

Bactericidal activity of ethanolic extracts of propolis against *Staphylococcus aureus* isolated from mastitic cows

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Abstract *Staphylococcus aureus* is an important pathogen for both humans and animals, and it has been an ubiquitous etiological agent of bovine mastitis in dairy farms worldwide. Elimination of *S. aureus* with classic antibiotics is difficult, and the current study aimed to evaluate the efficacy of ethanolic extracts of propolis (EEP) against *S. aureus* cultivated in complex media or milk. EEP (0–0.5 mg ml⁻¹) decreased growth of *S. aureus* in BHI media and 1 mg ml⁻¹ was bactericidal against washed cell suspensions (10⁷ CFU ml⁻¹). Propolis extracts also killed *S. aureus* cells resuspended in milk, but the bactericidal dose was at least 20-fold greater. Cultures that were transferred for at least 60 generations with sub-lethal doses of propolis did not change much their sensibility to EEP. Atomic force microscopy images revealed changes in morphology and cell size of *S. aureus* cells exposed to EEP (0.5 mg ml⁻¹). Our results indicate that propolis extracts might be effective against mastitis-causing *S. aureus* strains in vivo, but milk constituents affect the inhibitory activity of propolis. Considering that propolis-resistance appears to be a phenotype not easily selected, the use of EEP combined or not with other antimicrobial agents might be useful for mastitis control in vivo.

Keywords *Baccharis dracunculifolia* · Mastitis · Udder infection · AFM · Dairy

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Introduction

Bovine mastitis, a disease caused by infection of cow udders, causes significant economical losses to dairy farmers and the dairy industry (Erskine et al. 2003; McDougall et al. 2009). Animals afflicted with mastitis often show reduced milk yield and changes in milk composition. *Staphylococcus aureus*, an important pathogen for both humans and livestock, is one of the most infectious and prevalent etiological agent of mastitis among ruminants animals (Aires-de-Sousa et al. 2007; Capurro et al. 2010). *S. aureus* causes clinical and, more frequently, subclinical infections that tend to become chronic and difficult to eradicate by conventional antimicrobial therapies (Sears and McCarthy 2003).

The treatment of mastitis often involves administration of intramammary antibiotic preparations either during lactation or during the dry (non-lactating) period (Erskine et al. 2003; McDougall et al. 2009). However, the widespread use of antibiotics in animal husbandry has been associated with selection of antibiotic-resistant pathogens and the presence of antibiotic residues in the food chain (Van Eenennaam et al. 1993; Nickerson 2009).

Problems with decreased therapeutic efficacy and the raise of antibiotic-resistant bacteria in livestock production have stimulated the research for new strategies to control mastitis (Varella Coelho et al. 2007; Wu et al. 2007). Previous studies indicated that propolis, a complex resinous material produced by honeybees from plant buds, exudates, beeswax, and bee secretions, was effective against *S. aureus* infections (Sayed et al. 2009; Raghukumar et al. 2010). Despite of the differences in chemical composition among propolis samples, the Brazilian green propolis produced in the Southeast States of Brazil has *Baccharis dracunculifolia* as its main botanical source and its chemical composition and biological activity have been well characterized

(Teixeira et al. 2005; Salomao et al. 2008). In recent years, there is a growing interest in propolis extracts and its constituents due to their potential application for medical and cosmetics purposes.

A large number of constituents have been identified in Brazilian propolis samples and the biological activity has been mainly associated with flavonoids, terpenes, caffeic, ferulic, cinnamic and coumaric acids and esters (Teixeira et al. 2005). The Brazilian green propolis is particularly rich in artepillin C (3,5-diprenyl-4-hydroxycinnamic acid), a phenolic compound with immunomodulatory and anti-inflammatory properties that could be useful for treatment of udder infections (Paulino et al. 2008; Messerli et al. 2009; Fischer et al. 2010).

Previous work indicated that ethanolic extracts of propolis could inhibit *S. aureus* strains, but the effect of the medium matrix on propolis activity or the selection of propolis-resistant cells was not examined (Fernandes Júnior et al. 2005; Lu et al. 2005; Salomao et al. 2008). In selecting antibacterial agents for mastitis therapy using in vitro methods, it is important to consider the effect of milk on the activity of the compounds in situ. In this study we aimed to (1) determine the bactericidal activity of ethanolic extracts of Brazilian green propolis against mastitis-causing *S. aureus* isolates growing in synthetic media and milk, (2) to monitor if propolis-resistant *S. aureus* cells would be selected by exposure to sub-inhibitory concentrations of the ethanolic extracts and (3) to verify the influence of propolis extracts on *S. aureus* cells by atomic force microscopy (AFM).

Materials and methods

Microorganisms and growth conditions

Three *Staphylococcus aureus* isolates that were previously reported as sensitive to propolis extracts were used in this study (Pinto 2008). *S. aureus* 2979 and *S. aureus* 4118 were isolated from mastitic cows belonging to two different bovine herds located in the States of Minas Gerais and Rio de Janeiro, Brazil. *S. aureus* ATCC 29213 was used as a reference strain in this study. The bovine isolates were previously characterized biochemically by Brito and Brito (1999), and were obtained from the Mastitis Pathogens Culture Collection maintained at EMBRAPA Gado de leite (Juiz de Fora, Minas Gerais State, Brazil). The isolates were routinely grown in BHI media (Brain heart infusion—Difco, Detroit, MI) at 37°C.

Preparation of ethanolic extracts of propolis (EEP)

The propolis samples used in this study were obtained from a commercial propolis producer located in the Zona da

Mata region of Minas Gerais State, Brazil. To prepare the EEP, propolis samples were first cut into small pieces and ground. Six grams of ground propolis was then extracted with 20 ml of 70% ethanol (v/v) at 45°C for 48 h. The ethanolic extract of propolis (300 mg ml⁻¹) was then filtered to remove waxes and the stock solution was stored at room temperature until use. The chemical composition of the ethanolic extract was previously determined by using gas chromatography-mass spectrometry (GC/MS) analysis (Pinto 2008). Quantification of flavonoids and phenolic compounds was performed colorimetrically and expressed as equivalents (%) of quercetin and gallic acid, respectively (Pinto 2008). The EEP used in this study contained 2.14 ± 0.05 mg flavonoids per gram of propolis and the concentration of phenolics compounds was 7.7 ± 0.65 mg g⁻¹. Artepillin C (3,5-diprenyl-4-hydroxycinnamic acid), a distinct antimicrobial compound isolated from Brazilian propolis, was found at highest concentration on the EEP (51.96%), as compared with other chemical constituents.

Antimicrobial assay of EEP

Minimum inhibitory concentration (MIC) was determined in 96-wells microtiter plates by means of the broth microdilution method described by the Clinical and Laboratory Standards Institute (CLSI, 2003). Twenty-five microliters of bacterial inoculum (10⁵ CFU ml⁻¹) was added to 175 µl of BHI broth containing different propolis concentrations previously prepared by twofold serial dilutions in 96-well plates. The propolis ethanolic extracts were assayed in the range of 73 × 10⁻³ to 150 mg ml⁻¹. After inoculation, microplates were incubated at 37°C for 48 h. The MIC value was defined as the lowest concentration of propolis that completely inhibited bacterial growth after 48 h of incubation.

Effect of EEP on *S. aureus* growth

The strains of *S. aureus* used in this study were inoculated into BHI broth (5% inoculum, v/v) added with increasing concentrations of EEP (0–1 mg ml⁻¹) and bacterial growth was monitored indirectly via changes in optical density (OD) at 600 nm in a Spectronic 20D+ spectrophotometer (Thermo Electron, Madison, WI, USA). The specific growth rate (h⁻¹) was estimated from differences in the natural logarithms of optical density and time. Lag time (h) was defined as the time before a detectable increase in optical density was observed. Maximum optical density values (OD_{600 nm}) were determined over a period of 24 h for each *S. aureus* isolate and for each concentration of EEP tested.

Viability of EEP-treated *S. aureus* cells in phosphate buffer and milk

To verify if EEP was bactericidal against *S. aureus* cells, stationary phase cultures were harvested at room temperature by centrifugation at $2,096 \times g$ for 15 min (Labor Muszeripari Muvek, Hungary). The cell pellet was washed with 5 mM potassium phosphate buffer (pH 6.5) and approximately 10^7 CFU ml⁻¹ were resuspended into tubes containing sterile UHT (Ultra High Temperature) milk or 5 mM phosphate buffer (pH 6.5). Concentrations of 0–10 or 0–20 mg ml⁻¹ of EEP were added to tubes containing phosphate buffer and milk, respectively, and incubated at 37°C. After 6 h of exposure, 100 µl samples were withdrawn and serially diluted (tenfold increments) into BHI media and spread-plated on solid BHI media. *S. aureus* colonies were enumerated (CFU ml⁻¹) after plates being incubated at 37°C for 24 h. To determine the effect of propolis extracts on *S. aureus* over time, an EEP dose of 1 and 20 mg ml⁻¹ was added to phosphate buffer or milk, respectively. At fixed time intervals, ranging from 0 to 24 h the viable cells were enumerated as described above.

Selection of propolis-resistant *S. aureus* cells

Staphylococcus aureus 2979 and 4118 were transferred every 12 h for approximately 60 generations into fresh BHI media containing sub-lethal doses of EEP. The sub lethal dose was defined as the concentration of ethanolic extract that reduced the bacterial growth rate in at least 25%, but only caused less than 5% decrease in the maximum OD_{600nm} of *S. aureus* cultures. Concentrations of 0.15 and 0.25 mg ml⁻¹ were used for isolates 2979 and 4118, respectively. After each transfer, the cultures were incubated at 37°C for 12 h. To verify the selection of resistant cells, the sensibility of each isolate to propolis was re-evaluated every 20 transfers by microdilution assays. The determination of the MIC values was performed as described above.

Atomic force microscopy (AFM)

The effect of ethanolic extracts of propolis on *S. aureus* cells was monitored with atomic force microscopy. Isolates 2979 and 4118 were grown overnight in BHI media, centrifuged ($2,096 \times g$, room temperature, 15 min), washed three times in phosphate buffer (5 mM, pH 6.5), and approximately 10^8 CFU ml⁻¹ were resuspended into tubes containing the same buffer. Propolis extracts diluted to 0.5 mg ml⁻¹ were added to the cell suspensions and the samples were incubated for 4 h at 37°C. Cell suspensions without the EEP additions were used as controls. After incubation, samples of 1 ml were collected from each treatment, centrifuged ($6,149 \times g$, room temperature,

15 min, Eppendorf 5415 C, Hamburg, Germany) and a smear of cells was prepared in a glass slide (1 × 1 cm). The slides were air dried and submitted to intermittent-contact atomic force microscopy—IT-AFM (NT-MDT Co., Ntegra Prima, Russia).

Statistical methods

All experimental determinations represent observations from at least duplicate samples obtained from two experiments performed independently. The mean, standard deviation and coefficients of variation were computed and data and error bars are expressed as mean ± SD (standard deviation), unless otherwise stated.

Results

Staphylococcus aureus strains 2979, 4118 and ATCC 29213 grew in BHI broth with a specific growth rate of 1.64, 1.61 and 1.48 h⁻¹, respectively. However, the addition of increasing concentration of EEP (0.050–0.5 mg ml⁻¹) reduced the specific growth rate and increased the doubling time and lag phase duration of all the isolates tested (Table 1). The number of viable cells also decreased rapidly if doses ≥ 0.25 mg ml⁻¹ were added to phosphate buffer (Fig. 1a). Control treatments maintained viability during the incubation period (6 h), but the enumeration of viable cells decreased at least 22.0, 99.0 and 99.9% at EEP concentrations of 0.1, 0.25 and 0.5 mg ml⁻¹, respectively (Fig. 1a).

Time killing assays indicated that *S. aureus* cells resuspended (10^7 CFU ml⁻¹) in phosphate buffer maintained their viability for at least 24 h of incubation (Fig. 1b), but the addition of propolis at 1 mg ml⁻¹ killed more than 90% of the cells after 12 h of incubation. After being exposed for 24 h to EEP, no viable cells could be enumerated even if enrichments were performed (Fig. 1b).

However, if similar doses of EEP were added to milk, the number of viable *S. aureus* cells remained approximately unaffected (Fig. 2a). The lower bactericidal activity of EEP in milk suggested that some propolis substances could interact with milk components, decreasing the activity of the EEP against *S. aureus*. Nonetheless, if EEP concentration was increased up to 20 mg ml⁻¹, viability reduced at least 99.9% for both *S. aureus* isolates tested (Fig. 2a). This decrease in viability was achieved after cultures had been exposed to propolis for 8 h or more (Fig. 2b) and no viable cells were detected after 12 and 24 h of incubation for *S. aureus* 4118 and 2979, respectively (Fig. 2b).

The MIC values for EEP were 0.292, 0.586 and 0.586 mg ml⁻¹ for *S. aureus* strains 2979, 4118 and ATCC 29213, respectively. After being exposed to sub-lethal

Table 1 Effect of ethanolic extracts of propolis on growth parameters of *S. aureus* strains cultivated in BHI broth for 24 h at 37°C

<i>S. aureus</i> strain	Propolis concentration (mg ml ⁻¹)	Specific growth rate (h ⁻¹)	Generation time (h)	Lag phase duration (h)	Maximal optical density (600 nm)
2979	0	1.64 ± 0.13	0.42 ± 0.03	0.63 ± 0.25	2.13 ± 0.13
	0.05	1.35 ± 0.18	0.51 ± 0.07	1.22 ± 0.19	2.49 ± 0.03
	0.1	1.21 ± 0.20	0.57 ± 0.10	1.59 ± 0.19	2.44 ± 0.04
	0.25	0.51 ± 0.07	1.36 ± 0.19	3.65 ± 0.47	2.24 ± 0.08
	0.5	0.36 ± 0.08	1.91 ± 0.40	5.20 ± 0.02	1.70 ± 0.09
	1	–	–	–	–
4118	0	1.61 ± 0.05	0.43 ± 0.01	0.98 ± 0.01	2.24 ± 0.09
	0.05	1.46 ± 0.03	0.47 ± 0.01	1.09 ± 0.12	2.33 ± 0.03
	0.1	1.41 ± 0.01	0.49 ± 0.01	1.31 ± 0.17	2.41 ± 0.01
	0.25	0.98 ± 0.01	0.70 ± 0.01	2.05 ± 0.07	2.16 ± 0.11
	0.5	0.68 ± 0.01	1.01 ± 0.02	2.22 ± 0.02	1.86 ± 0.11
	1	–	–	–	–
ATCC 29213	0	1.48 ± 0.21	0.47 ± 0.07	1.08 ± 0.25	2.09 ± 0.09
	0.05	1.24 ± 0.21	0.56 ± 0.10	1.37 ± 0.25	2.02 ± 0.10
	0.1	0.97 ± 0.13	0.71 ± 0.09	1.63 ± 0.20	2.09 ± 0.06
	0.25	0.43 ± 0.09	1.62 ± 0.32	2.53 ± 0.47	1.62 ± 0.07
	0.5	0.21 ± 0.01	3.27 ± 0.24	6.13 ± 0.06	1.21 ± 0.16
	1	–	–	–	–

– No growth

doses of EEP for at least 60 generations, *S. aureus* 2979 showed an increase in the MIC value for EEP from 0.293 to 0.586 mg ml⁻¹, while *S. aureus* 4118 did not alter its sensibility to propolis, showing an MIC value of 0.586 mg ml⁻¹ before and after exposure to sub-lethal doses of EEP. Additionally, cultures with increased MIC values could not sustain this phenotype if transferred in the absence of the EEP. These results suggest that resistance to propolis is not a phenotype easily selected among sensitive bacterial populations, a characteristic that might be explained by the multiple biological activities reported for propolis extracts.

Atomic force microscopy images of untreated *S. aureus* cells cultivated in complex media indicated that cells maintained their typical near-spherical shape and the characteristic arrangement in clusters of cocci (Fig. 3a, c). However, EEP-treated *S. aureus* cells had expressive morphological changes when exposed to propolis extracts. The cells showed irregular cell surface and lost their characteristic arrangement and size (Fig. 3b, d), suggesting major changes in cell physiology and structure (Fig. 3).

Discussion

Previous studies have proposed the use of propolis as an effective agent against *S. aureus* (Fernandes Júnior et al. 2005; Lu et al. 2005). The effect of propolis and some of its

components on *S. aureus* cells appears to be bactericidal, but the activity of ethanolic extracts of green propolis (the most commercialized preparation of propolis in Brazil) is less evident (Park et al. 2005; Iio et al. 2010). Our results indicated that EEP increased the duration of the lag phase and reduced the growth rate of *S. aureus* strains on BHI media. The ethanolic extracts were bactericidal against *S. aureus* in phosphate buffer, but 20 times as much EEP was required to achieve similar killing effects against *S. aureus* in milk.

The lower bactericidal activity of EEP in milk suggested that some propolis substances could interact with milk components, decreasing the activity of the EEP against *S. aureus*. In a previous work, Kuang et al. (2009) demonstrated that tetracycline, an antibiotic that has been widely used for mastitis therapy, could bind to casein and heat-sensitive substances in raw milk. The authors concluded that these components had a role decreasing the activity of tetracycline in raw milk. An earlier work by Owens and Watts (1987) indicated the effect of milk components on a broader spectrum of antibiotics. In their work, the antimicrobial susceptibility testing performed in vitro with *S. aureus* in Mueller–Hinton milk agar indicated reduction of disc diffusion zone diameters, which changed the interpretation of the results from susceptible to intermediate or resistant.

In addition, propolis components that are biologically active on the cytoplasmic membrane could be less effective against *S. aureus* cultures if cell homeostasis (e.g. ion

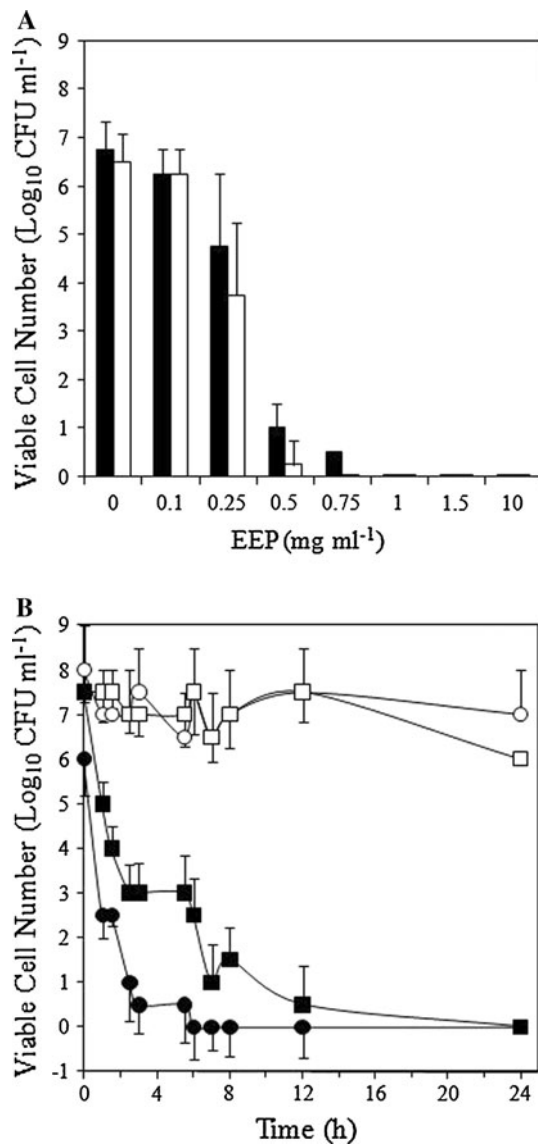


Fig. 1 Viability of washed cell suspensions of *S. aureus* 2979 and *S. aureus* 4118 treated with ethanolic extracts of propolis (EEP) in phosphate buffer. **a** Increasing concentrations of EEP (0–10 mg/ml) were added to cell suspensions of *S. aureus* 2979 (black bars) and *S. aureus* 4118 (white bars). **b** EEP was added at 1 mg ml⁻¹ to washed cell suspensions of *S. aureus* 2979 (filled circles) and *S. aureus* 4118 (filled squares) resuspended in phosphate buffer. Control treatments without EEP are also shown (open circles and squares). The cell suspensions (approximately 10⁷ CFU ml⁻¹) were incubated at 37°C for 6 h (**a**) or at different time intervals (**b**), before viable cell number was enumerated

gradients, intracellular pH) could be better maintained in a milk medium.

In our assays, *S. aureus* cells were incubated with EEP in milk for only 6 h, but previous reports demonstrated that *S. aureus* can produce biofilm and is less susceptible to antibiotics when grown in milk (Ali-Vehmas et al. 1997). Although the mechanism affecting the activity of EEP on milk is not yet clear, studies should be conducted to

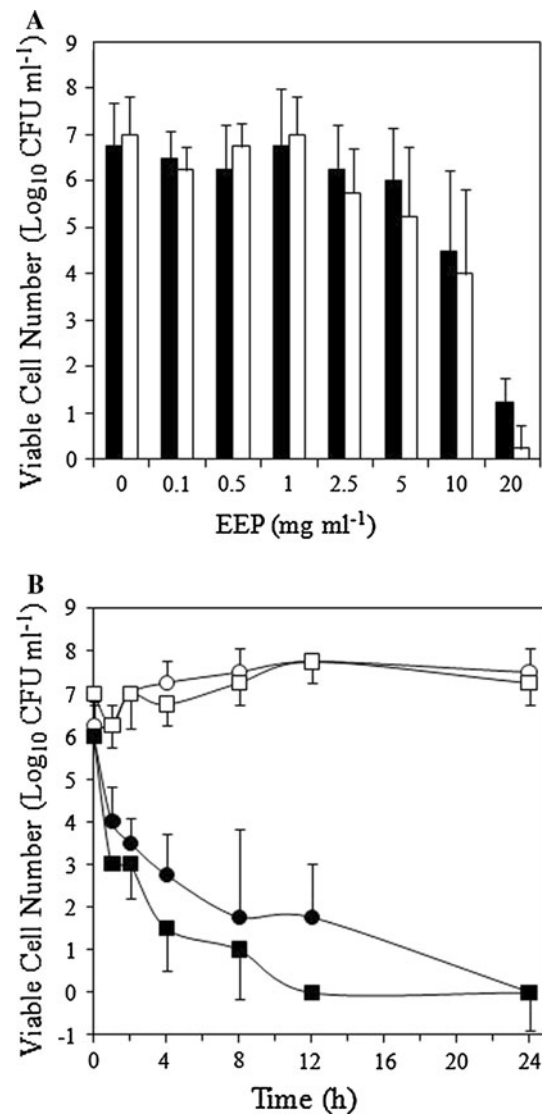
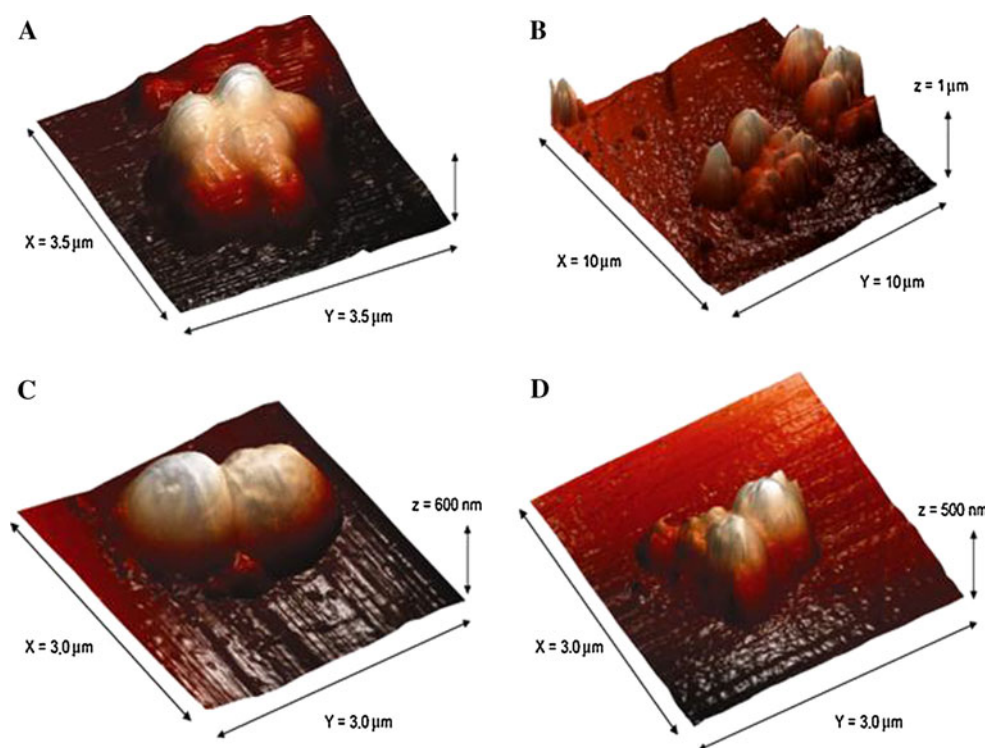


Fig. 2 Viability of washed cell suspensions of *S. aureus* 2979 and *S. aureus* 4118 treated with ethanolic extracts of propolis (EEP) in milk medium. **a** Increasing concentrations of EEP were added (0–20 mg ml⁻¹) to a milk medium containing washed cell suspensions of *S. aureus* 2979 (black bars) and *S. aureus* 4118 (white bars). **b** EEP was added at 20 mg ml⁻¹ to washed cell suspensions of *S. aureus* 2979 (filled circles) and *S. aureus* 4118 (filled squares) resuspended in milk. Control treatments without EEP are also shown (open circles and squares). The cell suspensions (approximately 10⁷ CFU ml⁻¹) were incubated at 37°C for 6 h (**a**) or at different time intervals (**b**), before viable cell number was enumerated

improve the activity of propolis in milk, since intramammary preparations are expected to retain their antimicrobial activity in the udder cistern for treatment of lactating dairy cows (Gehring and Smith 2006).

Resistance to antibiotics is a challenging feature of *Staphylococcus aureus*. Reports of antibiotic-resistant *S. aureus* strains abound in the literature (Gundogan et al. 2005; Gould et al. 2010; Zoraghi et al. 2010). However, to our knowledge, the resistance of *S. aureus* to propolis or

Fig. 3 Atomic force microscopy (AFM) images of *S. aureus* cells. Cells from *S. aureus* 2979 (**a**, **b**) and 4118 (**c**, **d**) were resuspended (approximately 10^7 CFU ml⁻¹) in phosphate buffer added with 0.5 mg/ml of EEP (**b**, **d**) and incubated at 37°C for 4 h. After the incubation period, cells were dispersed in a glass slide and submitted to IT-AFM. Control treatments without EEP are shown (**a**, **c**)



propolis constituents had not yet been tested. Our results suggest that selection of the resistance phenotype did not occur or did not persist under our experimental conditions, as would be expected if resistant cells were present as a subpopulation in the *S. aureus* culture. Because propolis is a complex substance containing many chemical constituents with distinct mechanisms of action and synergistic interactions (Salomao et al. 2008), resistance might be a complex phenotype.

Atomic force microscopy has been a useful technique to determine morphological and chemical changes in the bacterial cell envelope (Eaton et al. 2008). AFM images of *S. aureus* cells that were EEP treated indicated changes in morphology and cell size. Treated cells appeared scarce in the samples, were generally thinner and often showed irregular shapes when compared to their untreated counterparts.

When Cushnie and Lamb (2005) investigated the effect of galangin—a flavonol present in propolis—on *S. aureus* cells, they verified an increased potassium efflux from sensitive cells. Their results appeared to be caused by direct damage of the cytoplasmic membrane or indirect damage through osmotic lysis. Quercetin, a flavonoid found in propolis (Bonvehí et al. 1994), can affect the cytoplasmic membrane of bacteria (Mirzoeva et al. 1997), while propolis phenolics are generally linked to protein denaturation (Denyer and Stewart 1998), leakage of cytoplasmic constituents and disruption of peptidoglycan or damage of the cell membrane (Juven et al. 1972). Because the integrity of the cytoplasmic membrane is

needed to maintain ion gradients and osmotic pressure and proper enzyme activity, cell homeostasis and growth should be affected by changes in the selective properties of the cytoplasmic membrane.

These results indicate that propolis could be effective to control mastitis-causing *S. aureus* strains, but the activity of this antimicrobial agent and its efficacy in milk requires further investigation. Considering that resistance to propolis appears not to be easily selected, its use alone or in combination with other antimicrobial agents should be examined *in vivo*.

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