosa* microorganisms (Brooks et al., 2012). Resistance is the best documented case of biological evolution, making it necessary to develop new therapeutic forms for the treatment of pathogenic microorganisms. In this context phytotherapy shows itself a search engine (Duarte, 2006; Silva et al., 2010).

Punica granatum Linn., popularly known as pomegranate, is used in popular medicine for the treatment of diarrhea, eye and upper airway inflammation, and the external treatment of infectious sores (Sartório, 2000; Reis, 2003).

Previous studies have already reported the *in vitro* antimicrobial activity of aqueous, ethanolic and hydroalcoholic extracts of *P. granatum* facing several strains of *Staphylococcus aureus* (Michelin et al., 2005; Catão et al., 2006; Silva et al., 2008; Pereira et al., 2010; Kadi et al., 2011). Pradeep, Manojbabu & Palaniswamy (2008) and Choi et al. (2011), showed the growth inhibition of *Salmonella typhimurium* and *S. typhi* facing the methanol and acetone extracts of *P. granatum*. The antimicrobial activities of pomegranate extracts against oral microorganisms (Janani & Estherlydia, 2013) and Shigella (Parra et al., 2011) also were proven. The antioxidant activity of the pulp and seeds of the species has also been reported, being related to the presence of phenolic compounds (Jardini & Filho, 2007).

Recent studies have also demonstrated the ability of extracts of *P. granatum* in protective effect against serum/glucose deprivation-induced PC12 cells injury (Forouzanfar et al., 2013), in aldose reductase inhibitory activity (Karasu et al., 2012), antioxidant capacity (Dassprakash et al., 2012; Karasu et al., 2012) and in potential reduction of metastases in breast cancer (Dikmen, Ozturk & Ozturk, 2011; Rocha et al., 2012).

The antimicrobial activity of pomegranate fruit peel is related to phytocompounds present, predominantly alkaloids and tannins, among which stands out the punicalagin compound, ellagitannin with proven antimicrobial activity (Machado et al., 2002). Phytochemical analyses revealed, also, the presence of flavonoids, anthocyanins, glycosides and fatty acids in fruit peel of this species (Nicoll, 2005), and the inhibitory potential of polyphenols and flavonoids also been reported (Ahmad & Beg, 2001; Naz et al., 2007; Al-Zoreky, 2009).

With the objective of finding new substances with antimicrobial potential for development of herbal medicines, this study evaluated the *in vitro* inhibitory effect of the crude ethanol extract of *P. granatum* peels, and different fractions of the extract, on pathogenic microorganisms.

MATERIAL AND METHODS

Plant material

Punica granatum Linn. fruits from Palotina, Paraná, Brazil (24°17′ S; 53°50′ W) were collected in March 2013. The specimen was identified on the Herbarium of the Pontifical Catholic University of Parana, and a voucher specimen was deposited in the Herbarium (HUCP), under code number 22859. Fruits were collected, rolled up in paper and packed in cardboard pouches. The fruits were then cleaned, their peels parts separated, dried with air circulation at 40 °C for three days. The dried peels were ground by knife grinder.

Preparation and fractionation of crude extract

Portions (75 g) of finely-powdered peels were extracted with 800 mL of ethanol 99.95% by Soxhlet extractor for 4 hours followed by concentration under reduced pressure at 40 °C. With the purpose of separating the components of the crude extract by polarity difference, the crude extract (64.35 g) was added to 350 mL of water and partitioned successively with 350 mL of hexane, 350 mL of dichloromethane and 350 mL of ethyl acetate. The organic solvent was evaporated in a vacuum evaporator at 40 °C to yield the hexane fraction (HF) (3.63 g), dichloromethane fraction (DF) (0.51 g), ethyl acetate fraction (EAF) (20.28 g). The aqueous fraction was lyophilized and thus the aqueous fraction was obtained (AF) (30.42 g).

Based on the activity, only the AF (4 g) was chomatographed on a column (1 x 30 cm) filled with Silica Gel 60 (Merck®, 70-230 mesh ASTM) eluated using a gradient of ethyl acetate:ethanol (8:2; 7:3; 6:4; 1:1; 4:6; 3:7; 2:8; 1:9) and ethanol, affording 124 fractions (10 mL each fraction). The collected fractions were analyzed by TLC on Silica Gel plates F254 (Merck®, 0.25mm thick), eluated with ethyl acetate:ethanol (7:3). The spots on TLC were visualized under UV at 254 nm and sprayed with vanillin/sulfuric acid, and were combined according to their TLC profiles in 15 sub-fractions.

Preliminary phytochemical screening

The presence or absence of the phytochemical constituents in the crude ethanolic extract was analyzed using standard procedures for carbohydrates (Molisch´s test), reducing sugars (Fehling´s test), saponins, tannins, flavonoids, alkaloids, anthraquinones, steroids, glycosides as described by Goyal et al. (2010). Furthermore, the total phenolic concentrations of the crude extract and in fractions (HF, DF, EAF and AF) were determined using the Folin-Ciocalteu methods described previously (Singleton & Rossi, 1965). Briefly, 200 µL of diluted extract was added to a test tube and then mixed with 1000 µL of Folin-Ciocalteu reagent (1:10). Thirty seconds later 800 µL Na_2CO_3 (7.5%) was added. The reaction mixture was incubated at 24 °C for 1 hour, before the absorbance was read at 765 nm using a UV–Vis spectrophotometer. The standard calibration (500–15.625 µg/mL) curve was plotted using gallic acid. All determinations were carried out in triplicate, and the total phenolic content was expressed as mg gallic acid equivalent/g of extract.

Antibacterial activity

The strains of the reference microorganisms tested against the crude extract of *P. granatum* and its fractions were: *Staphylococcus aureus* (ATCC 25922), *Escherichia coli* (ATCC 25923), *Salmonella typhimurium* (ATCC 14028) and *Pseudomonas aeruginosa* (ATCC 27853), provided by the microbiology laboratory of PUCPR-Toledo.

The antibacterial activity was determined by disk diffusion method (NCCLS, 2002), measuring the inhibition halos formed (Barry & Thornsberry, 1991). The extracts were diluted in sterile water at a concentration of 0,2 g mL^{-1} and applied in three volumes (10, 20 and 30 µL) onto filter paper discs measuring 6 mm in diameter. The 15 sub-fractions obtained by column chromatography of the AF were evaluated only at the dose of 10 µL, at a concentration of 0,2 g mL^{-1}.

Discs impregnated with the dilutions were placed on the surface of Petri dishes containing Muller Hinton Agar inoculated previously with a bacterial suspension with turbidity equivalent to the tube 0.5 of McFarland scale (1.5 x 10^8 CFU mL^{-1}). The cultures were incubated at 37 °C for 24 h (Carvalho et al., 2002). As negative control were used disks impregnated with sterile water. As positive control penicillin 10 µg (PEN), gentamicin 10 µg (GEN), ceftriaxone 30 µg (CRO) and tetracycline 30 µg (TET) were used. All evaluations were performed in triplicate. The extract or fraction was considered active when the lowest concentration of compounds that produced an 80% reduction in visible growth compared with control (Holetz et al., 2002).

Statistical analysis

Results are expressed as mean ± S.E.M. of triplets. The groups were compared by ANOVA post hoc Tukey´s test, using GraphPad Prism, Version 5.0 (GraphPad Software, San Diego, CA, USA). P-values < 0.05 were considered significant.

RESULTS

Four microorganisms were tested for sensitivity to the crude extract and fractions of *P. granatum*. Antimicrobial activity was determined by agar diffusion method. Table 1 presents diameters/zones of inhibition exerted by the extract, fractions, and standard antimicrobials against the organisms challenged. The results show that the fractions that presented had the best results were AF and AEF, not differing statistically between themselves.

It was observed an increase in the inhibition of the microorganisms with increasing dosage (from 10 to 30 µL), however this difference was only significant for the crude extract and AEF against *S. typhi*murium, leading one to believe that the higher the concentration of the extract or fraction of *P. granatum* greater the antimicrobial activity *in vitro* at doses of up to 30 µL.

Regarding the susceptibility to inhibition of microorganisms, *P. aeruginosa* was more sensitive facing the extract and fractions of *P. granatum*. The AF and AEF against *S. aureus* showed significant antimicrobial activity, close to antimicrobial gentamicin. AEF and AF still showed effect similar to penicillin on *E. coli*. The crude extract and AF showed inhibitory effect similar to the antimicrobial tetracycline for *P. aeruginosa*, with no significant difference between both. However, the non-polar fractions, such as HF and DF, did not inhibit significantly the growth of tested microorganisms, when compared to ethanolic extract and aqueous fraction.

Verified the promising results of AF, this was listed to perform semi-purification by column chromatography, in an attempt to enhance the antimicrobial activity of *P. granatum* by isolating compounds. The results obtained from the diffusion test of the 15 sub-fractions resulting from the chromatography process are shown in Table 2.

Accordingly to the activity of the extracts, the Gram-negative bacteria *P. aeruginosa* was the most susceptible one when the 15 sub-fractions of AF were tested. It was possible to observe activity in fractions 1-5 and 10-15, while the fractions 6-9 were not active. The fraction that showed the best result compared to the commercial antimicrobial used as positive control was the FR13. However, these halo diameters obtained with the fractions are inferior to the inhibitory effect of non-partitioned extracts at a volume of 10 µL, suggesting that the antimicrobial activity of AF is the result of the synergistic action of several compounds present in the fraction of the extract of *P. granatum*.

The preliminary phytochemical screening of the crude ethanolic extract revealed the presence of several bioactive compounds, predominantly tannins, convergent with results found by Machado et al., (2002). Results of phytochemicals tests are summarized in Table 3.

After drawn the standard curve of gallic acid (Equation of the line: y=0.0073x+0.1498; R2=0.9948), the concentration of phenolic compounds in the extract and fractions were determined (Table 4), in order to verify the relationship between the concentration of phenolic compounds and antimicrobial activity. A small variation, not significant, regarding the concentration of total phenolic compounds in the different fractions was verified; however it is possible to observe that the AF had the highest concentration of phenolic compounds in its constitution, and the better efficiency in the inhibition of microorganisms *in vitro*, while HF showed a lower antimicrobial activity and lower concentrations of total phenolics as has been shown in the Table 1.

DISCUSSION

Previous studies with extracts of *P. granatum* have already demonstrated efficient antimicrobial inhibition, especially against Gram positive bacteria such as *S. aureus*. This pathogen has caused serious problems in human health for its acquired resistance to antibiotics (Menezes et al., 2008). *S. aureus* is a pathogen in potential to cause disease and is most commonly found in the nasopharynx region and nasal cavity (Trindade, Fonseca & Juiz, 2009). Justifying the fact of the use in the phytotherapy of teas of the pomegranate peel, in the form of gargle, against inflammation of the upper airways.

In relation to the constituents of cell wall of Gram negative and positive bacteria, no relation was observed in this study, since there was inhibition of the extracts of *P. granatum* in both situations. The results observed by other authors (Michelin et al., 2005; Catão et al., 2006; Trindade, Fonseca & Juiz, 2009; Gi Choi et al., 2009; Pereira et al., 2010) corroborate the results obtained in this study, showing high antimicrobial activity of the extracts of *P. granatum*, against a range of microorganisms, both Gram positive and negative.

In the present study *P. aeruginosa* showed itself to be the most susceptible organism to inhibition facing extract and fractions of *P. granatum*. These results diverge from those found by Pradeep, Manojbabu & Palaniswamy (2008) in which the authors reported the inactivity of the crude methanolic extract of *P. granatum* against *P. aeruginosa*.

Pereira et al. (2005) and Schreiner et al. (2009) suggest the use of the species as an alternative way in treatments in the dental office, as in the decrease of microorganisms in orthodontic elastic bandages or in the use of a dentifrice based on *P. granatum*.

Non-polar fractions, HF and DF, did not significantly inhibit the growth of the tested microorganisms. These results agree with previous tests using less hydrophobic solvents such as n-hexane and chloroform (Cowan, 1999; Alzoreky & Nakahara, 2003; Negi & Jayaprakasha, 2003; Voravuthikunchai et al, 2005).

Positive results against bacteria that cause gastrointestinal infections are due to possible secondary metabolites present in the extracts of *P. granatum* (Pradeep et al., 2008). In the present study it was verified an intense activity mainly for fractions of medium and high polarity,

Table 1 - Antibacterial activity of different extracts/fractions obtained from *Punica granatum* fruit peels using agar diffusion method.

Extract/Fraction	Dose (µL)	Microorganisms			
		S. aureus	*E. coli*	*S. typhi***murium**	*P. aeruginosa*
Ethanolic	**10**	3.33 ± 3.33	4.00 ± 4.00	0	14.67 ± 0.88
	20	6.66 ± 6.66	6.66 ± 3.33	6.33 ± 3.18	18.00 ± 1.00#
	30	13.00 ± 0.57*	5.66 ± 2.84	9.66 ± 0.88**	18.00 ± 1.15#
Hexane	**10**	0	0	0	0
	20	0	0	0	0
	30	3.66 ± 3.66	3.33 ± 3.33	0	0
Ethyl acetate	**10**	6.33 ± 3.18	13.66 ± 2.40#	2.00 ± 2.00	13.33 ± 0.33
	20	15.00 ± 2.64*#	12.33 ± 1.45#	10.00 ± 0.00*	13.00 ± 1.00
	30	14.66 ± 0.88*	9.66 ± 5.78#	10.00 ± 2.00*	7.66 ± 4.09
Dichloromethane	**10**	2.00 ± 2.00	3.00 ± 3.00	0	9.33 ± 1.20
	20	5.33 ± 5.33	2.33 ± 2.33	0	11.00 ± 0.57
	30	0	0	2.33 ± 2.33	11.67 ± 0.88
Aqueous	**10**	4.33 ± 4.33	0	10.00 ± 0.00	17.33 ± 0.66
	20	7.33 ± 7.33	7.66 ± 5.78*	11.33 ± 0.66	20.67 ± 0.66#
	30	15.00 ± 0.57**#	13.00 ± 2.00**#	12.00 ± 2.00	18.33 ± 1.20#
Sterile water		0	0	0	0
PEN	**10**		10.00 ± 0.88#		
GEN	**10**	21.00 ± 0.66#			
CRO	**30**			40.00 ± 0.80	
TET	**30**				23.00 ± 2.00#

The results were expressed by mean ± S.E.M. of inhibition zones (mm). *P < 0.05; **P < 0.01 compared with lower doses of the same extract/fraction. #No significant difference (P < 0.05) between size of inhibition zone formed by the extract/fraction and standard antibiotic. (PEN): Penicillin; (GEN): Gentamicin; (CRO): Ceftriaxone; (TET): Tetracycline.

Table 2 - Antibacterial activity of fractions from water extract obtained by column chromatography from *Punica granatum* fruit peels using agar diffusion method.

Fractions	Microorganisms			
	S. aureus	*E. coli*	*S. typhi***murium**	*P. aeruginosa*
FR1[a]	2.33 ± 2.33[b]	0	0	7.33 ± 3.71
FR2	0	0	9.00 ± 0.00	8.66 ± 4.66
FR3	0	0	10.33 ± 0.33	10.33 ± 5.17
FR4	0	3.66 ± 3.66	6.66 ± 3.33	10.33 ± 5.78
FR5	0	0	0	9.66 ± 4.84
FR6	0	0	0	4.66 ± 4.66
FR7	3.00 ± 3.00	0	0	3.66 ± 3.66
FR8	0	0	0	4.00 ± 4.00
FR9	0	0	0	3.66 ± 3.66
FR10	0	0	10.00 ± 0.00	13.33 ± 1.76
FR11	0	6.33 ± 3.28	2.66 ± 2.66	10.67 ± 5.36
FR12	0	0	3.00 ± 3.00	10.00 ± 0.00
FR13	0	10.00 ± 0.00	6.33 ± 3.18	15.33 ± 0.33
FR14	0	6.00 ± 3.05	5.33 ± 2.66	14.00 ± 0.00
FR15	0	0	0	14.33 ± 1.33
Sterile water	0	0	0	0

The results were expressed by mean ± S.E.M. of inhibition zones (mm).

[a]Each fraction was applied onto paper disks (10 µL of 0,2 mg mL^{-1}).

[b]Inhibition zones (mm).

Table 3 - Preliminary phytochemical test of the crude ethanolic extract of *P. granatum* fruit peels.

Test/Chemical compounds	Result
Reducing sugars	-
Alkaloids	-
Anthraquinone	+
Carbohydrates	+
Steroids	-
Flavonoid	++
Glycosides	+
Saponinas	-
Tannins	+++

(-): Compound not detected; (+): Compound detected at low concentration; (++): Compound detected in intermediate concentration; (+++) Compound detected in high concentration.

Table 4 - Phenolic compounds concentration from crude extract and fractions of *Punica granatum*.

Extract	Mean (mg/g) ± S.D.
Crude ethanolic extract	0.1655 ± 0.0002
Hexane fraction	0.1577 ± 0.0000
Dichloromethane fraction	0.1628 ± 0.0001
Ethyl acetate fraction	0.1621 ± 0.0002
Aqueous fraction	
13th sub-fraction of aqueous fraction	0.1624 ± 0.0002

while non-polar fractions did not inhibit the growth of tested microorganisms. It is known that the inhibitory action of EAF probably is due to the fact that in the extracting solvent ethyl acetate usually are extracted flavonoid compounds. Studies have shown that there are several classes of flavonoids with significant anti-inflammatory (Calixto, 2000) and antimicrobial action (Sartori et al., 2003). The antimicrobial action of flavonoids is, probably, related to the capacity of this compound to complex extracellular and soluble proteins as well as structures of the bacterial cell wall (Chabot et al., 1992).

The activity of AF may be due to the presence of tannins, since this class of secondary metabolites shows great solubility in water and property of precipitating proteins, and consequently antimicrobial action (Monteiro et al., 2005). In the fruits of *P. granatum*, tannins are important constituents and represent about 25% of the constituents of the peels (Voravuthikunchai et al., 2005). The peels of the fruits are rich in ellagitannins and derivatives of gallic acid, flavonoids, anthocyanins, glycosides and fatty acids (Nicoll, 2005).

Differences in the activity between the extracts and fractions could also be partially explained by qualitative and quantitative variations of phenolic compounds and of the sensitivity of the strains used. The antimicrobial activity as well as the antioxidant activity and other biological activities with phenolic compounds, including tannins, was already demonstrated previously (Cowan, 1999; Alzoreky & Nakahara, 2003; Machado et al., 2003; Voravuthikunchai et al., 2004; Reddy et al., 2007; Shan et al., 2007; Fan et al., 2008). Segundo Haslam (1996), the phenolic compounds disrupt the bacterial cell walls, destroying the cell.

The results obtained in this study showed the good antimicrobial activity of the extracts and fractions of *P. granatum*, as reported by several authors and by popular medicine, which has been using *P. granatum* for many generations. However to obtain clearer and more accurate results, studies of isolation and identification of the active compounds responsible for the activity are made necessary.

CONCLUSION

The pomegranate extract and fractions showed significant antimicrobial activity. The aqueous fraction had greater relevance in the activity when compared with the other fractions. Thus, the semi-purification of this fraction by column chromatography allowed infering that the antibacterial activity is a synergistic relationship between several compounds present in fruit peels, since the activity of the aqueous fraction was higher then the sub-fractions. Phytochemical tests allowed detection of anthraquinones, carbohydrates, flavonoids, glycosides and tannins in the crude ethanolic extract of peels of the fruits of *P. granatum*. Differences in the activity between the extracts and fractions can be partly explained by qualitative and quantitative variations in the secondary metabolites present in the extracts and semi-purified fractions.

RESUMO

Atividade antibacteriana do extrato bruto e fracionado de cascas dos frutos de Punica granatum Linn.

Atualmente está clara a necessidade do desenvolvimento de novos antimicrobianos buscando resolver problemas como a resistência a antibióticos, neste contexto, as plantas medicinais vem utilizando um lugar de destaque, e os conhecimentos da medicina popular mostram-se uma ferramenta de busca promissora. Cascas dos frutos de *Punica granatum* **são utilizadas popularmente para o tratamento de diarreias, inflamações oculares e das vias aéreas superiores, e no tratamento externo de feridas infecciosas. Assim, este estudo teve o objetivo de avaliar o efeito inibitório** *in vitro* **do extrato bruto etanólico de cascas de** *P. granatum***, três frações orgânicas e de frações obtidas por cromatografia em coluna, sobre microrganismos referência (***Staphylococcus aureus***,** *Escherichia coli***,** *Salmonella typhimurium* **e** *Pseudomonas aeruginosa***) pelo método de disco difusão. Os resultados obtidos evidenciaram que as frações acetato de etila e aquosa frente a** *S. aureus* **e** *E. coli* **demonstraram atividade significativa, próximo ao antimicrobiano gentamicina e penicilina, respectivamente. Já o extrato bruto etanólico**

de *P. granatum* e a fração aquosa apresentaram efeito inibitório semelhante ao antimicrobiano tetraciclina frente a *P. aeruginosa*. Observou-se um aumento na inibição dos microrganismos conforme o aumento do volume de extrato (de 10 para 30 µL), sendo que *S. aureus* e *P. aeruginosa* foram os microrganismos mais suscetíveis. Diferenças na atividade entre os extratos e frações podem ser parcialmente explicadas por variações qualitativas e quantitativas de metabólitos secundários presentes no extrato e frações.
Palavras-chave: Romã. Plantas medicinais. Ação antimicrobiana.

REFERENCES

Ahmad I, Beg AZ. Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. J Ethnopharmacol. 2001;74:113-133.

Alzoreky N, Nakahara K. Antibacterial activity of extracts from some edible plants commonly consumed in Asia. Int J Food Microbiol. 2003;80:223–30.

Al-Zoreky NS. Antimicrobial activity of pomegranate (*Punica granatum* L.) fruit peels. Int J Food Microbiol. 2009;134:244–8.

Barry AL, Thornsberry C. Susceptibility tests: diffusion test procedures. In: Balows A, Hauser WJ, Hermann KL, Isenberg HD, Shamody HJ (Eds). Manual of clinical microbiology. 5th. ed. Washington: American Society for Microbiology; 1991.

Brooks GF, Carroll KC, Butel JS, Morse AS, Mietzner TA. Microbiologia Médica de Jawetz, Melnick e Adelberg. 25. ed. Porto Alegre: AMGH; 2012.

Calixto JB. Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phythoterapeutic agents). Braz J Med Biol Res. 2000;33(2):179-89.

Carvalho AAT, Sampaio MCC, Sampaio FC, Melo AFM, Sena KXFR, Chiappeta AA, Higino JS. Atividade antimicrobiana *in vitro* de extratos hidroalcoólicos de Psidium guajava L. sobre bactérias gram-negativas. Acta Farm Bonaer. 2002;21:255-8.

Catão RMR, Antunes RMP, Arruda TA, Pereira MSV, Higino JS, Alves JA, Passos MGVM, Santos VL. Atividade antimicrobiana "*in vitro*" do extrato etanólico de *Punica granatum* linn. (romã) sobre isolados ambulatoriais de *Staphylococcus aureus*. RBAC. 2006;38(2):111-4.

Chabot S, Bel-Rhlid R, Chenevert R, Piché Y. Hyphal growth promotion *in vitro* of the VA mycorrhizal fungus. Gigaspora margarita Becker and Hall, by the activity of structurally specific flavonoid compounds under CO enriched conditions. New Phytol. 1992;122:461-7.

Choi JG, Kang OH, Lee YS, Chae HS, Oh YC, Brice OO, Kim MS, Sohn DH, Kim HS, Park H, Shin DW, Rho JR, Kwon DY. *In vitro* and *in vivo* antibacterial activity of *Punica granatum* peel ethanol extract against Salmonella. e-CAM. 2011:1-8.

Cowan MM. Plant products as antimicrobial agents. CMR. 1999;12:564–82.

Dassprakash MV, Arun R, Abraham SK, Premkumar K. *In vitro* and *in vivo* evaluation of antioxidant and antigenotoxic potential of *Punica granatum* leaf extract. Pharm Biol. 2012;50(12):1523-30.

Dikmen M, Ozturk N, Ozturk Y. The antioxidant potency of *Punica granatum* L. Fruit peel reduces cell proliferation and induces apoptosis on breast cancer. J Med Food. 2011;14(12):1638-46.

Duarte MCT. Atividade Antimicrobiana de Plantas Medicinais e Aromáticas Utilizadas no Brasil. Rev MultiCiências. 2006;1(7):1-16.

Fan W, Chi Y, Zhang S. The use of a tea polyphenol dip to extend the shelf life of silver carp (Hypophthalmicthys molitrix) during storage of ice. Food Chem. 2008;108:148–53.

Forouzanfar F, Afkhami Goli A, Asadpour E, Ghorbani A, Sadeghnia HR. Protective Effect of *Punica granatum* L. against Serum/Glucose Deprivation-Induced PC12 Cells Injury. e-CAM. 2013;2013:1-9.

Gi Choi J, Kang OH, Lee YS, Chae HS, Oh YC, Brice OO, Kim MS, Sohn DH, Kim HS, Park H, Shin DW, Rho JR, Kwon DY. *In vitro* and *In vivo* Antibacterial Activity of *Punica granatum* Peel Ethanol Extract against Salmonella. e-CAM. 2009;2011:1-8.

Goyal AK, Middha SK, Sen A. Evaluation of the DPPH radical scavenging activity, total phenols and antioxidant activities in Indian wild Bambusa vulgaris "Vittata" methanolic leaf extract. J Nat Pharm. 2010;1(1):34-39.

Haslam E. Natural Polyphenols (Vegetable tanniins) as drugs: possible modes of action. J Nat Prod. 1996;59(2):205-15.

Holetz FB, Pessini GL, Sanches NR, Cortez DAG, Nakamura CV, Filho BPD. Screening of some plants used in the Brazilian folk medicine for the treatment of infections diseases. Mem Inst Oswaldo Cruz. 2002;97(7):1027-31.

Janani J, Estherlydia D. Antimicrobial activities of *Punica granatum* extracts against oral microorganisms. Int J PharmTech Res. 2013;5(3):973-7.

Jardini FA, Filho JM. Avaliação da atividade antioxidante em diferentes extratos da polpa e sementes da romã (*Punica granatum*, L.). Rev Bras Cien Farm. 2007;43(1)137-47.

Kadi H, Moussaoui A, Benmehdi H, Lazouni HÁ, Benayahia A, Nahal Bouderba N. Antibacterial activity of ethanolic and aqueous extracts of *Punica granatum* L. Back. J Appl Pharm Sci. 2011;1(10):180-2.

Karasu C, Cumaoğlu A, Gürpinar AR, Kartal M, Kovacikova L, Milackova I, Stefek M. Aldose reductase inhibitory

activity and antioxidant capacity of pomegranate extracts. Interdiscip Toxicol. 2012;5(1):15-20.

Machado TB, Leal ICR, Amaral ACF, Santos KRN, Silva MG, Kuster RM. Antimicrobial ellagitannin of *Punica granatum* fruits. J Braz Chem Soc. 2002;13(1):606-10.

Machado TB, Pinto A, Pinto M, Leal I, Silva M, Amaral A, Kuster R, Santos KN. *In vitro* activity of Brazilian medicinal plants, naturally occurring naphthoquinones and their analogues, against methicillin-resistant *Staphylococcus aureus*. Int J Antimicrob Agents. 2003;21:279–84.

Menezes SMS, Pinto DM, Cordeiro LN. Atividades biológicas *in vitro* e *in vivo* de *Punica granatum* L. (romã). Rev Bras Med. 2008;65(11):388-91.

Michelin DC, Moreschi PE, Lima AC, Nascimento GGF, Paganelli MO, Claud MV. Avaliação da atividade antimicrobiana de extratos vegetais. Rev Bras Farmacogn. 2005;15(4):316-20.

Monteiro JM, Albuquerque UP, Araújo EL, Amorim ELC. Taninos: uma abordagem da química à ecologia. Quim Nova. 2005:28(5)892-6.

Naz S, Siddiqi R, Ahmad S, Rasool S, Sayeed S. Antibacterial activity directed isolation of compounds from *Punica granatum*. J Food Sci. 2007;72:341–5.

Negi PS, Jayaprakasha GK. Antioxidant and antibacterial activities of *Punica granatum* peel extracts. J Food Sci. 2003;68:1473–7.

Nicoll M. Nanotecnologia potencializa poder curativo da romã: FAPERJ, 2005. Available in: <www.faperj.br/boletim_interna.phtml?obj_id=2427>

NCCLS. Performance standards for antimicrobial disk and dilution susceptibility test for bacteria isolated from animals: approved standard. Wayne, USA; 2002. (NCCLS document. M31-A2).

Parra VIR, Gaudioso C, Cecilia M, Silvia C. Evaluation of the antimicrobial activity of aqueous pomegranate (*Punica granatum* L.) extract against Shigella. Res J Biol Sci. 2011;6(5):205-12.

Pereira JV, Pereira MSV, Silva VA, Azevedo TKB, Pereira MV, Trevisan LFA. Atividade antimicrobiana *in vitro* do extrato da romã (*Punica granatum* Linn) e alecrim-pimenta (Lippia sidoides Cham.) sobre plasmídeos de *Staphylococcus aureus* de origem animal. Rev Biol Farm. 2010;4(1):5-10.

Pereira JV, Pereira MSV, Higino JS, Sampaio FC, Alves PM, Araújo CRF. Estudos com o extrato da *Punica granatum* Linn. (romã): efeito antimicrobiano *in vitro* e avaliação clínica de um dentifrício sobre microrganismos do biofilme dental. Rev Odonto Cien. 2005;20(49):262-9.

Pradeep BV, Manojbabu MK, Palaniswamy M. Antibacterial Activity of *Punica granatum* L. Against Gastro Intestinal Tract Infection Causing Organisms. Ethnobot Leaflets. 2008;1:1085-9.

Reis P. Farmácia em casa: prevenção, cura e rejuvenescimento. 4. ed. São Paulo: Ave - Maria editora; 2003.

Reddy M, Gupta S, Jacob M, Khan S, Ferreira D. Antioxidant, antimalarial and antimicrobial activities of tannin-rich fractions, ellagitannins and phenolic acids from *Punica granatum* L. Planta Med. 2007;73:461–7.

Rocha A, Wang L, Penichet M, Martins-Green M. Pomegranate juice and specific components inhibit cell and molecular processes critical for metastasis of breast cancer. Breast Cancer Res Treat. 2012;136(3):647-58.

Sartori MRK, Pretto JB, Cruz AB, Bresciani LFV, Yunes RA, Sortino M, Zacchino AS, Cechinel Filho V. Antifungal activity of fractrions and two pure compounds of flowers from Wedeliapaludosa (Acmelabrasiliensis) (Asteraceae). Pharmazie. 2003;58(8):567-9.

Sartório ML. Cultivo orgânico de Plantas Medicinais. Viçosa: Aprenda Fácil Editora; 2000.

Silva MAR, Higino JS, Pereira JV, Siqueira-Junior JP, Pereira MSV. Antibiotic activity of the extract of *Punica granatum* Linn. over bovine strains of *Staphylococcus aureus*. Braz J Pharmacogn. 2008;18(2):209-12.

Silva VA, Freitas AFR, Pereira MSV, Pereira AV. Avaliação "*in vitro*" da atividade antimicrobiana do extrato da Lippia sidoides Cham. sobre isolados biológicos de *Staphylococcus aureus*. Rev Bras Plantas Med. 2010;12(4):452-5.

Singleton VL, Rossi Jr JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Viticult. 1965;16(3)144–58.

Schreiner F, Retzlaff G, Siqueira MFR, Rezende EC, Simão LC, Kozlowski-Junior VA, Santos LC. Uso do chá de *Punica granatum* (romã) no controle da Aderência de bactérias orais em ligaduras ortodônticas. ROBRAC. 2009;18(45):56-61.

Shan B, Cai YZ, Brooks J, Corke H. The *in vitro* antibacterial activity of dietary species and medicinal herb extracts. Int J Food Microbiol. 2007;117:112–9.

Trindade MP, Fonseca L, Juiz PJL. Atividade antimicrobiana da tintura da casca de romã (*Punica granatum*) sobre cepas de *Staphylococcus aureus* e Streptococcus pyogenes: estudo *in vitro*. RBPS. 2009;11(4):49-54.

Voravuthikunchai S, Lortheeranuwat A, Jeeju W, Sririrak T, Phongpaichit S, Supawita T. Effective medicinal plants against enterohaemorrhagic *Escherichia coli* O157:H7. J Ethnopharmacol. 2004;94:49–54.

Voravuthikunchai S, Sririrak T, Limsuwan S, Supawita T, Iida T, Honda T. Inhibitory effects of active compounds from *Punica granatum* pericarp on verocytotoxin production by enterohemorrhagic *Escherichia coli* O157: H7. J Health Sci. 2005;51:590-6.

Received on December 31th 2013

Accepted on February 11th 2014