The effects of Brazilian propolis on etiological agents of mastitis and the viability of bovine mammary gland explants

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ABSTRACT

The objective of this study was to evaluate in vitro the antimicrobial activity of Brazilian propolis from Urupema, São Joaquim, and Água Doce (Santa Catarina State) and green propolis from Minas Gerais State, and the effects of propolis on bovine mammary gland explant viability. The propolis samples differed in flavonoid content and antioxidant activity. Green propolis showed the highest content of flavonoids, followed by the sample from São Joaquim. The propolis from Urupema showed the lowest flavonoid content along with the lowest antioxidant activity. The total phenolics were similar across all studied samples. Despite phytochemical differences, the propolis samples from Minas Gerais, São Joaquim, and Urupema presented the same level of antimicrobial activity against *Staphylococcus aureus* strains. The reduction in *S. aureus* growth was, on average, 1.5 and 4 log₁₀ times at 200 and 500 µg/mL, respectively. At concentrations of 1,000 µg/mL, all propolis reduced bacterial growth to zero. On the other hand, when the propolis were tested against strains of *Escherichia coli*, the samples presented weak antimicrobial activity. Mammary explants were maintained in culture for 96 h without a loss in viability, demonstrating the applicability of the model in evaluating the toxicity of propolis. The origin and chemical composition of the propolis had an effect on mammary explant viability. We encountered inhibitory concentrations of 272.4, 171.8, 63.85, and 13.26 µg/mL for the propolis from Água Doce, Urupema, São Joaquim, and Minas Gerais, respectively. A clear association between greater antimicrobial activity and toxicity for mammary explants was observed. Of all propolis tested, the Urupema sample was noteworthy, as it showed antimi-

crobial activity at less toxic concentrations than the other samples, reducing bacterial growth to an average of 9.3×10^2 cfu/mL after 6 h of contact using 200 µg/mL of extract. The results demonstrate the potential for Brazilian propolis in the treatment of mastitis, although effectiveness is dependent on geographical origin and concentration. The results from the mammary gland explant assays are promising for the investigation of other natural products with antimicrobial and anti-inflammatory properties that can be used in the intramammary treatment of subclinical mastitis and during dry cow therapy.

Key words: phytotherapy, antimicrobial activity, toxicity, mammary gland explants

INTRODUCTION

Mastitis is the most significant disease in dairy herds, causing reductions in milk production and quality, increases in treatment costs, and a reduction in animal welfare (Nielsen et al., 2010). Despite being disease with many etiologies, the majority of mastitis cases are caused by bacteria (Watts, 1988). *Staphylococcus aureus* and *Escherichia coli* have been documented as the principal etiological agents of bovine mastitis (Passey et al., 2008; Rall et al., 2014).

The prevalence of mastitis in dairy herds has resulted in the extensive use of antibiotics (Bradley, 2002). However, treatment with antibiotics during lactation not only has limited efficacy, but also leads to increased residues found in commercial milk samples (Mukherjee et al., 2005). On the other hand, the use of dry cow therapy, which consists of an antibiotic preparation infused into each quarter immediately after the last milking of a lactation, has helped to cure recurrent infections and prevent new infections (Bradley, 2002; Robert et al., 2006). In such cases, the greatest risk lays in the development of antibiotic-resistant bacteria, but it also presents an obstacle for organic milk production (USDA, 2015).

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For mastitis, the search for an antimicrobial product that possesses a wide spectrum of biological properties is directly related to the fact that the bacterial infection is accompanied by an inflammatory response. As such, therapeutic agents with anti-inflammatory, antioxidant, and immunostimulatory properties are needed to improve the overall health of not only the mammary gland, but also the animal. With this goal in mind, plant extracts from magnolol, oregano, macela, and cranberry have been investigated in the treatment of mastitis (Sperotto et al., 2012; Diarra et al., 2013; Wei et al., 2015). Similarly, propolis extracts, which are recognized for their antimicrobial, anti-inflammatory, immunostimulatory, and antioxidant properties (Sforcin, 2007; Bachiega et al., 2012), have also been studied in the treatment of mastitis (Santana et al., 2012). Despite reports of curing mastitis with intramammary propolis use (Mirolyubov and Barskov, 1980; Meresta et al., 1989), Romvary et al. (1993) noted the occurrence of inflammation in the gland after its use. Contradictory results in relation to the use of propolis stems from fact that the product has a highly variable chemical composition that is mainly the result of the flora in the region where the propolis originates and the season. Thus, it has been suggested that the therapeutic properties of propolis are subject to change (Silva et al., 2006; Velazquez et al., 2007).

The study of new therapeutic agents for the treatment of mastitis must begin with the screening of a potential product for its antimicrobial effects on the principal etiological agents causing the disease. Such analysis has been done using in vitro tests with standard bacterial strains and samples of mastitic milk (Silva et al., 2006; Santana et al., 2012); studies have also been done on animal models, such as rats, goats, and cows (Chen et al., 2013; Wei et al., 2015). However, because of the ethical concerns of using animal models in research, the implementation of more effective in vitro models is necessary. Recently, Rabot et al. (2006) assessed the use of bovine mammary explants (alveolar tissue sections) in the study of inflammatory reactions and gland immunity. The results were similar to those found during in vivo studies, suggesting that this model is suitable for the analysis of inflammatory reactions of the mammary gland.

Considering the risks associated with intramammary application of a product that has not been tested for possible tissue damage, the current study investigates both the antimicrobial activity of Brazilian propolis as well as the effects of propolis on the viability of in vitro mammary explants. Our study demonstrates a need to consider the results of in vitro antimicrobial tests in conjunction with tests on mammary explants in selecting potential new substances as substitutes for antibiot-

ics for the intramammary treatment of bovine mastitis or during dry cow therapy. In the current study, we evaluated in vitro the effects of Brazilian propolis from different locations (typical of Santa Catarina and Minas Gerais States) on standard strains and wild-types of *S. aureus* and *E. coli*, as well their effects on the viability of bovine mammary tissue.

MATERIALS AND METHODS

Origins of the Propolis Samples

Propolis samples from the municipalities of Urupema, São Joaquim, and Água Doce in Santa Catarina State, southern Brazil, and green propolis from Minas Gerais were collected during the winter of 2011 (Figure 1). The municipality of Água Doce, located in western Santa Catarina (Figure 2) at approximately 970 m above sea level, is characterized by a humid mesothermal climate, without a dry season, and with cool summers and cold winters, often with frosts and snow. The vegetation is dense and several large tree species, such as *Araucaria angustifolia*, *Parapiptadenia rigida*, *Mimosa scabrella*, and *Cinnamomum zeylanicum*, are typical of the region. Urupema and São Joaquim are neighboring municipalities located in Santa Catarina's mountain range, at approximately 1,500 m above sea level (Figure 2). The climate of the region is classified as humid temperate, with cool summers and harsh winters, and the region is considered one of the coldest in Brazil. The flora of the region is a transition zone between forest interspersed with angiosperms, mainly of the genera *Pinus* spp. and *Araucaria* spp., and natural grasslands, characterized by several grass species of the genera *Avena* spp., *Hemarthria* spp., *Axonopus* spp., and *Trichocline* spp., among others (IBGE, 2012).

Propolis from Minas Gerais (green propolis) was included in our study because it has a unique chemical composition. Green propolis is recognized worldwide for its diverse biological properties and, as such, the majority of its production is destined for the export market. Previous studies have attributed the diverse biological activities of green propolis to the presence of the compound Artepillin C, a prenylated cinnamic acid derivative whose main source is the plant known as field rosemary (*Baccharis dracunculifolia*; Park et al., 2002; Silva et al., 2006; Sforcin, 2007; Bachiega et al., 2012).

Propolis Extraction

For extraction, crude propolis samples from each location were ground with 70% (vol/vol) ethanol (Synth, São Paulo, Brazil) at a ratio of 1:10 (wt/vol). The ex-

Figure 1. Crude Brazilian propolis from the municipalities (A) São Joaquim, (B) Água Doce, (C) Urupema (Santa Catarina State), and (D) Minas Gerais. Color version available online.

tract was vacuum filtered and maintained at -20°C for 24 h. Subsequently, the sample was centrifuged ($672 \times g$, 10 min, at room temperature) to collect the supernatant, which was then dried in a forced air oven at 60°C

to completely remove the solvent. The residues were stored at -20°C .

Total Phenolic Content

The total phenolic contents of the propolis were determined based on the Folin-Ciocalteu colorimetric method (Singleton and Rossi, 1965), using a standard curve of gallic acid (Sigma-Aldrich, St. Louis, MO; 20–100 $\mu\text{g/mL}$; $y = 0.0107x$; $R^2 = 0.9773$). Results were expressed as milligrams of gallic acid equivalents per milligram of propolis.

Total Flavonoids

The flavonoid contents were determined using a colorimetric reaction with aluminum chloride, as described by Popova et al. (2004). We expressed the results in micrograms of quercetin equivalent (Sigma-Aldrich; $y = 0.0129x$; $R^2 = 0.9881$) per milligram of propolis.

Antioxidant Activity

The antioxidant potential of the propolis were assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH)

Figure 2. Geographical origin of propolis samples produced in Santa Catarina State.

method (Sigma-Aldrich; Choi et al., 2002). Results were expressed as the percentage inhibition (%) of the DPPH per milligram of propolis.

Antimicrobial Tests

The propolis were tested on standard strains of *S. aureus* ATCC 25923 and *E. coli* ATCC 8739, as well as 2 wild-types from each species isolated from mastitic milk. The isolated wild-types, provided by the Desidério Finamor Veterinary Research Institute (Rio Grande do Sul, Brazil), were derived from milk samples used to diagnose the etiologic agent and were kept lyophilized until the time of the experiment. Bacterial suspensions were prepared by the direct colony method. The turbidity of initial suspension was adjusted by comparing with 0.5 McFarland standard, analogous to a suspension containing 1 to 2×10^8 cfu/mL (CLSI, 2009). During the tests, we used a logarithmic dilution of 10^5 cfu/mL of inoculum in Mueller Hinton broth (Himedia, Mumbai, India). Initially, we prepared serial dilutions of propolis ranging from 0 to 1,000 µg/mL in ethanol:H₂O (8:92, vol/vol). Bacterial activity was evaluated using the pour-plate technique, with modifications (Sperotto et al., 2012). In Eppendorf tubes, we added 900 µL of propolis dilutions to 100 µL of inoculum. After 2 incubation periods (6 and 24 h), the solution was placed in petri dishes, 20 mL of the Mueller Hinton agar (Difco, Franklin Lakes, NJ) was added, and the dishes were kept in an oven at 37°C. For *S. aureus* the colony count was done 48 h after adding the medium; *E. coli* counts were taken after 24 h, proceeding only with the plate counts that presented up to 300 cfu/mL (Sanders, 2012).

Because the Urupema propolis presented the best results in antimicrobial and mammary gland explants viability tests, in a subsequent test the propolis from Urupema was also tested at a concentration of 200 µg/mL in ethanol:H₂O (8:92, vol/vol). For this test, we used the same isolates and standard strains of *S. aureus*, but with a bacterial load of 10^3 cfu/mL (a dose capable of causing clinical and subclinical mastitis; Schukken et al., 1999). The same method of contact described above was used in Eppendorf tubes for 6 and 24 h. However, the antimicrobial activity was tested using the surface-scattering technique by spreading 0.1 mL of the suspension on a petri dish containing Mueller Hinton agar using a Drigalski handle. We used serial log dilutions to determine population density or initial infecting dose and growth reductions for each treatment. Bacterial counts were taken from plates presenting between 30 and 300 cfu/mL. We conducted 2 independent experiments for each test, with 2 replicates for each experiment.

Source and Manipulation of Mammary Gland Explants

To obtain bovine mammary gland explants, we used the method described by Rabot et al. (2006). In sterile conditions, mammary gland samples were obtained from 18 cows soon after slaughter at a commercial meat processing plant. The material was sectioned (7×3 cm) with the use of surgical tweezers and a number 21 scalpel, washed with PBS (Gibco, Seaforth, Ontario, Canada), and supplemented with penicillin G (Gibco; 100 µg/mL), streptomycin (Gibco; 100 µg/mL), and amphotericin B (Sigma-Aldrich; 2.5 µg/mL). The sections were placed in tubes with basal culture medium DMEM/F12 (Sigma-Aldrich), supplemented with insulin (Lilly, Indianapolis, IN; 10 µg/mL), hydrocortisone (Sigma-Aldrich; 0.5 µg/mL), penicillin G (Gibco; 100 µg/mL), streptomycin (Gibco; 100 µg/mL), and amphotericin B (Sigma-Aldrich; 2.5 µg/mL), and immediately transported to the laboratory. Under a laminar flow hood, the udder sections were again manipulated with the aid of the PUNCH (Keys, São Paulo, Brazil) biopsy instrument to obtain explants of approximately 5×2.5 mm. For the viability tests, we conducted 2 independent experiments for each propolis, with 3 repetitions per test, totaling 6 repetitions for each extract tested. Explants from the same animal were submitted to all concentrations of the same extract.

Effects of Propolis on Viability of Bovine Mammary Gland Explants

After collection, explants were weighed and transferred to 12 well plates containing culture medium and different concentrations of propolis, ranging from 0 to 560 µg/mL, the maximum concentration established during preliminary tests (data not shown). At concentrations above the maximum, tissue viability was zero. Explants were maintained at 37°C and 5% CO₂ for 48 h. Afterward, the analysis of explant cell viability was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich; 5mg/mL) test (Mosmann, 1983). The negative control groups consisted of (1) the culture medium and (2) the culture medium with dimethyl sulfoxide (DMSO, 2%; vehicle used to solubilize propolis; Synth, São Paulo, Brazil). After the incubation period with MTT, the culture medium was removed and DMSO was added to dissolve the formazan crystals. Absorbance was quantified using a 540-nm spectrophotometer. To compare the absorption values for explants of different weights, the following formula was used: (absorbance $\times$ 1,000)/explant weight in milligrams (Peres et al., 2009). Results were expressed as a percentage of viable cells,

Table 1. Total phenolic and flavonoid contents¹ and antioxidant activity of propolis produced in southern Brazil (Água Doce, São Joaquim, and Urupema) and Minas Gerais State

Item	Phenolics (μg of GAE/mg)	Flavonoids (μg of EQ/mg)	DPPH ² (% inhibition of DPPH/mg)
Água Doce	116.29 $\pm$ 6 ^a	10.25 $\pm$ 0.82 ^c	85.39 $\pm$ 3.57 ^a
São Joaquim	116.32 $\pm$ 21.5 ^a	13.07 $\pm$ 0.11 ^b	85.64 $\pm$ 0.51 ^a
Urupema	113.58 $\pm$ 3.27 ^a	7.33 $\pm$ 0.23 ^d	72.76 $\pm$ 0.48 ^b
Minas Gerais	97.97 $\pm$ 10.17 ^a	15.91 $\pm$ 0.8 ^a	81.89 $\pm$ 1.56 ^a

^{a-c}Different letters within a column indicate statistical difference ($P < 0.05$).

¹All values are presented as means $\pm$ SD of 3 independent extractions with 6 repetitions per experiment. GAE = gallic acid equivalents; EQ = quercetin equivalent.

²2,2-Diphenyl-1-picrylhydrazyl.

considering the control group without the addition of DMSO as 100% cell viability.

Analysis of Mammary Explant Viability During the Time of Culture

The viability of mammary tissue was evaluated over a period of 96 h. The readings were done every 24 h, beginning at time zero, when the explants were produced. The culture medium was replenished every 24 h and at this time MTT solution was also added (Sigma-Aldrich; 5 mg/mL) for the group corresponding to the reading day (time 0, 24, 48, 72, and 96 h), as described previously. For this test, we also conducted 2 independent experiments with 3 repetitions per experiment, wherein explants from the same animal were kept in culture for all evaluated time periods.

Statistical Analyses

The total phenolic content, flavonoids, and antioxidant activity were submitted to ANOVA and expressed as an average $\pm$ standard deviation. The difference between paired samples was determined using LSMEANS. For the antimicrobial tests, data were log-transformed and assessed using the Kruskal-Wallis test and Dunn's multiple comparisons post-test. The results of the cellular viability test was submitted to ANOVA using mixed procedure (PROC MIXED), considering the origin of the propolis, the concentration, and the interaction between origin and concentration. Because significant effects of origin by concentration were noted, we used this interaction to compare differences, through LSMEANS, with a Tukey adjustment. We also determined the half-maximal inhibitory concentration (IC_{50}), the concentration responsible for reducing half of the cellular viability, through a linear regression statistic. The viability data were submitted to ANOVA using GLM. For all comparisons, significance was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Phytochemical Analysis

The propolis samples showed similar results for total phenolic content (Table 1). However, the flavonoid content differed between the samples ($P < 0.05$; Table 1), with the highest content found in the propolis from Minas Gerais followed by the propolis from São Joaquim, Água Doce, and Urupema ($P < 0.05$). As well as having the lowest flavonoid content, the Urupema propolis also showed the lowest percentage inhibition of the DPPH (i.e., the lowest level of antioxidant activity). Of the remaining propolis, no differences for antioxidant activity were found (Table 1).

Despite the higher content of flavonoids from propolis sampled from temperate zones in comparison with those from tropical climates, our results showed that both the propolis from Minas Gerais and the sample from São Joaquim possess significant levels of flavonoids. In fact, green propolis from Minas Gerais is recognized worldwide for its diverse therapeutic properties associated with the presence of Artepillin C, a prenylated derivative of cinnamic acid (Sforzin et al., 2000; Silva et al., 2006). These results are important, as propolis characterization studies in Santa Catarina are rare, despite the importance of propolis production in the state (IBGE, 2010). Recently, a pioneering study to characterize propolis was undertaken in Santa Catarina; as expected, the preliminary results show clear differences in samples based on their geographic origin but also show that differences are a result of seasonality (Maraschin et al., 2012). In the current study, the selection of propolis samples was based on the Maraschin et al. (2012) study and the chosen samples showed chemical profiles typical for the state with changes only in the amounts of chemical compounds as a result of the season (M. Maraschin, Federal University of Santa Catarina, Florianópolis, Santa Catarina, Brazil, personal communication).

Table 2. Count¹ (Log₁₀) of standard *Staphylococcus aureus* ATCC 25923 (Log₁₀) and 2 wild-types from mastitic milk (mean ± SD), using an initial population density of the inoculum of 10⁵ cfu/mL, after exposure to different concentrations of studied propolis

Propolis (µg/mL)	Minas Gerais	São Joaquim	Urupema	Água Doce
0	UNC ^{2,B,a}	UNC ^{B,a}	UNC ^{B,a}	UNC ^{B,a}
100	4.4 ± 0.66 ^{B,a}	4.6 ± 0.55 ^{B,a}	4.4 ± 0.58 ^{B,a}	UNC ^{B,a}
150	4.2 ± 0.68 ^{B,a}	3.9 ± 0.79 ^{B,a}	3.8 ± 0.28 ^{B,a}	UNC ^{B,a}
200	3.48 ± 0.54 ^{A,a}	3.4 ± 1.09 ^{A,a}	3.5 ± 0.14 ^{A,a}	UNC ^{B,b}
250	2.82 ± 1.2 ^{A,a}	3.4 ± 1.32 ^{A,ab}	2.9 ± 1.35 ^{A,a}	4.1 ± 0.30 ^{B,b}
500	0.3 ± 0.79 ^{A,a}	1.8 ± 1.45 ^{A,ab}	0.3 ± 0.55 ^{A,a}	2.9 ± 1.14 ^{A,b}
750	0.3 ± 0.79 ^{A,a}	0.4 ± 0.87 ^{A,a}	0.3 ± 0.80 ^{A,a}	1.6 ± 1.16 ^{A,a}
1000	0.1 ± 0.35 ^{A,a}	0.3 ± 0.88 ^{A,a}	0.0 ± 0.00 ^{A,a}	0.7 ± 1.00 ^{A,a}

^{A,B}Different uppercase letters indicate significant differences between the concentrations and the control within the same propolis ($P < 0.05$).

^{a,b}Different lowercase letters indicate significant differences between the propolis samples ($P < 0.05$).

¹Average values represent the counts performed after 6 and 24 h of incubation.

²UNC = uncountable.

Variations in the content of bioactive compounds of propolis in relation to geographic origin have been extensively studied (Bankova et al., 1995; Calhella et al., 2014). Silva et al. (2006), for example, compared commercial extracts of propolis from various municipalities in the southeast and northeast of Brazil and showed wide variation among the total phenolic content, flavonoids, and antioxidant activity as a result of local production. In relation to the content of bioactive compounds, propolis produced in different regions of the world, such as Argentina, Mexico, and Spain, contain similar levels of phenolics and flavonoids as those found in the current study (Velazquez et al., 2007; Chaillou and Nazareno, 2009; Bonvehi and Gutierrez, 2011). In samples from Argentina, the antioxidant activity was less significant, between 49 and 66% inhibition of DPPH, despite elevated levels of phenolic compounds (Chaillou and Nazareno, 2009). The same was found for Mexican and Spanish propolis samples, in which the antioxidant activity was not always associated with higher levels of phenolics and flavonoids (Velazquez et al., 2007; Bonvehi and Gutierrez, 2011). Variations in propolis samples are correlated with variations in botanical composition in the regions of production, as well as climatic differences. It is important to note that propolis extracts are complex matrices and along with phenolics and flavonoids, they may contain other compounds that can exert a significant influence on antioxidant and other biological activities. Therefore, a need to compare propolis of different origins exists for bioprospecting studies.

Antimicrobial Activity

At concentrations of 750 and 1,000 µg/mL, all of the propolis reduced the growth of *S. aureus* to an average

of zero (ranging from 0 to 4,000 cfu/mL; Table 2). At other concentrations, the rate of bacterial growth was correlated with the origin of the propolis. Concentrations above 200 µg/mL of the Urupema, Minas Gerais, and São Joaquim propolis, for example, generated an average growth reduction of 1.5 log₁₀ times. The propolis from Água Doce, on the other hand, showed a growth reduction of the original inoculum (average of 1.2 log₁₀) only at concentrations above 300 µg/mL. The Urupema and São Joaquim propolis showed bactericide effects similar to green propolis from Minas Gerais, at concentrations above 500 µg/mL (Table 2).

The effects on bacterial growth has been attributed to diverse compounds found in propolis, principally flavonoids (Cushnie and Lamb, 2005), phenolic acids (Chaillou and Nazareno, 2010), esters (Velazquez et al., 2007), and terpenoids (Bankova et al., 1995; Silva et al., 2006). Among these, some studies have suggested that flavonoids have the greatest influence on *S. aureus* growth inhibition (Santos Neto et al., 2009). In our study, the propolis with the highest content of flavonoids (Minas Gerais and São Joaquim) showed strong antimicrobial activity (Table 2). On the other hand, we found that the propolis from Urupema, despite having a lower level of flavonoids and antioxidant activity, showed antimicrobial effects against *S. aureus* identical to the propolis from São Joaquim and Minas Gerais. Silva et al. (2006) and Velazquez et al. (2007) found similar bactericide properties using propolis samples with lower levels of flavonoids and antioxidant activity. Clearly, it is not possible to make a direct association between the phenolic or flavonoid content and effects on microbial growth. In our study, the propolis samples containing higher levels of flavonoids showed strong antimicrobial properties along with the propolis from Urupema that, despite having lower flavonoid levels,

must possess a unique (nonflavonoid) chemical profile that results in higher antimicrobial activity.

Beyond the results found for *S. aureus*, all the studied propolis, independent of their origin, showed a weak response against *E. coli* (Table 3). No differences were found between the groups exposed to the propolis at concentrations below 500 µg/mL and the control group. At this concentration, the Minas Gerais propolis reduced significantly the bacterial growth by 2 log₁₀ times (*P* < 0.05). However, the bacterial growth in the presence of the other propolis at 500 µg/mL did not differ from the control. Only at higher concentrations (750 and 1,000 µg/mL) did we see a reduction in bacterial counts of the groups treated with propolis compared with the control (*P* < 0.001), with no differences found among them (Table 3), independent of length of contact. However, although a reduction was noted, the bacterial count remained high in the face of the highly pathogenic *E. coli*. Higher concentrations of propolis were not tested due to the likely toxicity for mammary glands. The limited effectiveness of propolis against *E. coli* has been described elsewhere (Kujumgiev et al. 1999; Sforcin et al., 2000). The efficacy of propolis is limited by the lipid content and higher chemical complexity of the cell wall of gram-negative bacteria, such as *E. coli* (Sforcin et al., 2000).

In relation to propolis concentrations with antimicrobial properties, a wide range of values have been reported, ranging from 400 mg/mL (Velazquez et al., 2007) to very low levels between 15.3 and 49.1 µg/mL (Moreno et al., 1999). This variation is related, in part, to differences in the chemical composition and vegetation of the region of origin that confer unique characteristics on the propolis; however, it may also be due to other factors, such as the method of extraction, purification, and means of solubilizing the extract, all of which could affect the bioavailability of compounds. Santana et al. (2012), using an extraction method

similar to that used in the current study, also observed growth inhibition for *S. aureus* treated with propolis from Minas Gerais at concentrations of up to 500 µg/mL and bactericide effects at concentrations of 1,000 µg/mL. Those authors note that for a natural product that functions in a complex way, such as propolis, a reduction in infectious load is a critical finding, as the product's compounds do not lead to changes in bacterial susceptibility over time. Santana et al. (2012) reached their conclusions after evaluating 60 generations of *S. aureus* strain cultivation that were subjected to sublethal doses of propolis.

Viability of Mammary Explants During the Time of Culture

Initially, to standardize the tests on mammary gland explants, the analysis of viability in the culture medium was monitored for 4 d. We did not find a significant loss of tissue viability after the test period in the culture conditions used (Figure 3). This finding corroborates the results presented by Baumrucker and Stemberger (1989), who showed that the specific functions of mammary tissue can be conserved in culture for a significant period of up to 96 h. This finding also validated our proposed experimental model. According to Baumrucker and Stemberger (1989), maintaining cell viability during culture is the main obstacle to the development of in vitro experimental models. Even though it may mimic in vivo conditions, the tissue may inevitably be subjected to hypoxia, resulting in cell death.

Effects of Propolis on Mammary Explant Viability

In analyzing the effects of different propolis on the viability of bovine mammary gland explants, we found the highest percentage of living cells at all concentrations tested after exposure to the Água Doce propolis,

Table 3. Count¹ (Log₁₀) of standard *Escherichia coli* ATCC 8739 and 2 wild-types from mastitic milk (mean ± SD), using an initial population density of the inoculum of 10⁵ cfu/mL, after exposure to different concentrations of studied propolis

Propolis (µg/mL)	Minas Gerais	São Joaquim	Urupema	Água Doce
0	UNC ^{2,B,b}	UNC ^{B,b}	UNC ^{B,b}	UNC ^{B,b}
250	UNC ^{B,b}	UNC ^{B,b}	UNC ^{B,b}	UNC ^{B,b}
500	3.9 ± 0.56 ^{A,a}	4.6 ± 0.50 ^{B,b}	4.3 ± 0.54 ^{B,b}	4.2 ± 0.57 ^{B,b}
750	3.64 ± 0.18 ^{A,a}	3.7 ± 0.17 ^{A,a}	3.5 ± 0.26 ^{A,a}	3.3 ± 0.49 ^{A,a}
1000	3.4 ± 0.43 ^{A,a}	3.6 ± 0.18 ^{A,a}	3.3 ± 0.53 ^{A,a}	2.5 ± 1.31 ^{A,a}

^{a,b}Different lowercase letters indicate significant differences between the propolis samples (*P* < 0.05).
^{A,B}Different uppercase letters indicate significant differences between the concentrations and the control within the same propolis (*P* < 0.05).
¹Average values represent the counts performed after 6 and 24 h of incubation.
²UNC = uncountable.

Table 4. Viability of bovine mammary explants¹ after 48 h of exposure to different concentrations of studied propolis

Propolis (µg/mL)	Minas Gerais	São Joaquim	Urupema	Água Doce
0	100 ± 9.47 ^{A,a}	100 ± 6.87 ^{A,a}	100 ± 6.45 ^{A,a}	100 ± 6.10 ^{A,a}
DMSO ²	97 ± 8.59 ^{A,a}	101 ± 5.52 ^{A,a}	98 ± 9.09 ^{A,a}	97 ± 3.93 ^{A,a}
17.5	35 ± 19.27 ^{B,b}	88 ± 9.31 ^{A,a}	89 ± 5.91 ^{A,a}	91 ± 6.73 ^{A,a}
35	28 ± 15.04 ^{B,b}	75 ± 14.06 ^{A,a}	87 ± 10.31 ^{A,a}	89 ± 10.23 ^{A,a}
70	15 ± 2.86 ^{B,c}	43 ± 12.76 ^{B,b}	80 ± 6.55 ^{A,a}	82 ± 12.46 ^{A,a}
140	15 ± 1.89 ^{B,c}	19 ± 4.88 ^{C,c}	58 ± 11.79 ^{B,b}	77 ± 8.68 ^{A,a}
280	16 ± 0.83 ^{B,b}	18 ± 2.89 ^{C,b}	26 ± 9.68 ^{B,c}	46 ± 10.77 ^{B,a}
560	15 ± 2.46 ^{B,a}	16 ± 2.25 ^{C,a}	16 ± 2.32 ^{C,a}	22 ± 7.20 ^{C,ac}

^{A-C}Different uppercase letters within a column indicate significant differences between the concentrations and the control for the same propolis ($P < 0.05$).

^{a-c}Different lowercase letters within a row indicate significant differences between propolis samples ($P < 0.05$).

¹Results are expressed as a percentage of viable cells ± SD.

²Dimethyl sulfoxide.

except at the highest tested concentration (560 mg/mL; Table 4). At 560 mg/mL, a reduction of approximately 80% of viable tissue cells occurred. For the Água Doce propolis, a reduction in explant viability was observed only above 280 µg/mL (Table 4). It is interesting to note that this same extract, even with a high content of phenolics and intermediate level of flavonoids (Table 1), showed the lowest level of bactericide activity against *S. aureus* strains (Table 2). The explants exposed to propolis from Minas Gerais showed the lowest percentage of cells with mitochondrial activity, with reduced viability even at the lowest test concentration (17.5 µg/mL). Similarly, the São Joaquim propolis appeared to be toxic at low concentrations, starting at 70 µg/mL. These 2 propolis stand out, as they showed the highest

levels of flavonoids and high levels of antimicrobial activity against *S. aureus* strains. Our results suggest that antimicrobial compounds, possibly flavonoids, are also responsible for toxic effects on bovine mammary tissue. The Urupema propolis showed intermediate toxicity, reducing the number of viable cells to approximately 50% at concentrations above 140 µg/mL (Table 4).

Thirugnanasampandan et al. (2012) evaluated the cytotoxicity of propolis and attributed the toxic effect on cancer cells and healthy lung cells to the major presence of the flavonoid quercetin. Their result corroborates a hypothesis in our study, that flavonoids have a toxic effect both on microorganisms and on healthy cells.

Using a nonlinear regression analysis, we assessed the IC₅₀ of propolis (Table 5). Through these values, it was clear that the Água Doce propolis was less toxic for bovine mammary tissue (IC₅₀ = 272.4 µg/mL), whereas the Minas Gerais extract showed an IC₅₀ 20 times greater. The Urupema propolis showed intermediate IC₅₀ levels (IC₅₀ = 171.8 µg/mL).

Zizic et al. (2013), evaluating the cytotoxicity of propolis from various regions on human colon cancer cell lines, recorded IC₅₀ levels varying between 26.33 and 143.09 µg/mL. However, propolis may have a different effect on cancer cells than on normal cells. In bladder carcinomas, for example, the ethanol extract of propolis at a concentration of 75 µg/mL inhibited the proliferation of tumor cells, whereas concentrations up to 300 µg/mL had no cytotoxic effect on normal epithelial cells (Orsolice et al., 2009). On the other hand, an evaluation of Portuguese propolis showed cytotoxic doses very similar for tumor and normal cells, with wide variations (between 9 and 182 µg/mL) of IC₅₀ among samples from different regions and between different types of carcinomas (Calhella et al., 2014). Thirugnanasampandan et al. (2012) also noted this pattern

Figure 3. Viability of bovine mammary gland explants maintained in culture for 96 h. Results are expressed as a percentage of viable cells ± SD ($P < 0.05$).

Table 5. Half-maximal inhibitory concentration (IC₅₀) of Brazilian propolis on bovine mammary gland explants

Item	São Joaquim	Minas Gerais	Água Doce	Urupema
IC ₅₀ (µg/mL)	63.85	13.23	272.4	171.8
SD	1.55	0.76	5.21	3.26

when evaluating propolis cytotoxicity on normal cells and cancerous lung cells. On cancerous cells, Thiruganasampandan et al. (2012) found an IC₅₀ of 13 µg/mL, whereas for normal cells the IC₅₀ was 10 µg/mL. Zizic et al. (2013) suggest that the cytotoxic effect is intimately linked to the chemical composition of the sample; Bankova et al. (1995) agreed that the cytotoxicity of propolis extracts from different regions around the world vary due to both chemical composition as well as the extraction solvent.

The analysis of all data together, as shown in Tables 2 and 5, show that concentrations of propolis with antimicrobial effects are toxic for bovine mammary explants as the antimicrobial activity is accompanied by higher toxicity. However, of the studied extracts, the Urupema propolis showed not only good antimicrobial properties against *S. aureus* (Table 2), but also a moderate toxicity (IC₅₀ = 171.8 µg/mL). As such, we assessed its potential use to control subclinical mastitis or during dry cow therapy, periods when the bacterial load is lower. With this objective, the exact bacterial reduction at a concentration of 200 µg/mL, a value close to the IC₅₀ of the Urupema propolis, was analyzed in a new antimicrobial test (Table 6). As such, it was possible to quantify the growth of *S. aureus* at 6 and 24 h of contact, which showed significant reductions when compared with the control ($P < 0.001$). Whereas the bacterial growth of the control group was, on average, 2.5×10^6 cfu/mL in 6 h, the group exposed to the

Urupema propolis showed a reduced average growth of 9.3×10^2 cfu/mL ($P < 0.05$). Similarly, after 24 h, the average growth of the control and the group exposed to the propolis were 7.9×10^7 and 1.67×10^5 cfu/mL ($P < 0.01$), respectively. There were no significant differences between the 2 contact periods.

The use of purified fractions of these extracts, containing isolated compounds or less complex extracts, could be an alternative to address the variation between antimicrobial and toxic concentrations. Nieva Moreno et al. (2005), evaluating the cytotoxic potential of Argentinian propolis using *Artemia salina* as the model, found an IC₅₀ of 100 µg/mL. However, this dose was not sufficiently toxic to control strains of *Salmonella typhimurium*. When analyzing the major component of the extract (flavonoid 2,4 dihydroxychalcone) in isolation, the IC₅₀ diminished to 0.5 µg/mL. At this concentration, the compound was a potent antimicrobial against gram-negative bacteria, with high anticarcinogenic properties.

Due to the variation between antimicrobial and toxic doses, a clear need exists for in vitro studies of new mastitis treatments before in vivo testing. Studies using mammary explants provide greater security in the development of alternative products for the treatment of mastitis and should be used widely before in vivo experiments, principally due to concerns about animal welfare. Furthermore, this type of study allows us to foresee the extent of damage to mammary glands

Table 6. Count¹ of standard *Staphylococcus aureus* ATCC 25923 (Log₁₀) and 2 wild-type isolated from mastitic milk (mean ± SD), using an initial population density of the inoculum of 10³ cfu/mL, after exposure (time 0 = initial infecting dose; 6 and 24 h) to 200 µg/mL of Urupema propolis

Item	Period of incubation (h)	Count (cfu/mL)	Control (cfu/mL)
<i>S. aureus</i> ATCC	0	5.95×10^3 A,a	1.0×10^3 A,a
	6	1.72×10^3 A,b	7.47×10^5 B,a
	24	2.36×10^4 A,b	4.07×10^7 B,a
<i>S. aureus</i> B (+)	0	4.02×10^3 A,a	2.0×10^3 A,a
	6	2.2×10^2 A,b	4.09×10^6 B,a
	24	4.7×10^5 A,b	1.12×10^8 B,a
<i>S. aureus</i> C (+)	0	1.18×10^4 A,a	9.0×10^3 A,a
	6	8.5×10^2 A,b	3.34×10^6 B,a
	24	7.5×10^3 A,b	8.48×10^7 B,a

^{A,B}Different uppercase letters within a column indicate significant differences between the periods of incubation and the propolis ($P < 0.05$).

^{a,b}Different lowercase letters within a row indicate significant differences between the propolis and the control ($P < 0.05$).

¹Average values represent the counts performed after 0, 6, and 24 h of incubation.

caused by the use of antimicrobial agents, which may compromise milk production in subsequent lactation. Currently, the majority of studies use in vitro models with isolated microorganisms and cells or in vivo experiments using rats (Diarra et al., 2013; Wei et al., 2015). In both situations, a limit exists to which results can be extrapolated to understand the actual effects of the product on the animals of interest. The complex relations between a cell and its metabolism in the tissue mimics more precisely what happens in the body. From this, we can better select products with active potential and determine effective dosage, thereby reducing the demand for animal models in research.

CONCLUSIONS

The propolis studied herein show high antimicrobial activity on the growth of *S. aureus*. However, this effect was accompanied by toxicity for mammary tissue, demonstrated by diminished viability. In both cases, the observed effects were influenced by the geographical origin and concentration of the propolis. We have shown that wide variation exists between an effective antimicrobial concentration and a concentration with low levels of toxicity for bovine mammary tissue. As such, implementing standard in vitro models using sections of bovine mammary tissue enables us to test potential substances for mastitis treatment and determine dosage before in vivo experiments. Such tests help to estimate possible damage to the mammary gland that can affect milk production in subsequent lactation. In our study, the results of the toxicity and antimicrobial tests demonstrate that Urupema propolis has potential to be used in the treatment of subclinical mastitis or during dry cow therapy when the bacterial count is comparable with the clinical condition; the Urupema propolis also represents a viable treatment option in organic milk production.

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REFERENCES

- Bachiega, T. F., C. L. Orsatti, A. C. Pagliarone, and J. M. Sforcin. 2012. The effects of propolis and its isolated compounds on cytokine production by murine macrophages. *Phytother. Res.* 26:1308–1313.

- Bankova, V., S. Christov, A. Kujumgiev, M. C. M. Arcucci, and S. Popova. 1995. Chemical composition and antibacterial activity of Brazilian propolis. *Z. Naturforsch. C* 50:167–172.
- Baumrucker, C. R., and B. H. Stemmerger. 1989. Insulin and insulin-like growth factor-I stimulate DNA synthesis in bovine mammary tissue in vitro. *J. Anim. Sci.* 67:3503–3514.
- Bonvehí, J. S., and A. L. Gutierrez. 2011. Antioxidant activity and total phenolics of propolis from Basque Country (northeastern Spain). *J. Am. Oil Chem. Soc.* 88:1387–1395.
- Bradley, A. 2002. Bovine mastitis: An evolving disease. *Vet. J.* 164:116–128.
- Calhelha, R. C., S. Falcão, M. J. Queiroz, M. Vilas-Boas, and I. C. Ferreira. 2014. Cytotoxicity of Portuguese propolis: The proximity of the in vitro doses for tumor and normal cell lines. *Biomed. Res. Int.* 2014:897361.
- Chaillou, L. L., and M. A. Nazareno. 2009. Bioactivity of propolis from Santiago del Este, Argentina, related to its chemical composition. *LWT Food Sci. Technol. (Campinas.)* 42:1422–1427.
- Chen, H., X. Mo, J. Yu, and Z. Huang. 2013. Alpinetin attenuates inflammatory responses by interfering toll-like receptor 4/nuclear factor kappa B signaling pathway in lipopolysaccharide-induced mastitis in mice. *Int. Immunopharmacol.* 17:26–32.
- Choi, J. S., M. J. Jung, J. Park, H. Y. Chung, and S. S. Kang. 2002. Further isolation of peroxynitrite and 1,1-diphenyl-2-picrylhydrazyl radical scavenging isorhamnetin 7-O-glucoside from the leaves of *Brassica juncea* L. *Arch. Pharm. Res.* 25:625–627.
- CLSI (Clinical and Laboratory Standards Institute). 2009. Performance Standards for Antimicrobial Disk Susceptibility Tests: Approved Standard. 10th ed. M02–A10. CLSI, Wayne, PA.
- Cushnie, T. P., and A. J. Lamb. 2005. Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Agents* 26:343–356.
- Diarra, M. S., G. Block, H. Rempel, D. B. Oomah, J. Harrison, J. McCallum, S. Boulanger, E. Brouillette, M. Gattuso, and F. Malouin. 2013. In vitro and in vivo antibacterial activities of cranberry press cake extracts alone or in combination with β -lactams against *Staphylococcus aureus*. *BMC Complement. Altern. Med.* 13:90–114.
- IBGE (Instituto Brasileiro de Geografia e Estatística). 2010. Produção da Pecuária Municipal. 38th ed. IBGE, Rio de Janeiro, Brazil.
- IBGE (Instituto Brasileiro de Geografia e Estatística). 2012. Manual Técnico da Vegetação Brasileira. 2nd ed. IBGE, Rio de Janeiro, Brazil.
- Kujumgiev, A., I. Tsvetkova, Y. Serkedjieva, V. Bankova, R. Chostov, and S. Popov. 1999. Antimicrobial, antifungal and antiviral activity of propolis of different geographic origin. *J. Ethnopharmacol.* 64:235–240.
- Maraschin, M., A. R. S. Zeggio, S. K. Oliveira, S. Kuhnen, M. M. Tomazzoli, A. C. M. Zeri, R. Carreira, and M. B. A. Rocha. 2012. A machine learning and chemometrics assisted interpretation of spectroscopic data—A NMR-based metabolomics platform for the assessment of Brazilian propolis. Pages 129–140 in *Pattern Recognition in Bioinformatics. Lecture Notes in Computer Science*, vol. 7632. T. Shibuya, H. Kashima, J. Sese, and S. Ahmad, ed. Springer, Berlin, Germany.
- Meresta, L., T. Meresta, J. Burdzinski, and P. Chmurzynski. 1989. Treatment of mastitis in cows using an extract of propolis. *Med. Weter.* 45:392–395.
- Mirolyubov, M. G., and A. A. Barskov. 1980. Propolis and mastitis. *Veterinariia* 2:45–46.
- Moreno, M. I. N., M. I. Isla, N. G. Cudmani, M. A. Vattuone, and A. R. Sampietro. 1999. Screening of antibacterial activity of *Amaiha delvalle* (Tucuman, Argentine) propolis. *J. Ethnopharmacol.* 168:97–102.
- Mosmann, T. 1983. Rapid colorimetric assay for growth and survival – application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65:55–63.
- Mukherjee, R., P. K. Dash, and G. C. Ram. 2005. Immunotherapeutic potential of *Ocimum sanctum* (L) in bovine subclinical mastitis. *Res. Vet. Sci.* 79:37–43.
- Nielsen, C., S. Østergaard, U. Emanuelson, H. Andersson, B. Berglund, and E. Strandberg. 2010. Economic consequences of masti-

- tis and withdrawal of milk with high somatic cell count in Swedish dairy herds. *Animal* 4:1758–1770.
- Nieva Moreno, M. I., I. C. Zampini, R. M. Ordoan, G. A. S. Jaime, M. A. Vattuone, and M. Isla. 2005. Evaluation of the cytotoxicity, genotoxicity, mutagenicity, and antimutagenicity of propolis from Tucuman, Argentina. *J. Agric. Food Chem.* 53:8957–8962.
- Orsolic, N., D. J. Starjcar, and I. Basic. 2009. Propolis and its flavonoid compounds on human urinary bladder transitional cell carcinoma in primary culture. *Period. Biol.* 111:113–121.
- Park, Y. K., S. M. Alencar, and C. L. Aguiar. 2002. Botanical origin and chemical composition of Brazilian propolis. *J. Agric. Food Chem.* 50:2502–2506.
- Passey, S., A. Bradley, and H. Mellor. 2008. *Escherichia coli* isolated from bovine mastitis invade mammary cells by a modified endocytic pathway. *Vet. Microbiol.* 130:151–164.
- Peres, L. A. B., V. D. A. Delfino, L. A. Tutida, B. A. Faris, D. S. Barbosa, and M. E. Favero. 2009. Efeitos do ramipril, captopril e sinvastatina na injúria da preservação a frio de rins de ratos. *J. Bras. Nefrol.* 31:39–47.
- Popova, M., V. S. Bankova, D. Butovska, V. Petkov, B. Nikolova-Damyanova, A. G. Sabatini, G. L. Marcuzzan, and S. Bogdanov. 2004. Validated methods for the quantification of biologically active constituents of poplar-type propolis. *Phytochem. Anal.* 15:235–240.
- Rabot, A., O. Wellnitz, H. H. D. Meyer, and R. M. Bruckmaier. 2006. Use and relevance of a bovine mammary gland explant model to study infection responses in bovine mammary tissue. *J. Dairy Res.* 74:93–99.
- Rall, V. L., E. S. Miranda, I. G. Castilho, C. H. Camargo, H. Langoni, F. F. Guimarães, J. P. Araújo Júnior, and A. Fernandes Júnior. 2014. Diversity of *Staphylococcus* species and prevalence of enterotoxin genes isolated from milk of healthy cows and cows with sub-clinical mastitis. *J. Dairy Sci.* 97:829–837.
- Robert, A., H. Seegers, and N. Bareille. 2006. Incidence of intramammary infections during the dry period without or with antibiotic treatment in dairy cows—a quantitative analysis of published data. *Vet. Res.* 37:25–48.
- Romvary, A., G. Szita, G. Csiko, Z. Gaspar, and I. Gaspar. 1993. Effectiveness of an intramammary infusion with no antibiotics. IV. Innocuity test of new intramammary infusion. *Magyar Allatorvosok Lapja* 48:493–497.
- Sanders, E. R. 2012. Aseptic laboratory techniques: Plating methods. *J. Vis. Exp.* 63:e3064 <http://dx.doi.org/10.3791/3064>.
- Santana, H. F., A. A. T. Barbosa, S. O. Ferreira, and H. C. Mantovani. 2012. Bactericidal activity of ethanolic extracts of propolis against *Staphylococcus aureus* isolated from mastitic cows. *World J. Microb. Biotechnol.* 28:485–491.
- Santos Neto, T. M., R. A. Mota, L. B. G. Silva, D. A. Viana, J. L. Lima-Filho, L. A. Sarubbo, A. Converti, and A. L. F. Porto. 2009. Susceptibility of *Staphylococcus* spp. isolated from milk of goats with mastitis to antibiotics and green propolis extracts. *Lett. Drug Des. Discov.* 6:63–68.
- Schukken, Y. H., K. E. Leslie, D. A. Barnum, B. A. Mallard, J. H. Lumsden, P. C. Dick, G. H. Vessie, and M. E. Kehrli. 1999. Experimental *Staphylococcus aureus* intramammary challenge in late lactation dairy cows: Quarter and cow effects determining the probability of infection. *J. Dairy Sci.* 82:2393–2401.
- Sforcin, J. M. 2007. Propolis and the immune system: A review. *J. Ethnopharmacol.* 113:1–14.
- Sforcin, J. M., J. R. A. Fernandes, C. A. M. Lopes, V. Bankova, and S. R. Funari. 2000. Seasonal effect on Brazilian propolis antibacterial activity. *J. Ethnopharmacol.* 73:243–249.
- Silva, J. F. M., M. C. Souza, S. R. Matta, M. R. Andrade, and F. V. N. Vidal. 2006. Correlation analysis between phenolic levels of Brazilian propolis extracts and their antimicrobial and antioxidant activities. *Food Chem.* 99:431–435.
- Singleton, V. L., and J. A. Rossi Jr.. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* 16:144–158.
- Sperotto, V. D., A. L. Murari, D. A. R. Silva, C. G. R. Possenti, J. M. Wiest, and C. A. M. Avancini. 2012. Activity of the decoction of *Achyrocline satureioides* DC (Lam.) - Asteraceae (“macela”) against standard and isolated bacteria from bovine mastitis. *Acta Sci. Vet.* 40:1052.
- Thirugnanasampandan, R., S. B. Raveendran, and R. Jayakumar. 2012. Analysis of chemical composition and bioactive property evaluation of Indian propolis. *Asian Pac. J. Trop. Biomed.* 2:651–654.
- USDA. 2015. Title 7 Part 205: National Organic Program. Accessed Feb. 13, 2015. <http://www.ecfr.gov/cgi-bin/text-idx?rgn=div5&node=7:3.1.1.9.32>.
- Velazquez, C., M. Navarro, A. Acosta, A. Ângulo, Z. Dominguez, R. Robles, R. Robles-Zepeda, E. Lugo, F. M. Gaycoolea, and E. F. Velazquez. 2007. Antibacterial and free-radical scavenging activities of Sonoran propolis. *J. Appl. Microbiol.* 103:1747–1756.
- Watts, J. L. 1988. Etiological agents of bovine mastitis. *Vet. Microbiol.* 16:41–66.
- Wei, W., L. Dejie, S. Xiaojing, W. Tiancheng, C. Yongguo, T. Zhengtao, and Z. Naisheng. 2015. Magnolol inhibits the inflammatory response in mouse mammary epithelial cells and mouse mastitis model. *Inflammation* 38:16–26.
- Zizic, J. B., N. L. Vukovi, M. B. Jadranin, B. C. Andelkovic, V. V. Tesevic, M. M. K. Kacaniova, S. B. Sukdolak, and S. D. Markovic. 2013. Chemical composition, cytotoxic and antioxidative activities of ethanolic extracts of propolis on HCT-116 cell line. *J. Sci. Food Agric.* 93:3001–3009.