

Seasonal variation of propolis from southern Brazil: phytochemical screening, antimicrobial activity, and effects on bovine mammary epithelial cells

Variação sazonal da própolis do Sul do Brasil: screening fitoquímico, atividade antimicrobiana e efeitos em células epiteliais mamária bovina

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ABSTRACT

Previous studies have reported on the phytotherapeutic potential of propolis from southern Brazil (Urupema, Santa Catarina State), in particular, its efficacy in the treatment of bovine mastitis. The present study evaluates the effect of seasonal variation on the chemical composition of propolis from southern Brazil and its resultant antimicrobial and cytotoxic activities in the context of treating bovine mastitis. Antimicrobial activity against *Staphylococcus aureus* was assessed, along with the cytotoxicity and induction of apoptosis in MAC-T bovine mammary epithelial cells. With the exception of spring propolis, the flavonoid quercetin was the main compound present in all samples. The minimum inhibitory concentration (MIC) of propolis against *S. aureus* from mastitic milk was 140 µg/mL for samples collected during the spring, autumn, and winter seasons, but 280 µg/mL for samples collected in summer. For MAC-T cells, the spring propolis extract was more toxic with an IC₅₀ of 120 µg/mL. However, for 120 µg/mL of spring propolis extract, only 0.77% of necrotic and 37% of apoptotic MAC-T cells were found, respectively. Nonetheless, the induction of cell death through apoptosis by propolis extract suggests less severe damage to bovine mammary glands. Moreover, only negligible seasonal variation was found in the chemical composition of propolis from southern Brazil, and no biological activities were determined to be harmful. Therefore, this propolis shows promise as an alternative to commercial antimicrobials in the control of bovine mastitis, offering support for organic milk production.

Keywords: Bovine mastitis. Brazilian propolis. MAC-T cells. *Staphylococcus aureus*.

RESUMO

Estudos prévios tem demonstrado o potencial terapêutico da própolis do Sul do Brasil (Urupema, Santa Catarina), em particular no tratamento da mastite bovina. O presente estudo tem como objetivo avaliar o efeito da variação sazonal sobre a composição química da própolis de Urupema do Sul do Brasil e suas atividades antimicrobiana e citotóxica visando o tratamento da mastite bovina. A atividade antimicrobiana contra *Staphylococcus aureus* foi avaliada, juntamente com a citotoxicidade e indução de apoptose em células epiteliais mamárias bovina da linhagem MAC-T. Com exceção da própolis da primavera, o flavonóide quercetina foi o composto majoritário em todas as amostras. A concentração inibitória mínima (CIM) da própolis contra *S. aureus* de leite mastítico foi 140 µg/mL para as amostras de primavera, outono e inverno e 280 µg/mL para a amostra coletada no verão. Para as células MAC-T, o extrato de própolis da primavera foi o mais tóxico, sendo a IC₅₀ 120 µg/mL. Entretanto, com 120 µg/mL do extrato de própolis primaveril, somente 0,77% de células MAC-T necróticas e 37% apoptóticas foram encontradas. Portanto, a indução da morte celular por apoptose pelo extrato de própolis sugere danos possivelmente menos graves a glândula mamária bovina. Além disso, somente uma pequena variação sazonal foi encontrada para a composição química da própolis do Sul do Brasil, a qual não prejudicou suas atividades biológicas. Portanto, esta própolis mostra como uma alternativa promissora ao uso de antimicrobianos comerciais no controle da mastite bovina e uma opção para subsidiar a produção orgânica de leite.

Palavras-chave: Células MAC-T. Mastite bovina. Propolis brasileira. *Staphylococcus aureus*.

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Introduction

Propolis, a complex mixture of diverse resinous substances collected from plants by bees, has become common in popular medicine, including the control of bovine mastitis (Fiordalisi et al., 2016; Santana et al., 2012). The ability of propolis to reduce the growth of diverse microorganisms, including *Staphylococcus aureus*, one of the main etiological agents of mastitis, is widely discussed in the literature (Fiordalisi et al., 2016; Santana et al., 2012). However, because it is a complex matrix, propolis is subject to seasonal fluctuations owing to local flora. The vegetation from which the resin is collected and the time of collection are factors that substantially affect chemical composition (El-Guendouz et al., 2018; Popova et al., 2005). Thus, variations in chemical composition of propolis can be influenced seasonally and its typical botanical composition. Lu et al. (2005), for example, showed higher antimicrobial activity against *S. aureus* to Taiwan propolis produced in August.

Recently, propolis was found to protect bovine mammary alveolar cells (MAC-T) against damage caused by pathogens that trigger mastitis (Zhang et al., 2016). MAC-T cells represent an isolated epithelial cell line in the alveolar tissue of bovine mammary glands, and these cells are commonly used in the study of mammary gland functions, such as hormonal regulation and inflammatory responses (Huynh et al., 1991; Piotrowska-Tomala et al., 2015). Since MAC-T cells are derived from an immortalized line of bovine alveolar tissue, they are an appropriate model with which to evaluate the effects of novel antimicrobials to control mastitis on bovine mammary cells. The use of a cell line, rather than *in vitro* tissue culture studies, offers a

number of advantages, such as genetic uniformity, lower risk of microbial contamination, and the possibility of studying phenomena that cannot be assessed with the use of intact tissues (Alberts et al., 1994).

For bovine mastitis, damage that provokes cell death can aggravate the inflammatory process brought on by infection. In attempts to combat inflammation, conjunctive tissue is formed in the affected area, causing a decrease in the alveolar area responsible for the synthesis of milk and, consequently, milk production. Once affected, mammary gland functionality can be compromised for several lactations. Reduction in milk production accounts for approximately 70% of the total costs incurred by the incidence of mastitis in a herd (Zhao & Lacasse, 2008).

The damage caused to the mammary gland can occur through cell necrosis or apoptosis. These two types of cell death can be distinguished by morphological, biochemical, and molecular changes. During mammary gland infection, tissue damage can be initially caused by bacteria or their products. Certain bacteria produce toxins that destroy cell membranes, while others, such as *S. aureus*, are capable of invading and multiplying within mammary epithelial cells before causing cell death. Necrosis is characterized by the loss of membrane integrity, release of cellular content, and tissue reaction. Apoptosis, on the other hand, usually occurs without membrane damage, but with DNA fragmentation, the formation of apoptotic bodies, and the absence of tissue reaction (Lamkanfi & Dixit, 2010). The induction of apoptosis has also been suggested as a strategy used by cells to combat pathogenic infection (Liu et al., 2014). As such, understanding the possible effects of intramammary administration of antimicrobials is necessary, essentially because toxicity, particularly at levels that causes necrosis, may worsen the animal's clinical condition and compromise glandular functionality for future lactation (Troncarelli et al., 2014).

Therefore, this study aimed to evaluate the effect of seasonal variation on the chemical composition of propolis from Urupema municipality (Santa Catarina State, Brazil) and its resultant antimicrobial and cytotoxic activities in the context of treating bovine mastitis, in particular, *in vitro* antimicrobial activity against *S. aureus* and the induction of apoptosis in MAC-T bovine mammary epithelial cells.

Material and Methods

The propolis samples used in this study were collected from Urupema municipality (Santa Catarina) (Figure 1) during all seasons of 2014. Urupema is located in the Santa Catarina mountain range at approximately 1500 meters above sea level and the climate of the region is temperate humid, according to the Köppen climate classification system

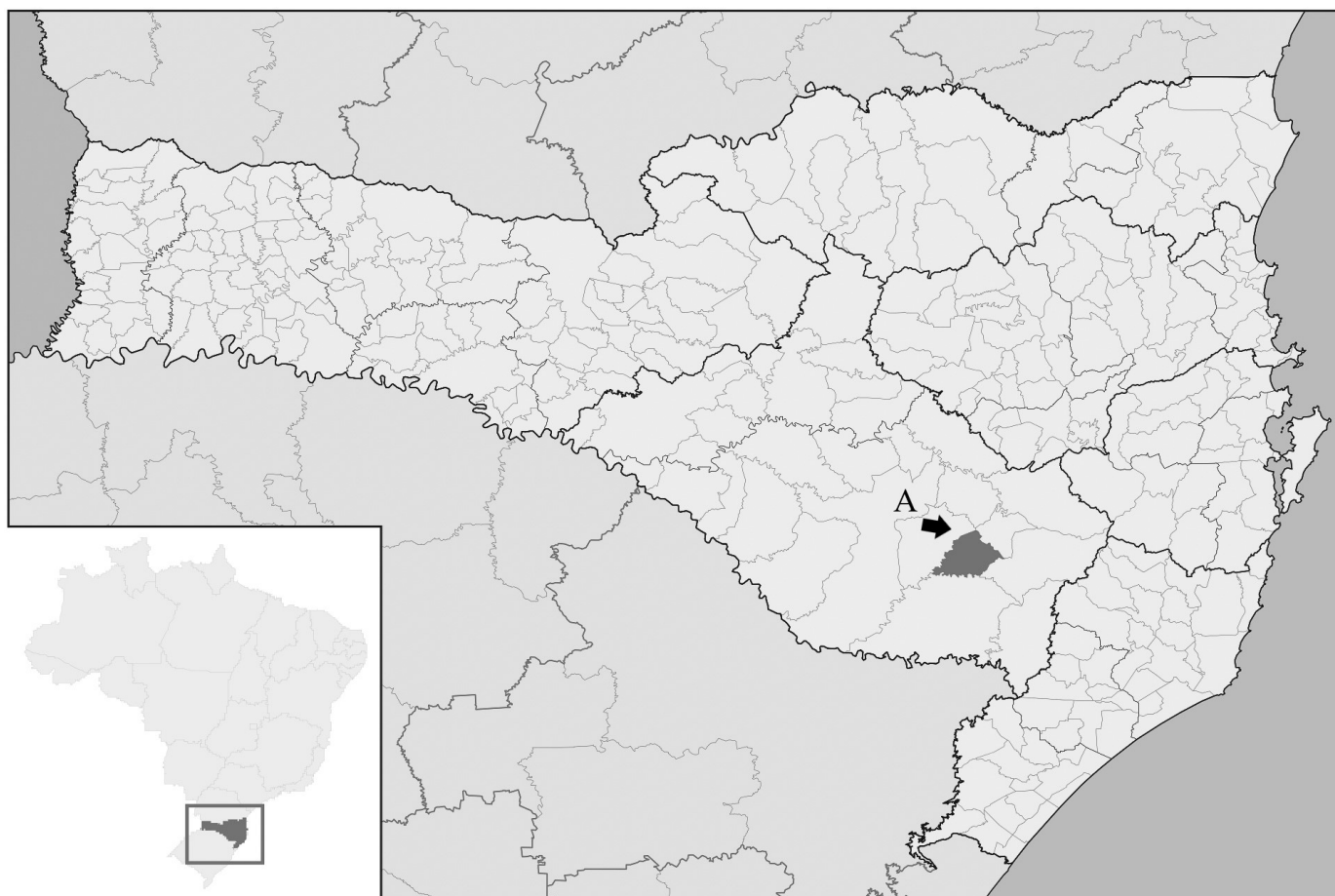


Figure 1 – Location of Urupema (A), a municipality in Santa Catarina State, Brazil.

(Martins-Ramos et al., 2011). After scraping all propolis from the honey production hives, the resin from each box was stored separately in plastic bags. The samples were frozen at -20°C and sent to the laboratory (LABIMA/UFSC). Afterwards, the samples were processed to remove impurities and then ground. Samples from at least three boxes per season were pooled together to prepare the crude extracts.

For extract preparation, ground crude propolis samples were macerated in ethanol 70% (v/v) (1:10, w/v) and stored in darkness for 24 h. Subsequently, the macerate was vacuum filtered and the extract frozen at -20°C for 24 h. The hydroalcoholic extract was centrifuged (10,000 rpm, 10 min), and the supernatant was collected and dried at 60°C . The residues were then stored in darkness at -20°C .

The total phenolic contents of the propolis extracts were determined using the Folin-Ciocalteu colorimetric method (Singleton & Rossi, 1965). The results were calculated using a standard curve of gallic acid (20 to 100 $\mu\text{g}/\text{mL}$) ($y = 0.0105x / r^2 = 0.9757$) and expressed in μg of gallic acid equivalents (Sigma-Aldrich) per mg of extract ($\mu\text{GAE}/\text{mg}$).

The total flavonoid contents were determined using a colorimetric reaction with aluminum chloride, following the method described by Popova et al. (2004). The results

were calculated using a quercetin calibration curve (Sigma-Aldrich) (10 to 100 $\mu\text{g}/\text{mL}$) ($y = 0.0287x / r^2 = 0.9954$), and the results were expressed in μg quercetin equivalents per mg of extract ($\mu\text{QE}/\text{mg}$).

The antioxidant potential of the propolis extracts was assessed using the DPPH test, according to Choi et al. (2002). The results were expressed as a percent inhibition DPPH radical/mg of extract.

Analysis of the phenolic composition of the propolis extracts was performed on a HPLC Thermo Scientific Ultimate 3000 RS Dual System (Thermo Fisher Scientific, San Jose, CA, USA), using a Thermo Scientific C18 reverse-phase column (4.6 X 250 mm; $5\mu\text{m}$; 120 \AA) (Acclaim™120, ThermoScientific®) at 40°C , operating at 240, 260, 280 and 320 nm. Methanol PA (A) and pH 2.3 MilliQ water (B) were used as mobile phase at a flow of 1 mL/min. The initial concentration of the mobile phase was 85% of A and 15% of B, modified to 100% of B at 40 min, and returning to the initial conditions at 50 min. The identification of phenolic compounds in the samples was based on a comparison of retention times and UV-Vis profiles (260 nm) obtained after the injection of standard compounds (quercetin, hesperidin, ferulic acid, hydrocinnamic acid, t-cinnamic acid; Sigma-Aldrich). These compounds

were quantified with an external quercetin calibration curve (12.5 to 100 µg/mL) ($y = 2.0234x / r^2 = 0.9993$). The values were expressed in mg of quercetin equivalents per mL (mgQE/mL).

Antimicrobial activity of the propolis extracts was evaluated using microdilution in Muller-Hinton broth (MHB/ Sigma-Aldrich), as described in the Clinical and Laboratory Standards Institute Manual (Clinical and Laboratory Standards Institut, 2015). The test was conducted against a standard strain of *S. aureus* ATCC 25923 and two lineages isolated from mastitic milk. After the addition of serial dilutions of the propolis extracts (1.120 to 70 µg/mL) in EtOH:H₂O (8:92, v/v), 10 µL of inoculum suspension, equivalent to a logarithmic dilution of 10⁵ CFU/mL, were added. The initial inoculum was prepared using a turbidity control equivalent to a McFarland standard solution of 0.5, which corresponds to a suspension containing 1 to 2 x 10⁸ CFU/mL. After 24 h of incubation at 37 °C, a visual determination of the Minimum Inhibitory Concentration (MIC) based on the appearance of turbidity was performed. Additionally, the microplates were read in a spectrophotometer (EL808, Bio-Tek Instruments, Inc.) at 600 nm, and the percentage of microbial growth inhibition was calculated as equal to $[1 - (Ac/A_0)] \times 100$ (Bonifácio, 2014), where Ac is the average absorbance of treatments with inoculum subtracted from the average of replicate treatments without inoculum, and A₀ is the average absorbance of wells with MHB and inoculum. Therefore, the value obtained represents the percentage of microbial cells that each propolis concentration could inhibit. Antimicrobial experiments were performed in triplicate.

Mammary epithelial cells of the MAC-T (mammary alveolar cells-T) lineage were maintained in culture, as indicated by the supplier (Banco de Células do Rio de Janeiro, Brazil). Briefly, MAC-T lineage cells were cultivated with MAC-T lineage cells cultivated in Dulbecco's modified Eagle's medium (DMEM/GIBCO), supplemented with 20% fetal bovine serum (FBS/GIBCO), 4 mM of L-glutamine (Synth), 4.5 g/L of glucose (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 1.5 g/L of sodium bicarbonate (Vetec), 5 µg/mL of insulin (Himulin`Lilly), and 1 µg/mL of hydrocortisone (Sigma-Aldrich). Cytotoxicity was evaluated by adding different concentrations of propolis extract, 8.75 to 560 µg/mL in DMSO 0.5%, to the culture medium containing the adherent cells. After 24 h of incubation at 37°C, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 0.5 mg/mL) was added, followed by two more hours of incubation. The amount of formazan formed was measured by spectrophotometry at 546 nm (EL808, Bio-Tek Instruments, Inc.) and considered directly proportional to the number of viable cells. The control, i.e., fresh medium without extract, was considered as 100% viable cells.

MAC-T cells were evaluated for the presence of necrosis and apoptosis after treatment with different concentrations of spring propolis at 60, 120, and 240 µg/mL. The test was performed by labeling the MAC-T cells with annexin V-FITC and propidium iodide (PI) and analyzing them for apoptosis and necrosis by flow cytometry following the manufacturer's protocol (BD Bioscience, San Diego, CA, USA). Cells were incubated with different concentrations of propolis extract for 24 h. Subsequently, trypsinization and labeling with annexin V and PI were performed. Apoptotic cells were identified using a FACSCalibur™ BD flow cytometer and counted in the upper and lower right quadrants, including the percentages of both early (annexin V-positive) and late apoptotic cells (annexin V- and PI-positive). Normal cells were counted in the lower left quadrant (annexin V- and PI-negative), and necrotic cells were counted in the upper left quadrant (PI-positive).

Data were expressed as mean ± standard deviation (SD) of at least three independent experiments. Total phenolic and flavonoid contents, percent inhibition of DPPH radical, content of the compounds identified by High-performance liquid chromatography, as well as the percentage of cell viability and cellular apoptosis induction were all subjected to analysis of variance (ANOVA) adjusted for Tukey's test using GraphPrism 5.0 software. For the percentage of microbial inhibition, the MIXED model was used in SAS software. The effects were considered statistically significant for P<0.05. The inhibitory concentrations capable of reducing cellular viability by 50% (IC50) were calculated using a nonlinear regression of data obtained from the cellular viability tests with GraphPad Prism 5.0 software.

Results and Discussion

The highest total phenolic and flavonoid contents were found in the propolis extracts from autumn, and the lowest were found for the winter samples (Table 1). Furthermore, the autumn samples showed greater efficiency in the inhibition of DPPH radicals and, therefore, greater antioxidant activity

Table 1 – Seasonal variation in total phenolic contents* (µg of GAE/mg), total flavonoid contents (µg QE/mg), and antioxidant activity (% of radical DPPH inhibition) of propolis extracts from Urupema (SC, Brazil)

	Total Phenolics (µg EAG/ mg)	Total Flavonoids (µg QE/ mg)	% radical DPPH inhibition
Spring	228.3 ± 18.40 ^b	12.45 ± 0.82 ^{bc}	46.86 ± 1.72 ^{ab}
Summer	251.6 ± 59.80 ^b	14.1 ± 2.43 ^{ab}	54.31 ± 12.64 ^{ab}
Autumn	314.7 ± 9.20 ^a	16.54 ± 1.11 ^a	63.61 ± 4.42 ^a
Winter	143.7 ± 8.60 ^c	10.21 ± 0.63 ^c	45.72 ± 5.96 ^b

* Values represent the mean of three independent extractions ± the standard deviation (SD) of the mean. Different letters in the column indicate significant difference among the propolis samples (P < 0.05).

compared to winter samples (Table 1). The total phenolic and flavonoid content and antioxidant activity found in the present study are similar to results described in the literature for ethanolic extracts of propolis from different geographic regions (Machado et al., 2016; Osés et al., 2016). Furthermore, in a previous study on Urupema propolis sampled in the winter of 2011, Fiordalisi et al. (2016) found contents very similar to those found herein, suggesting a standardization in the extracts that is independent of the year of production. Nevertheless, seasonality seems to have a more prominent effect on the content of those compounds in propolis from southern Brazil (Table 1). Similarly, Zeggio (2016), evaluating the effect of seasonality on the chemical composition of Santa Catarina propolis, also found that variation in phenolic composition and antioxidant activity correlated with the season of sample collection.

Evidence of the effect of seasonality on the concentration of phenolic compounds and antioxidant activity of propolis is abundantly reported in the literature. For example, El-Guendouz et al. (2018), comparing extracts of propolis from different cities in the southeastern and northeastern regions of Brazil, found wide variation between the total phenolic and flavonoid contents and antioxidant activity according to the location of sample collection and the time of year. Specifically, the authors found antioxidant activity ranging from 12.5 to 51% inhibition of DPPH radical, a result which the authors attributed mainly to the season in which the samples were collected, as well as their geographic origin. Flora available for resin production during the year in a particular geographic location is, thus, further causally implicated. In the present study, the Ombrophilous Mixed Forest predominates in the Urupema region in which species of conifers, such as *Araucaria angustifolia* (Bertol) Kuntze, are found to be associated with other species at different successional stages (Leite & Klein, 1990). Thus, it is plausible that such wide variability of plant sources in the production of propolis would be associated with variations

in the contents of phenolic and flavonoid compounds, as well as antioxidant activity, of seasonally collected samples.

Seven phenolic compounds identified in the seasonal Urupema propolis samples (Table 2) are also found in samples of propolis from different wide-ranging origins (Kujumgiev et al., 1999; Popova et al., 2005; Frozza et al., 2013). In the present study, all samples showed a similar phenolic profile (Table 2). With the exception of spring propolis, the flavonoid quercetin was the main compound present in all samples. In a previous study, quercetin was identified as the main compound in autumn propolis collected from a town near Urupema (São Joaquim, SC, Brazil) (Meneghelli et al., 2013). However, in the spring extract, the principal compound could not be identified with HPLC. This compound, which was absent from the summer and autumn samples, also appeared at low levels in the winter extract (Table 2). It is possible that this chemical compound, which commenced production in winter with a peak in the spring, is associated with the collection of vegetative material typical of these seasons. In the study region, *A. angustifolia* is precisely in pollination phase during August, September and October, months which constitute the end of winter and height of spring. In this period, pollen cones (male flower) are mature and have a sticky, resinous substance that appears on the surface of the strobili, mainly in the recesses of the bracts (Soares & Mota, 2004). Zeggio (2016) found an association between the chemical profile of propolis extracts and the colorless resin from *A. angustifolia*. Thus, we can suggest that the compound in question may be the result of the material collected from this plant, which has not yet been identified.

All extracts caused inhibition of at least 90% microbial growth at 280 µg/mL (Table 3). Among the samples studied, spring propolis was the most efficient, inhibiting 100% growth of *S. aureus*. The MIC was confirmed by the presence or absence of turbidity. For the extracts from summer, autumn, and winter, the MIC was 280 µg/mL,

Table 2 – Contents* of the main phenolic compounds in the seasonal extracts of Urupema propolis (Santa Catarina, Brazil)

	Retention time (min)	Spring (µgQE/mg)	Summer (µgQE/mg)	Autumn (µgQE/mg)	Winter (µgQE/mg)
Syringic acid	17.81	1.51 ± 0.25 ^C	4.75 ± 0.23 ^A	4.39 ± 0.6 ^A	3.61 ± 0.08 ^B
P-coumaric acid	19.64	N.D.	3.20 ± 0.2 ^A	2.98 ± 0.66 ^A	N.D.
Ferulic acid	21.23	1.90 ± 0.21 ^B	4.73 ± 0.19 ^A	5.48 ± 0.76 ^A	5.05 ± 1.16 ^A
Hesperidin	22.91	1.86 ± 1.41 ^B	2.45 ± 0.08 ^{AB}	3.18 ± 0.62 ^A	2.72 ± 0.07 ^{AB}
N.I.*	26.23	34.55 ± 1.53 ^A	N.D.	N.D.	2.1 ± 0.15 ^B
Quercetin	26.71	6.41 ± 1.13 ^D	72.21 ± 3.10 ^A	47.94 ± 0.92 ^B	39.11 ± .00 ^C
Luteolin	27.79	3.37 ± 0.74 ^C	8.91 ± 0.25 ^A	6.85 ± 1.85 ^B	5.42 ± 0.15 ^B
3-O-methylquercetin	28.42	1.25 ± 0.53 ^B	1.49 ± 0.01 ^B	1.82 ± 0.23 ^B	3.81 ± 0.14 ^A
Total		50.85	97.74	72.64	61.82

* Values represent the mean of three independent extractions ± SD. Different letters in the rows indicate significant difference among the extracts from different seasons throughout the year (P < 0.05). N.I.* = unidentified compound. N.D. = not detected.

and for spring, 140 µg/mL (Table 3). Relating antimicrobial activity to chemical composition, it can be seen that the spring propolis extract is precisely when the unidentified compound is the principal compound, while in the other extracts, the main compound is quercetin.

The antimicrobial activity of propolis extracts has been attributed to the presence of several classes of compounds, particularly flavonoids (Mesbah & Samia, 2011), phenolic acids (Mushtaq et al., 2016), and terpenoids (Bankova et al., 1995), while it is not often attributed to a single class of compounds. Fiordalisi et al. (2016), when comparing the antimicrobial activity of propolis extracts from different regions in Santa Catarina, found that Urupema winter extract, despite a lower flavonoid content, had the same effect against *S. aureus* isolated from mastitic milk as that of extracts with higher levels of flavonoids. In that study, 200 µg/mL of Urupema propolis extract caused a significant reduction in *S. aureus* growth, and 250 µg/mL reduced bacterial growth by 2 log 10. This result is very similar to that presented herein (Table 3). Santana et al. (2012) also found antimicrobial activity consistent with that of the present study, with propolis extracts containing lower levels of flavonoids and phenolics (2.4 and 7.7 mg/g, respectively). In the literature, the MIC values of ethanolic propolis extracts against *S. aureus* vary widely. While Alencar et al. (2007) found a MIC lower than 100 µg/mL

for red propolis, Dias et al. (2012) and Campos et al. (2014) found higher values, ranging from 240 to 3100 µg/mL, depending on the origin.

In the present study, seasonality was not meaningfully correlated with antimicrobial activity in the context of compounds present in propolis extracts (Table 3). This similarity between propolis extracts can be explained by the composition of the samples from summer, autumn, and winter, which consist mainly of quercetin. In the spring extract, this flavonoid is the second most abundant. Previous studies have demonstrated the antimicrobial effect of quercetin against *S. aureus*, including multi-resistant strains (Diniz-Silva et al., 2017). Yet, inconsistencies are reflected in the literature in relation to the MIC of this flavonoid. For example, Parkar et al. (2008) found a MIC of 62.5 µg/mL, while Kang et al. (2006) found values higher than 300 µg/mL. In general, antimicrobial effect of flavonoids results from the presence of hydroxyl groups that have affinity for proteins. As such, they act as bacterial enzyme inhibitors, interfering with synthetic pathways and compromising metabolism (Flambó, 2013).

All studied extracts caused a significant reduction in cellular viability beginning at 70 µg/mL (Table 4). However, the spring propolis extract was more toxic to this cell type, followed by the winter sample. The spring extract reduced viable cells to less than half at a concentration of 140 µg/mL.

Table 3 – Percentage* inhibition (%) of *S. aureus* microbial growth after exposure to the seasonal extracts of Urupema propolis (SC, Brazil).

Propolis Concentration (µg/mL)	Spring	Summer	Autumn	Winter
70	57.60 ± 7.36 ^{Aa}	59.80 ± 7.36 ^{Aa}	73.20 ± 7.36 ^{Aa}	58.10 ± 7.36 ^{Aa}
140	80.90 ± 7.36 ^{Ba}	63.40 ± 7.36 ^{Aa}	78.20 ± 7.77 ^{Aa}	74.20 ± 7.36 ^{Ba}
280	100 ^{Ca}	87.30 ± 7.36 ^{Ba}	97.50 ± 7.77 ^{Aa}	95.30 ± 7.36 ^{Ca}
560	100 ^{Ca}	100 ^{Ca}	100 ^{Aa}	100 ^{Ca}
1120	100 ^{Ca}	92.90 ± 7.36 ^{Dab}	82.70 ± 7.36 ^{Bb}	100 ^{Ca}

* Mean ± SD of a standard *S. aureus* (ATCC 25923) and two strains isolated from mastitic milk. Different lowercase letters in the rows represent significant differences among extracts at the same concentration. Different uppercase letters in the columns represent significant differences among concentrations of the same extract ($P < 0.05$).

Table 4 – Percentage of viability* of MAC-T cells after exposure to different concentrations of Urupema propolis extracts collected during different seasons of the year

Propolis Concentration (µg/mL)	Spring	Summer	Autumn	Winter
0	100 ^{Aa}	100 ^{Aa}	100 ^{Aa}	100 ^{Aa}
DMSO 0.5%**	92 ± 3.4 ^{Aa}	75 ± 9.98 ^{Bc}	100 ± 6.45 ^{Aa}	91 ± 18.75 ^{Aa}
8.75	92 ± 8.86 ^{Aa}	95 ± 13.35 ^{Aa}	100 ± 5.20 ^{Aa}	97 ± 21.18 ^{Aa}
17.5	88 ± 9.8 ^{Ba}	100 ± 7.46 ^{Aa}	98 ± 8.79 ^{Aa}	92 ± 12.77 ^{Aa}
35	86 ± 6.39 ^{Bab}	88 ± 7.92 ^{Bb}	93 ± 7.42 ^{Aa}	95 ± 13.22 ^{Aa}
70	69 ± 6.86 ^{Bb}	90 ± 6.56 ^{Aab}	75 ± 7.42 ^{Bbc}	70 ± 14.19 ^{Bb}
140	36 ± 9.38 ^{Cc}	62 ± 7.72 ^{Ac}	55 ± 6.30 ^{Bc}	51 ± 12.63 ^{Bc}
280	25 ± 9.93 ^{Bcd}	36 ± 4.22 ^{Ad}	41 ± 4.93 ^{AcD}	35 ± 4.35 ^{Ad}
560	19 ± 7.45 ^{Bd}	33 ± 10.03 ^{Ad}	37 ± 2.32 ^{Ad}	34 ± 8.17 ^{Ad}

* Mean ± SD of three independent repetitions. Different uppercase letters in the columns represent significant differences among concentrations of the same extract. Different lowercase letters in the rows represent significant differences among extracts at the same concentration ($P < 0.05$). ** Negative control.

At a concentration of 280 µg/mL, all other extracts presented the same toxicity to MAC-T cells, reducing viability by approximately 50%. Through a nonlinear regression, it was possible to determine the concentration responsible for reducing cell viability by 50%, as shown in Table 5. Apart from having a higher toxic effect on MAC-T cells, it is interesting to note that the spring extract also showed greater antimicrobial activity against *S. aureus*. The predominant presence of the unidentified compound in this sample and its presence at a smaller quantity in the winter sample probably gave these extracts a possible boost in toxicity.

Our results are similar to those reported by Fiordalisi et al. (2016) who found an IC₅₀ of 171.8 µg/mL for a Urupema propolis using *in vitro* models with bovine mammary gland explants. The consistency between this mammary gland explant model, which is a primary culture, and the MAC-T cells model in the present study, validates the use of *in vitro* testing to assess the toxicity of potential products for bovine mastitis treatment. Such *in vitro* models may provide greater reliability and safety in the indication of phytotherapeutic doses for application and/or validation of *in vivo* models. This is an innovative approach, since the use of MAC-T cells for cytotoxicity studies of natural products is uncommon. Recently, Wang et al. (2016) evaluated the toxicity of Chinese propolis on MAC-T cells. Although the authors did not determine the IC₅₀ of the extract, they concluded that concentrations below 15 µg/mL were safe for MAC-T cells, since they reduced the loss of viability caused by exposure to LPS and *E. coli* and *S. aureus* bacteria.

Studies on the cytotoxic potential of propolis extracts on tumor cells are more frequent in the literature. In such studies, extracts should have low IC₅₀, thus demonstrating high cytotoxic potential. However, the IC₅₀ values found using these models vary widely, depending on the cell type studied and the origin and composition of the propolis sample. Some studies have found values that are much lower than those reported herein. Alencar et al. (2007), evaluating the effect of propolis on human HeLa tumor cells, found an IC₅₀ of 7.5 µg/mL. Frozza et al. (2013), also using HeLa and a propolis from another geographic region, found an IC₅₀ value almost 10 times higher (81.40 µg/mL).

Table 5 – Inhibitory concentration (IC₅₀)* of propolis extracts from different seasons of the year for MAC-T bovine mammary epithelial cells

Spring (µg/mL)	Summer (µg/mL)	Autumn (µg/mL)	Winter (µg/mL)
120 ± 2.80	252.6 ± 6.08	236 ± 5.46	188.6 ± 5.70

* The IC₅₀ values were calculated using a nonlinear regression of data obtained from the MTT assays.

Unlike studies on tumor cells, our model requires extracts with high antimicrobial potential, but low cytotoxicity to udder cells, aiming at a lower risk of harmful effects on bovine mammary gland health. In the present study, the antimicrobial effect was accompanied by a moderate toxic effect on the cells. These results suggest caution in intramammary administration of propolis extracts, and they are consistent with reports that internal use may increase somatic cell count (SCC) and proinflammatory reaction (Troncarelli et al., 2014). Nevertheless, we must consider that other *in vivo* factors, such as the presence of milk and the cellular complexity of the mammary gland, can affect the release of the active principles, thus mitigating this toxicity. In addition, the *S. aureus* bacterium can colonize and survive within mammary cells, resulting in low cure rates (Anderson & Azizoglu, 2014). As such, the death of some cells, by either necrosis or apoptosis, could help in the fight against chronic infection by releasing pathogens and making them accessible to antimicrobials.

Cytotoxicity analyses are commonly used in the literature to describe damage to cells and tissue (Frozza et al., 2013). Yet, we can use further tests to identify the type of cell death that causes loss of cellular viability. This information may be important in evaluating the potential of a product to treat mastitis, considering that induction of necrosis worsens the clinical condition of the animal. As such, by marking MAC-T cells with annexin V/PI, the type of damage that caused the loss of cellular viability after incubation with Urupema spring propolis extract was analyzed.

The graphical representation and percentage of MAC-T cell death after treatment with different concentrations of spring extract is shown in Figure 2. No significant differences in the percentage of necrotic cells occurred for any of the tested concentrations. However, with 120 µg/mL of propolis extract, a value similar to the IC₅₀, a minimal damage to the cellular membrane of MAC-T cells was found. On the other hand, an increase in concentrations of propolis showed an increase in the percentage of apoptotic MAC-T cells. During apoptosis, various alterations in cell morphology occur, such as chromatin condensation, cellular retraction, and loss of adherence to the extracellular matrix and neighboring cells (Häcker, 2017). The potential of propolis extracts to induce apoptosis has been previously studied as a treatment for infectious and neoplastic diseases (Begnini et al., 2014). It is possible that this induction occurs through the liberation of cytochrome c from within the mitochondria for cytoplasm, beginning the caspase cascade and, hence, activating pro-apoptosis proteins (Sawicka et al., 2012).

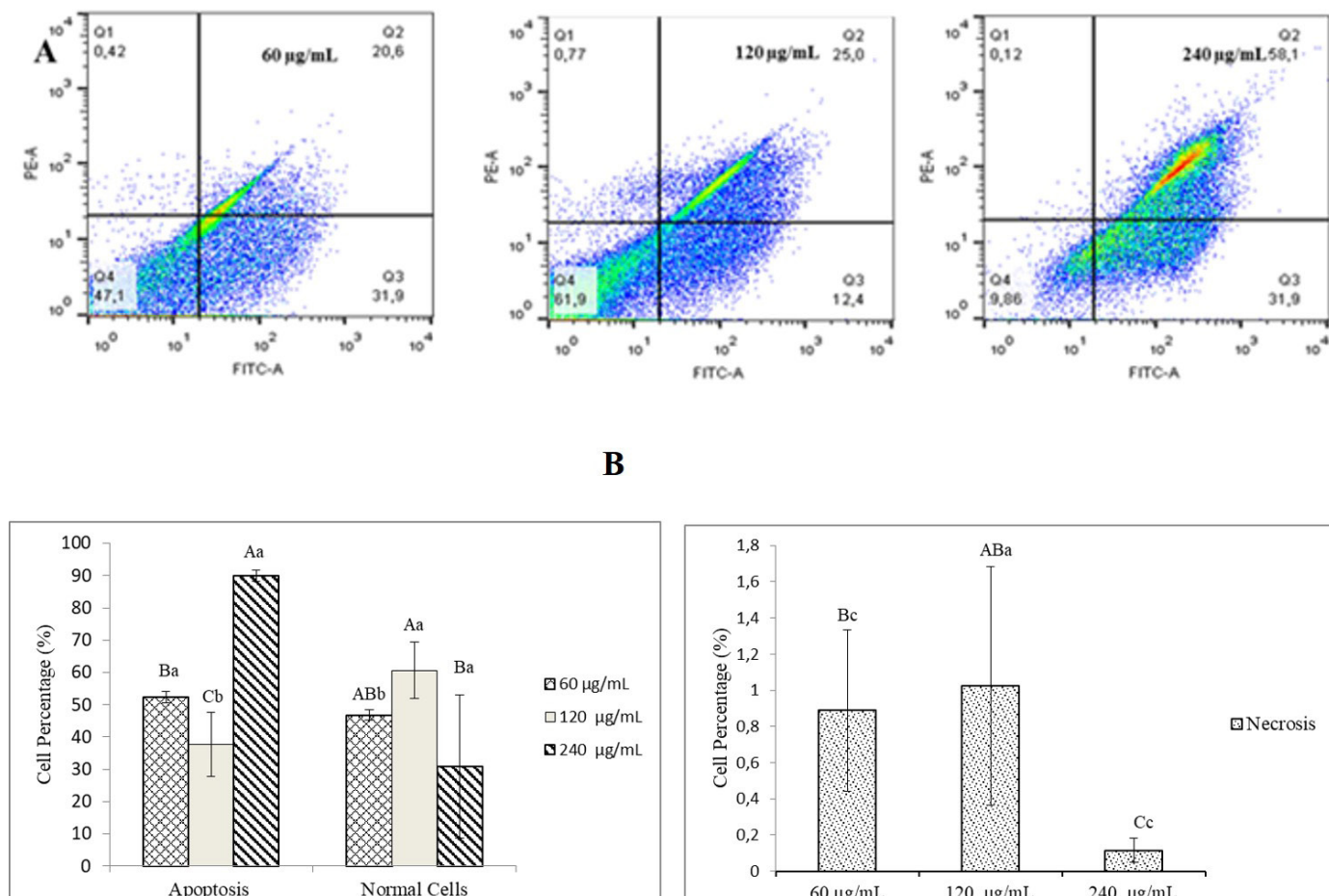


Figure 2 – (A) Graphical representation of the percentage of apoptotic (upper and lower right quadrant), necrotic (upper left quadrant), and normal cells (lower left quadrant) after treatment with three different concentrations of Urupema propolis. (B) Mean of nine replicates distributed across three independent experiments \pm SD of the percentage of necrotic, apoptotic, and unchanged cells after 24 h of treatment with 60, 120, and 240 $\mu\text{g/mL}$ of Urupema propolis. Uppercase letters represent significant differences in the percentage of the same type of cellular alteration at different tested concentrations; lowercase letters indicate significant differences in the percentage of distinct cellular changes for the same tested concentration ($p < 0.05$).

While reports in the literature have shown an increase in apoptosis after treatment with propolis, Wang et al. (2016) detected a significantly lower number of apoptotic MAC-T cells after pretreatment with Chinese propolis and subsequent induction of cell stress when exposed to inactivated *E. coli* and *S. aureus* bacteria. Furthermore, the authors suggested that propolis has a modulating effect on the apoptosis cascade, such as blocking caspase activation. These proteins signal the apoptosis process. By cleaving the cell membrane, they lead to nuclear condensation and fragmentation and the externalization of membrane phospholipids, indicating to the macrophages which cell should be phagocytosed (Nicholson & Thornberry, 1997).

During the induction of cellular apoptosis, the extravasation of cellular content is blocked, which also restricts the beginning of the inflammatory reaction, allowing the pathogen to later evade host defenses (Boutet et al., 2004). In this process, the production of granulocyte-macrophage

colony-stimulating factor (GM-CSF) stimulates and affects the functionality of defense cells. This contributes to the delay of apoptosis of these cell types, causing persistent accumulation of neutrophils and macrophages in the affected region and impeding antimicrobial activity (Boutet et al., 2004). However, it is worth considering that apoptosis induction may also represent a strategy for host cells to defend themselves against pathogenic bacteria by preventing the release and dissemination of intracellular bacteria (Chen et al., 2017).

In the present study, the modulating activity of propolis on MAC-T cells could explain the higher percentage of normal cells after treatment with 120 $\mu\text{g/mL}$ of Urupema propolis compared to that of 60 $\mu\text{g/mL}$. Furthermore, an increase in the percentage of apoptotic cells at concentrations greater than 240 $\mu\text{g/mL}$ may not represent substantial damage to mammary gland cells, since apoptosis does not cause a tissue reaction. As mentioned above, *S. aureus* colonizes the mammary epithelium in an intracellular manner, and

this gram-positive, round-shaped bacterium can produce toxins that destroy cell membranes, directly affecting the milk-producing tissue and inducing necrosis in the mammary gland (Jensen et al., 2013). Initially, bacterial influx damages the lining of the tissues of the teats and interior of the gland cisterns. They then invade the duct system and establish colonies in the alveoli, forming abscesses. If the infection is not eliminated, bacterial levels in the mammary gland tend to rise, thereby causing damage to the mammary epithelium. In a persistent infection, the number of somatic cells in milk increases, while tissue damage is aggravated. Alveoli in the gland lose integrity, and the barrier between milk and blood ruptures, causing extracellular fluid extravasation and milk contamination (Zhao & Lacasse, 2008).

Thus, in correlating the results obtained through the analysis of cellular apoptosis induction by Urupema propolis in MAC-T cells with its antimicrobial action against *S. aureus*, this product can be characterized as promising for the treatment of mastitis caused by this pathogen. In mastitis caused by *S. aureus*, loss of cell viability through the induction of apoptosis after exposure to propolis concentrations may not be detrimental to the host. In this process, the cellular content is retained, thus helping to prevent the spread of pathogens in the mammary gland.

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Conclusion

Seasonality of Urupema propolis samples had little influence on phenolic and flavonoid contents of propolis extract and, hence, showed no correlation with antimicrobial activity against *S. aureus* or cytotoxicity on MAC-T cells. The loss of cell viability caused by the extracts occurred because of apoptosis induction and may represent a strategy to prevent the spread of bacteria within the mammary gland. Thus, it can be suggested that Urupema propolis extracts are a promising treatment of bovine mastitis caused by *S. aureus* and that the efficacy of such treatment is independent of the time of resin production.

Conflict of interest

The authors declare that there were no conflicts of interest.

Ethics Statement

This article does not contain any studies with human participants or animals.

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