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RESEARCH ARTICLE

Aloe barbadensis Miller leaf exudate is a potential treatment for bovine mastitis [version 1; referees: awaiting peer review]

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Abstract

Background: *Aloe barbadensis* Miller is a well-known phytotherapeutic, and parts of its leaves are used for a wide range of medicinal purposes. This study seeks to assess the *in vitro* antimicrobial and cytotoxic effects of leaf exudate (LE) from *A. barbadensis* leaves against *Staphylococcus aureus* and MAC-T bovine mammary epithelial cells.

Methods: Seasonal LE samples were collected, and the effect on total phenolic and aloin contents was determined. Antimicrobial activity of LE was evaluated using the broth microdilution method, and toxicity to MAC-T cells was determined by MTT assay.

Results: Samples collected during different seasons of the year showed a seasonal effect on the chemical profile of LE ($P < 0.05$). However, despite these chemical variations, we found no differences in antimicrobial activity against *S. aureus*. For all studied samples, the minimum inhibitory concentration (MIC) was 1,000 $\mu\text{g/ml}$. Furthermore, we found an elevated cytotoxic effect of LE on MAC-T cells with a significant reduction in cellular viability at 7.8 $\mu\text{g/ml}$ ($P < 0.05$) and an IC_{50} of 91.89 $\mu\text{g/ml}$.

Conclusions: Despite the antimicrobial effects of LE, the high toxicity for MAC-T cells suggests that it is unsuitable for intramammary use, but does have potential as a topical antimicrobial.

Keywords

phytotherapy, *Staphylococcus aureus*, MAC-T cells

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Introduction

Bovine mastitis, which is characterized by inflammation of the mammary gland, is the most frequent infection found in dairy herds worldwide¹. The treatment recommended for mastitis is the administration of intramammary antimicrobials. However, control of infections caused by *Staphylococcus aureus*, the principal etiological agent of bovine mastitis², is very difficult. In addition to inactivating several antimicrobials, this microorganism can also survive in the intracellular environment after phagocytosis. As a consequence, the cure rate of mastitis caused by *S. aureus* is low, with a high incidence of recurrence³. As such, interest in the search for methods of control and prevention has increased, including the identification of new antimicrobials^{4–7}.

In vivo methods are still commonly used to study bovine mastitis, but *in vitro* testing has been recommended⁸. Based on *in vitro* models, studies have produced a wide range of results, from identifying the prevalence of etiological agents of mastitis to evaluating the direct effects of products on the susceptibility of studied microorganisms^{9,10}. Among these, *in vitro* tests on antimicrobials are some of the most widely used⁸. *In vitro* studies with bovine mammary gland explants or mammary epithelial cells (MEC) are commonly used to assess the different functions of mammary glands, such as the response to initial infection^{11,12}. Recently, primary cultures of mammary explants and MECs were also suggested as adequate models in the search for new therapeutic agents^{5,13}. In the case of mastitis, such *in vitro* methods can help evaluate the toxicity of antimicrobials, enabling the determination of safe doses and minimizing the potential risks during *in vivo* validation.

Aloe vera (*Aloe barbadensis* Miller) is a plant widely used and recognized for its antimicrobial, anti-inflammatory, wound-healing, antitumor, and antioxidant pharmacological properties¹⁴. Yet, until now, few studies have reported on its potential as a treatment for bovine mastitis. Most research on the pharmaceutical potential of *Aloe vera* has studied the mucilaginous gel, commonly known as aloe vera gel, that is rich in complex carbohydrates, particularly **acemannans**^{15,16}. However, along with the gel, a yellow exudate with a strong odor and bitter taste, known as leaf exudate (LE), can also be extracted from the leaves¹⁷. Its release occurs as soon as the leaves are cut and it can be found within the phloem vessels^{18,19}. Despite being composed of large amounts of 1,8-dihydroxyanthraquinone derivatives and their glycosides, the industry that uses aloe vera gel as a raw material considers LE a residue. Among the anthraquinones found in LE, the major compounds are a mixture of two readily oxidizable diastereomers, **aloin A** and **aloin B**, which are sometimes undesirable because of their toxic and cathartic potential. However, these compounds may be of therapeutic interest in the control of antimicrobial and tumor cell proliferation^{20,21}.

Thus, the current study seeks to investigate the potential of LE from *Aloe vera* leaves in the control of bovine mastitis through *in vitro* models that evaluate the antimicrobial effects against *S. aureus* and cytotoxicity to MAC-T cells.

Methods

Sampling and extraction of leaf exudate

A total of 30 plant samples were collected from 3-year-old *Aloe vera* (*Aloe barbadensis*) at random from a commercial grower (Naturama Sucos Integrals do Brasil Ltda®; Paulo Lopes, SC, Brazil) in March, June, September and December of 2015, and one leaf was taken from the mid-position of each plant. Thus, 30 leaves in total were collected for each month, representing each season. Leaves were cut at the base and maintained vertically for 3 h in a beaker to collect the LE at room temperature. Subsequently, the LE was lyophilized and stored at **-20°C**. LEs of six plants were combined for a total of five repetitions for each season of the year.

Chemical profile of leaf exudate

Total phenolics. The total phenolic content was determined using the colorimetric method of Folin-Ciocalteu¹⁴ and an external standard curve of gallic acid (10–100 **µg/ml**) ($y = 0.0197x / r^2 = 0.987$). The results were expressed in **µg** of gallic acid equivalents (GAE)/mg of extract (**µg** of GAE/mg). All tests were performed in triplicate.

Aloin. The aloin content in LE was obtained on an UHPLC Thermo Scientific UltiMate 3000 RS Dual System (Thermo Fisher Scientific, San Jose, CA), using a Thermo Scientific C18 reverse-phase column (4.6 x 250 mm; 5 **µm**; 120Å (Acclaim™120, Thermo Scientific®) at 40°C, operating at 240, 260, 280 and 320 nm. The mobile phase was eluted at 1 **ml/min** flow rate, using a methanol/water (70/30, **v/v**) mixture²². The identification of aloin was based on a comparison of the chromatographic profile and retention time with the commercial standard (Sigma-Aldrich, St. Louis, MO, USA/ B6906). After the addition of the standard, samples were co-chromatographed to confirm identification of the compound. Aloin content was determined through an external standard curve of barbaloin ($y = 302.73x / r^2 = 0.9822$) and the result expressed in **µg** of aloin per mg of the sample (**µg/mg**).

Antimicrobial activity

Antimicrobial activity was evaluated using a broth microdilution method according to the Clinical and Laboratory Standards Institute Manual²³. We tested six different concentrations of LE (**4000 to 125 µg/ml**) against the standard strain of *S. aureus* ATCC 25923 (Collection of Reference Microorganisms on Health Surveillance, Fundação Oswaldo Cruz, Fiocruz, Brazil) and seven strains of mastitic milk isolates. Milk samples from cows were submitted to the California Mastitis Test (CMT). CMT-positive milk samples were plated on blood agar supplemented with 5% sterile ovine blood and incubated for **24–48h at 37°C**. Gram-positive, catalase-positive, and rabbit plasma coagulase-positive samples were biochemically confirmed as *Staphylococcus aureus*²⁴. Each strain was considered one repetition of the experiment with five replicates/repetition. As such, we conducted eight repetitions for each LE sample.

The minimum inhibitory concentration (MIC) was determined through visual analysis of turbidity after **24 hours** of incubation on plates at **37°C** in addition to spectrophotometric reading at

600 nm to determine the percentage of inhibition of bacterial growth, using a previously described method²³.

Because LE is an exudate with a yellow color that easily oxidizes to a dark coloration¹⁴, we confirmed the MIC through a colorimetric method. We added 50 μl of resazurin dye (100 $\mu\text{g/ml}$, Sigma-Aldrich, St. Louis, MO, USA) to each well after reading the plates by spectrophotometer (600 nm). The plates were left to incubate at 37°C for an additional 30 minutes²⁵.

Cytotoxicity of leaf exudate to MAC-T cells

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 cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with 100 U/ml of penicillin, 100 $\mu\text{g/ml}$ of streptomycin, 20% (V/V) heat-inactivated fetal bovine serum (FBS, Gibco, CA, USA), 4 mM L-glutamine (Synth), 4.5 g/L glucose (Sigma-Aldrich, St. Louis, MO, USA), 1 mM of sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), 1.5 g/L sodium bicarbonate (Vetec™ Sigma-Aldrich, St. Louis, MO, USA), 5 $\mu\text{g/ml}$ insulin (Sigma-Aldrich, St. Louis, MO, USA), and 1 $\mu\text{g/ml}$ hydrocortisone (Sigma –Aldrich, St. Louis, MO, USA) at 37°C and 5% CO₂ in a humidified incubator. We changed the medium every 48 h. After reaching confluence, the cells were treated with 0.25% trypsin with 1 mM EDTA (Gibco, CA, USA) to prepare the cellular suspension (10⁵ cells/ml). The suspension was transferred a 96-well microplate (100 μl /well), followed by incubation (24 h) in culture conditions for adherence. Subsequently, varying concentrations (2000, 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8 and 3.9 $\mu\text{g/ml}$) of LE from the summer samples were added for 24 h, and cytotoxicity was determined based on the MTT method (Sigma-Aldrich, St. Louis, MO, USA)²⁶. The formed formazan was dissolved with dimethyl sulfoxide (DMSO) to give a purple color with characteristic absorption at 540 nm. Intensity of purple color is directly proportional to the cell number, thus indicating cell viability. The experiments were performed in triplicate.

Statistical analysis

Data were expressed as the mean \pm standard deviation (SD), of at least three independent experiments. We analyzed the data using analysis of variance with a Tukey adjustment (GraphPad Prism 5.0). We considered the effects 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. The average accumulated precipitation (mm) in the study region was calculated using available data²⁷.

Results and discussion

Leaf exudate chemical profile

The total phenolic and aloin content of LE from the aloe vera leaves varied based on the season during which the samples were collected. The highest levels were found in the samples taken during the summer and the lowest levels from the spring

samples ($P < 0.05$). The accumulation of total phenolics in the summer LE seems to be associated with the climatic conditions during the collection period (Figure 1a, b). Precipitation indices were lower during the summer months (January to March)²⁷ (Figure 1a), possibly causing hydric stress in the plants. In the spring, the lower total phenolic content of LE coincides with a period of greatest precipitation that year. Aloe is a plant comprised of 96% water; thus, its chemical composition is heavily influenced by precipitation levels²⁸, as well as other factors, such as the period of flowering²⁹.

The husk of *Aloe* leaves has greater levels of total phenolics compared to the leaf interior and internal parenchyma. In the literature, these values range from 12.06 to 20.86 μg GAE/mg leaf, depending on the species¹⁴. Among the various phenolic compounds in the leaves of *Aloe*, anthraquinones are noteworthy, particularly aloin. Anthraquinones are free in phloem vessels directly below the leaf epidermis, and aloin, in particular, is distributed throughout the plant as part of its defense mechanism³⁰. In the present study, we found the highest levels of aloin in the summer samples and the lowest in the spring samples (Figure 1 b, c). These results are correlated with the total phenolic content found in the studied samples (Figure 1). Previous studies have also suggested the effect of seasonality on aloin content, and its synthesis is strongly influenced by precipitation levels. Dry periods have been correlated with greater content of aloin in the analysis of aloe vera leaves^{15,31,32}. However, other factors can influence aloin content of aloe vera leaves, including cultivation conditions, age, and plant health³³. For example, higher levels of barbaloin, isobarbaloin, and aloin in *Aloe* sp. plants were found during periods of the year with higher temperatures³².

Antimicrobial activity

Despite significant differences in the levels of total phenolics and aloin in the LE samples (Figure 1b, c), these levels did not influence antimicrobial activity against *S. aureus*. For all LE samples, the MIC was 1,000 $\mu\text{g/ml}$, as confirmed by resazurin oxidation. The lowest tested concentration of 500 $\mu\text{g/ml}$ was incapable of reducing bacterial growth to values greater than 80%. The effect of other concentrations between 500 and 1,000 $\mu\text{g/ml}$ was not included in the study (Figure 2).

The effectiveness of LE from aloe vera leaves as an antimicrobial agent has been demonstrated for a wide variety of Gram-positive and Gram-negative bacteria, including *S. aureus* and others^{34,35}. In the literature, the MIC of aloe vera extracts against *S. aureus* varies. Previous studies have shown lower (195 $\mu\text{g/ml}$), similar (1560 $\mu\text{g/ml}$), and higher (5,000 $\mu\text{g/ml}$) MIC values compared to those in the present study^{36–38}. This variation may be related to diverse factors, such as the *Aloe* species studied, the part of the aloe leaf used in the tests, and the type of extraction and resuspension vehicle used. In the current study, the LE samples were collected directly from the cut leaf without any type of posterior extraction of the compounds of interest. Some solvents are capable of extracting certain compounds that may possess greater antimicrobial activity than others³⁹; however, resuspension in water may be the easiest way to use aloe vera leaf subproducts, making it accessible, even to the producer.

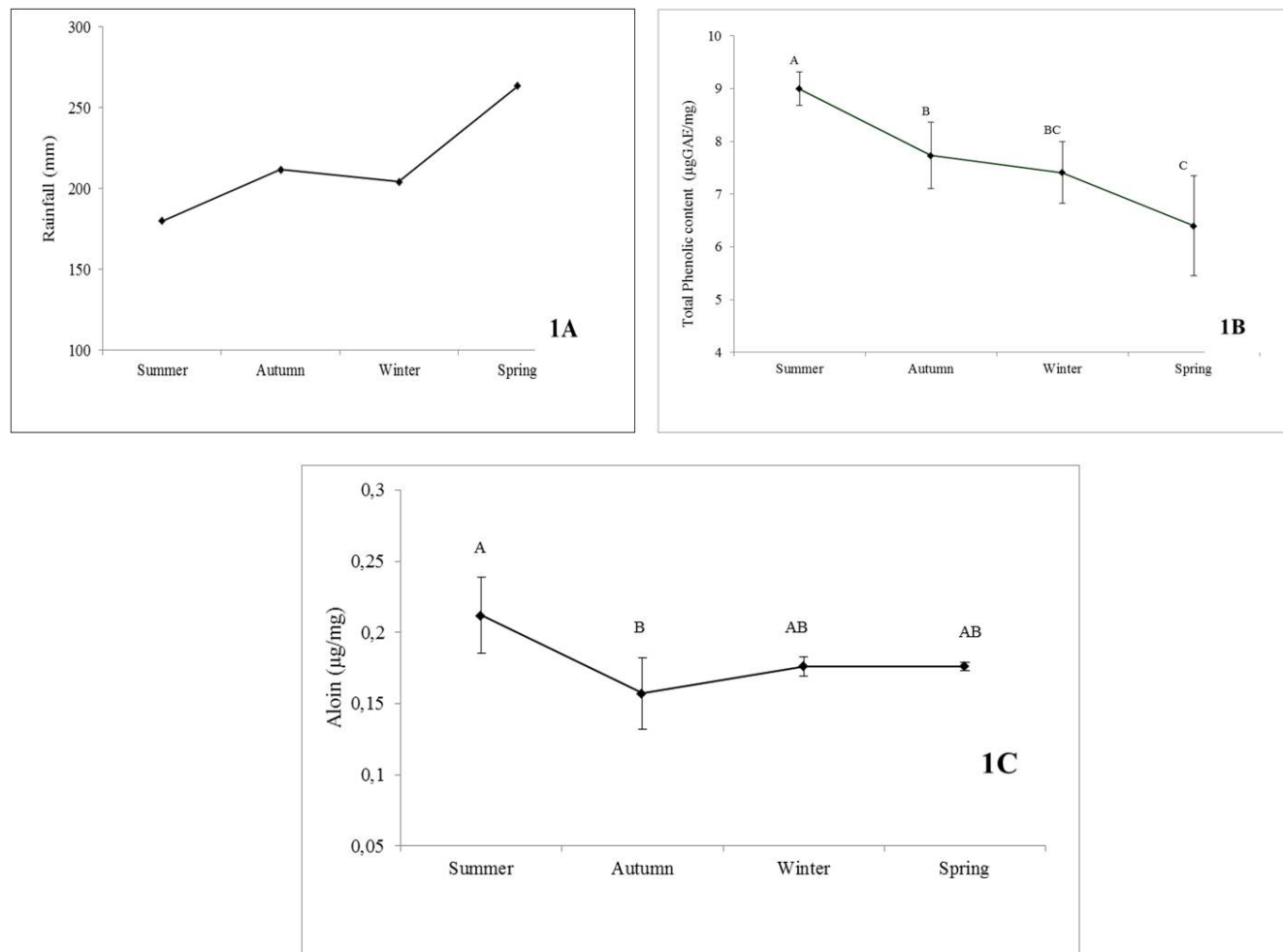


Figure 1. Seasonal differences in rainfall, phenolic content and aloin content. (A) Average accumulated precipitation (mm) in the study region (Paulo Lopes, SC, Brazil) during 2015; Source: INMET²⁶. (B) Total phenolic content (µg GAE/mg) of Aloe vera leaf LE from different seasons of the year (average of five repetitions \pm SD) ($P < 0.05$). (C) Average content (µg/mg) of aloin (average of three independent injections \pm SD) in samples of LE collected from Aloe vera leaves during different seasons of the year. Data points with the same letter above them are not significantly different from each other ($P < 0.05$ indicates a significant difference).

An interesting aspect to consider in the present study is that the concentration of total phenolics and aloin in the LE samples does not seem to affect antimicrobial activity. By contrast³⁶, the antimicrobial and anti-inflammatory activity of aloe vera LE has been associated with the concentration of phenolic and aloin compounds, suggesting that older leaves have higher levels of these compounds, and as such, have greater biological activity and defense against microorganisms and herbivores.

For Fabry *et al.*⁴⁰, the potentially useful activity defined for crude plant extracts with organic solvents is considered good when MIC values are < 8000 µg/ml, while Gibbons⁴¹ suggests that phytochemical isolates must have MIC values $< 1,000$ µg/ml. As such, the antimicrobial action of the LE samples in the present study can be considered good, even though neither extraction nor isolation of the principal components took place.

Cytotoxicity of leaf exudate

The LE showed high toxicity to MAC-T lineage cells, causing significant reduction in cellular viability at concentrations greater than 7.8 µg/ml (Figure 3). At higher concentrations, such as 500 µg/ml, the reduction in the percentage of viable cells was greater than 80%. The IC_{50} was 91.89 µg/ml. It is worth noting that the MIC of *S. aureus* growth was 1,000 µg/ml (Figure 2), a concentration that had a strong effect on the viability of mammary epithelial cells (Figure 3). This result is significant because it suggests that caution must be exercised when considering the intramammary use of LE in order to avoid inflammation, owing to the death of epithelial cells.

In an *in vivo* situation, the administration of a toxic product to bovine mammary glands can result in the development of inflammation⁴², which is more severe than that caused by the

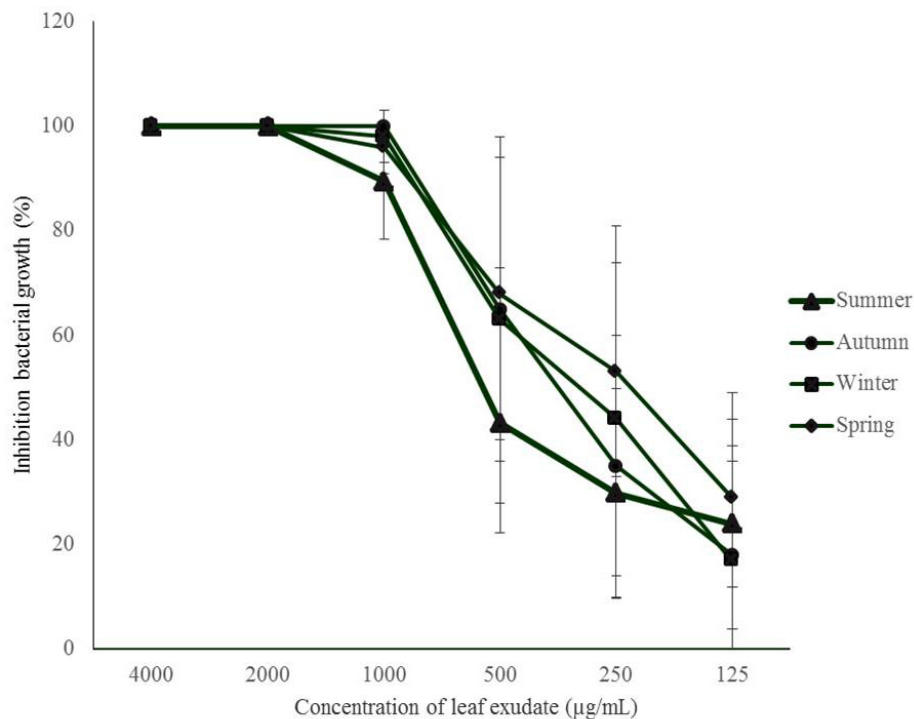


Figure 2. Percentage of inhibition (mean \pm SD) of the standard strain of *S. aureus* ATCC (25923) and seven strains of mastitic milk isolates by different concentrations of LE samples taken during different seasons of 2015.

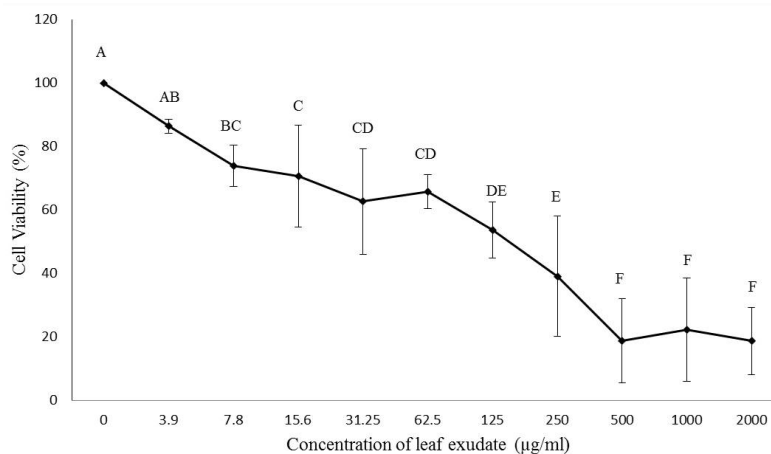


Figure 3. Percent of cellular viability (mean \pm SD) of the MAC-T lineage after exposure to different concentrations of LE samples in the summer of 2015. Data shown are an average of three independent experiments. Data points with the same letter above them are not significantly different from each other ($P < 0.05$ indicates a significant difference).

infection of pathogens⁴². In these cases, the attempt to combat inflammation leads to the formation of connective tissue at the affected site, which can diminish the alveolar area responsible for the synthesis of milk and, consequently, reduce milk production. In more severe cases, the loss of complete mammary glandular function, or even death of the animal, can occur^{43,44}.

The MAC-T cellular lineage⁴⁵ is an established model that has been frequently used in the investigation of mammary glandular functions and mediators of inflammatory processes⁴⁶.

Nonetheless, studies reporting on the effects of *Aloe* sp. extract, or fractions on this type of cell, are scarce. The toxic effects of aloe vera LE on other types of cells are discussed in the literature and have been associated with the presence of aloin and aloe emodin⁴⁷. These anthraquinones induce the apoptosis of cells caused by a reduction in the proportion of cells in the mitotic phase⁴⁸. Another hypothesis is that disruptions to the cell cycle and cellular differentiation, stimulation of the immune system, and antioxidant activity also have an anti-proliferative effect⁴⁹.

While the results found for MAC-T cells show that aloe vera LE has a high toxic potential for bovine mammary glands, the topical use of this product on the external area of the udder, for example pre- and post-dipping, or on instruments used during the management of milking, can be recommended. In this case, its potential as a disinfectant should be investigated.

Furthermore, it is important to highlight that the compounds present in the *Aloe vera* LE liquid oxidize easily in the presence of light, oxygen, and at room temperature⁵⁰. As such, very high concentrations are required in order to achieve antimicrobial efficacy against *S. aureus*, concentrations that would be toxic to mammary epithelial cells. Thus, we suggest the standardization of a methodology that can preserve and conserve these oxidative compounds, such as nanoencapsulation, which can maintain the desired antimicrobial activity and diminish the toxic effects.

Dataset 1. Raw data concerning the phytochemical characteristics of leaf exudate and its antimicrobial/cytotoxicity activity

<https://dx.doi.org/10.5256/f1000research.15671.d213901>

Conclusion

Although seasonality interferes with the chemical composition of aloe vera LE, the seasonal samples we evaluated did not

differ in relation to antimicrobial activity with a MIC of 1,000 ($\mu\text{g/ml}$) found for all samples. At this concentration, aloe vera LE shows strong toxic effects on bovine mammary epithelial cells of the MAC-T lineage. Despite the demonstrated antimicrobial activity of aloe vera LE, we suggest caution in recommending its intramammary use to treat bovine mastitis; instead, the topical use of this product on an external area, such as the udder, may be both efficacious and safe.

Data availability

Dataset 1. Raw data concerning the phytochemical characteristics of leaf exudate and its antimicrobial/cytotoxicity activity. DOI: <https://doi.org/10.5256/f1000research.15671.d213901>⁵¹.

Competing interests

No competing interests were disclosed.

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