

## Full Length Research Paper

## ***In vitro* antibacterial activities of pomegranate extract against standard microorganisms of bovine mastitis**

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Bovine mastitis is characterized by inflammation of the mammary gland, usually due to bacterial infection, compromising quantity and quality of milk production. This study aimed to determine the antibacterial activity, *in vitro*, of the hydroalcoholic extract of the pomegranate peel at 10% against standard strains of bovine mastitis. The colonies were adjusted to the concentration of  $10^7$  mL<sup>-1</sup> using UV-visible spectrophotometry, and the extracts were evaluated in quintuplicate in concentrations of 1000, 500, 250, 75, 50 and 25 µg mL<sup>-1</sup>. The sensitivity of the strains was determined using the minimum inhibitory concentration and disk diffusion test. Additionally, antioxidant activity and total phenolic content was evaluated. The extract, at concentrations of 500 and 1000 µg mL<sup>-1</sup>, inhibited *Staphylococcus aureus* (ATCC 25923), *S. Saprophyticus* (ATCC 15305), *S. Epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 11229), *Enterobacter cloacae* (ATCC 23355 ) and *Bacillus cereus* (ATCC 33018), but it was not effective for *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella enterica* subspecie, *enterica* serovar Typhi (ATCC 19214). Antioxidant activity was observed from 50 µg mL<sup>-1</sup> reaching a plateau at 500 mg mL<sup>-1</sup> with 64.90%, and the concentration that causes 50% of the inhibition (IC<sub>50</sub>) corresponded to 378.80 µg L<sup>-1</sup>. Perhaps the presence of other substances in the extract may have been responsible for the antioxidant activity detected. That way, the antioxidant and antibacterial activities of EHPG 10% may represent an important therapeutic potential, particularly for animal health in organic and agroecological production systems.

**Key words:** Agroecology, medicinal plants, organic animal production, *Punica granatum* Linn.

### INTRODUCTION

Bovine mastitis is an inflammation frequently caused by bacterial infection which determines important economic impact (Deb et al., 2013). The clinical presentation is mainly caused by *Escherichia coli*, *Klebsiella pneumonia*

and *Pseudomonas aeruginosa*, and the subclinical form is mainly caused by *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis*. Treatment usually occur by

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**Table 1.** Average values of inhibition halo (mm) of growth of *S. aureus* (ATCC 25923), *S. saprophyticus* (ATCC 15305), *S. epidermidis* (ATCC 12228), *E. coli* (ATCC 11229), *E. cloacae* (ATCC 23355) and *B. cereus* (ATCC 33018), submitted to different hydroalcoholic extract of *P. granatum* Linn at 10%.

Extract ( $\mu\text{g ml}^{-1}$ )	<i>S. aureus</i> ATCC 25923	<i>S. saprophyticus</i> ATCC 15305	<i>S. epidermidis</i> ATCC 12228	<i>E. cloacae</i> ATCC 23355	<i>E. coli</i> ATCC 11229	<i>B. cereus</i> ATCC 33018
500	10.2	9.6	-	15.8	-	-
1000	16.6	17.6	17.6	20.6	11.8	13.6

the use of antimicrobial chemicals, however, during lactation the use of these drugs is rare, due to the low efficiency and the presence of milk residues (Vlieghe et al., 2012). In addition, this kind of drug can determine residue in milk, and for this reason the use of chemical antibiotics are not allowed in organic or biodynamic systems of production. Thus, searches were determined for natural solutions that approach the problem without milk disposal. There was significant increase in production of organic and use of medicinal plants as encouraged by the World Health Organization (WHO) because it is accessible economically.

*Punica granatum* Linn., known as pomegranate, is cultivated worldwide and could be considered a functional food because it has compounds in different parts that display functional and medicinal effects (Bhandari, 2012; Ismail et al., 2012). Its constituents are alkaloids (peletierin, isopeletierin, metilpeletierin), tannins, phenolic compounds (anthocyanins, quercetin, phenolic acid) and flavonoids (Duman et al., 2009; Moorthy et al., 2013; Silva et al., 2013). Antimicrobial properties have been demonstrated (Moorthy et al., 2013), on *S. aureus* (Silva et al., 2013; Moorthy et al., 2013; Santos et al., 2014; Moreira et al., 2014). Antibacterial properties are particularly important for the treatment of bovine mastitis. So this study aimed to determine the antibacterial activity, *in vitro*, of the hydroalcoholic extract of the pomegranate peel at 10% (HEP10%) against standard strains of bovine mastitis.

## MATERIALS AND METHODS

A sample of the plant material was identified in the Forest Institute of Assis/SP- Brazil, and a voucher specimen was deposited in the Herbarium of the Institute under number SPSF 40136. For the preparation of the extract, 30 g of fruit peels dry with kiln forced ventilation air at 40°C was used, 80 mL of distilled water, and 190 mL of absolute ethyl alcohol (99.5%). The extraction occurred by constant mechanical stirring in 24 h, vacuum filtration and adding new hydroalcoholic solution in the same volume. This procedure was repeated for three consecutive times. After that, the extract was concentrated using rotary vaporizador (60°C), frozen and lyophilized. The antimicrobial activity was evaluated by using Kirby-Bauer disk diffusion technique (CLSI, 2011). 1000  $\mu\text{g mL}^{-1}$  of the extract was added in nutrient broth and the following dilutions were

held: 500, 250, 100, 75, 50 and 25  $\mu\text{g mL}^{-1}$ . The disk diffusion test was performed in five replications by impregnating an aliquot of 40  $\mu\text{L}$  of each concentration of HEP 10%, in filter paper discs (7 mm).

After drying, the discs were fixed in agar plates Mueller - Hinton previously seeded by  $10^7$  CFU  $\text{mL}^{-1}$  of *S. aureus* (ATCC 25923), *S. saprophyticus* (ATCC 15305), *S. epidermidis* (ATCC 12228), *E. coli* (ATCC 11229), *P. aeruginosa* (ATCC 27853), *S. enterica* subsp. *enterica* serovar *Typhi* (ATCC 19214), *E. cloacae* (ATCC 23355) e *B. cereus* (ATCC 33018). The incubation was performed in microbiological incubator at 37°C for 24 to 48 h. To determine the Minimum Inhibitory Concentration (MIC), the extract was resuspended in nutrient broth under concentrations of 1000, 500, 250, 100, 75, 50 and 25  $\mu\text{g mL}^{-1}$ . The bacterial inoculation ( $5 \times 10^5$  CFU  $\text{mL}^{-1}$ ) proceeded for 20 h at 35°C (CLSI, 2011). The antioxidant activity was evaluated in triplicate, using the same concentrations above. The antioxidant activity was determined according to Blois (1958) and the determination of the sample concentration that causes 50% of the inhibition of the initial concentration of DPPH ( $\text{IC}_{50}$ ) was calculated by linear regression of the points plotted graphically. The mean values obtained by the DPPH test were used to plot the points as Di Mambro and Fonseca (2005).

## RESULTS AND DISCUSSION

The disk diffusion test and the MIC test demonstrated that 500 and 1000  $\mu\text{g mL}^{-1}$  of the HEP 10% presented an antimicrobial activity against Gram positive bacteria. Regarding the dosage of 250  $\mu\text{g mL}^{-1}$ , only the MIC test was able to demonstrate antimicrobial activity (Table 1, 2). Santos et al. (2014) also examined the inhibitory effects of pomegranate peel extract on *S. aureus* isolates from cases of bovine mastitis, which has also been checked by us in preliminary studies (Moreira et al., 2014). However, for the conditions evaluated by the present study, antimicrobial activity for Gram-negative bacteria was not observed. These bacteria are more resistant to antimicrobials based on natural extracts (Carvalho et al., 2013) because they have phospholipid external layer that is impermeable for solute lipophilic. Additionally, the porins create a barrier against hydrophilic solutes, restricting the penetration of antimicrobial compounds. On the other hand, the Gram-positive bacteria have only peptidoglycan on the cell wall (CLSI, 2003; Rabêlo et al., 2014). Moorthy et al. (2013) reported antibacterial activity of pomegranate extract for

**Table 2.** Minimum Inhibitory Concentration (MIC) for the growth of *S. aureus* ATCC 25923, *S. saprophyticus* (ATCC 15305), *S. epidermidis* (ATCC 12228) and *B. cereus* (ATCC 33018) and antioxidant activity in different concentrations of hydroalcoholic extract of *P. granatum*.

Extract ( $\mu\text{g ml}^{-1}$ )	<i>S. aureus</i> ATCC 25923	<i>S. saprophyticus</i> ATCC 15305	<i>S. epidermidis</i> ATCC 12228	<i>B. cereus</i> ATCC 33018	Antioxidant (%)
250		G	G	G	35.59
500				G	64.90
1000					78.35

G: Bacterial growth / I: Growth Inhibition

Gram positive and negative bacteria, but they used the pericarp for making the extract. In this study, the use of the peel was prioritized as residue in order to promote the economic sustainability of the activity.

This extract showed antioxidant activity with values corresponding to 4.62% in the concentration of  $50 \mu\text{g mL}^{-1}$ , reaching 64.90% in the concentration of  $500 \mu\text{g mL}^{-1}$ , and the  $\text{IC}_{50}$  corresponding to  $378.80 \mu\text{g mL}^{-1}$  (Table 2). Karaaslan et al. (2014) found phenolic compounds, but they use the fruit to do the extract. Silva et al. (2013) also evaluated the extract confectioned from the pomegranate peel, and just as the present study, they found a high antioxidant activity, but without correlation to phenolic content. The pomegranate has complex composition; perhaps other alkaloids may have been responsible for the antioxidant activity. Noda et al. (2002) and Duman et al. (2009) associated the antioxidant activity with anthocyanins. Moorthy et al. (2013) reported the punicalagin as a major antimicrobial constituent that was found in pomegranate.

## CONCLUSION

The inhibition of Gram positive bacteria allows us to conclude that the hydroalcoholic extract of pomegranate showed therapeutic potential for bovine mastitis control.

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## Conflicts of interest

Author has none to declare.

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