Tannin-rich Tree Bark Extracts Inhibit the Development of Bacteria Associated with Bovine Mastitis

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Condensed tannins were quantified in the barks of selected tree species, and the antibacterial activity of these substances against clinical and subclinical isolates of bovine mastitis were evaluated. Tree barks from *Mimosa tenuiflora*, *Mimosa caesalpiniifolia*, *Anacardium occidentale*, and *Stryphnodendron adstringens* were used, as well as commercial tannin from *Acacia mearnsii*. The tannins were extracted using a mixture of ethyl alcohol and acetone. The moisture content (MC), Stiasny index (SI), total solids content (TSC), and condensed tannins (CT) were measured. The evaluation of antimicrobial activity was determined by applying the disk diffusion test. The species that showed the highest CT were *M. tenuiflora* and *A. occidentale*, with 37.3% and 37.3%, respectively. The highest SI were obtained by the species *A. occidentale* and *M. caesalpiniifolia*, with values above 90%. The bacterium with the lowest resistance to the use of tannins was *Streptococcus uberis*. *A. mearnsii* was the only one capable of inhibiting the growth of *Escherichia coli*, with a mean inhibition halo of 9 mm. All species under study showed high values of condensed tannins in their barks. In addition to showing good performance as antimicrobial agents, these tannins indicated potential applications in the development of natural medicines for the treatment of bovine mastitis.

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INTRODUCTION

Bovine mastitis is considered one of the main health problems in animals destined for milk production worldwide and it is responsible for triggering economic losses on dairy farms, leading to a decrease in milk production, an increase in veterinary care, and in slaughter and death rates of animals (Cheng and Han 2020; Pedersen *et al*. 2021). This disease is characterized by inflammation of the mammary gland caused by pathogenic microorganisms, of which 90% are bacteria, specifically those of the coliform group and those of the *Streptococcus* and *Staphylococcus* genera (Cheng and Han 2020).

The excessive, and often uncontrolled, use of these substances, however, has led to the rapid emergence of resistant strains, thus becoming a public health problem that has drawn the attention of the World Health Organization (WHO), which recently launched the *OIE Strategy on Antimicrobial Resistance* program (WHO 2016).

Given this scenario, the search for therapeutic alternatives to reduce the use of antimicrobials in dairy production, thus hindering the emergence of antimicrobial resistance, is a priority in veterinary medicine and in public health (Krömker and Leimbach 2017). Some alternatives have already been proposed for the treatment of bovine mastitis, such as the use of essential oils, lactic acid bacteria, substances of animal origin, such as propolis, and compounds derived from plants (Cheng and Han 2020; Lopes *et al*. 2020). In this context, plant-derived tannins stand out above the rest.

Tannins are polyphenols that belong to the group of secondary metabolites, chemically classified as hydrolysable and condensed, and are widely found in wood, bark, leaves, and fruits of several plant species, mainly of the woody type (Pizzi 2019). Tannic substances are presented as an option for the manufacture of phytopharmaceuticals, due to their antioxidant, antiviral, antitumor, antifungal, and antibacterial properties (Freitas *et al.* 2018; Pizzi 2019; Ucella-Filho *et al.* 2022).

Currently, the species *Stryphnodendron adstringens* stands out because of the use of tannins from its bark in the manufacture of herbal remedies. The discovery of new species is needed because it may lead to more effective alternate treatment for several diseases, including pathologies in domestic animals. Thus, it is important to increase the investigation of new antimicrobial agents of natural origin—herbal medicines and with proven efficacy—as therapeutic options for the treatment of animals infected with microorganisms that cause bovine mastitis.

Therefore, this research aims to: i) quantify the concentration of condensed tannins in the bark of forest tree species, and ii) determine the antibacterial properties of tannins against clinical and subclinical isolates of bovine mastitis.

EXPERIMENTAL

Collection and Preparation of Tree Bark

Tannin samples were collected from the barks of four individual tree species commonly found in dry forests in Brazil: *Mimosa tenuiflora* (Willd.), *Mimosa caesalpiniifolia* Benth., *Anacardium occidentale* L., and *Stryphnodendron adstringens* (Mart.) Coville. On average, these trees have a diameter at breast height (DBH) of 13.8, 10.2, 44.8, and 15.9 cm, respectively. The commercial tannin of the *Acacia mearnsii* Wild. was also used as a control. After collection, the barks were dried in a solar oven and then subjected to grinding in a Wiley-type mill. The fraction obtained between the 35- and 60-mesh sieve (0.25 mm) was used.

Determination of the Moisture Content of the Bark, Total Phenols, Extraction, and Quantification of Tannins

To determine the moisture content (MC), approximately 2 g of bark of each species were weighed and kept in an oven with air circulation at 105 °C ± 3 °C for 24 h. After this period, the dry weight was determined, and the MC was estimated (Eq. 1),

$$\mathrm{MC} = \left(\frac{\text{Dry weight}}{\text{Wet weight}}\right) \times 100 \tag{1}$$

where *MC* is moisture content (%).

The Folin-Ciocalteu method was employed to quantify the concentration of total phenols (Singleton and Rossi 1965). An aliquot of 100 µL of the bark extract was mixed with 4 mL of the Folin-Ciocalteu reagent; and after 6 min, 4 mL of a 7% Na_2CO_3 solution were added. After 15 min of incubation in a 45 °C bath, the absorbance at 760 nm was read *versus* a prepared blank. A calibration curve was constructed using gallic acid as a standard (0 to 150 g/mL). The total phenolic content was expressed in milligrams of gallic acid equivalent (mg GAE)/g of dried bark extract.

For the extraction of tannins, 10 g of the skins or barks of each species was mixed with 100 mL of a solution composed of ethanol and acetone (80:20 V/V) in a glass beaker. This was left to condition for 24 h in room temperature. After the extraction, the material was vacuum filtered with a porosity-2 sintered glass funnel. To study the antimicrobial action, the extracted liquid was taken in a 250-mL glass flask and concentrated in a rotary evaporator at 60 °C. Finally, the liquid was frozen and lyophilized to obtain powdered tannins. The quantification of the total solids content (TSC) of condensed tannins (CT) and Stiasny index (SI) was performed following the methodology proposed by Sartori *et al.* (2018).

To determine the TSC, three aliquots of 20 mL of each extract were placed in an oven at 103 ± 2 °C for 48 h and then weighed on a precision scale to obtain the final mass value (Eq. 2),

$$\text{TSC (\%)}=\frac{M1\text{ - }M2}{M2}\times 100 \tag{2}$$

where *TSC* is the total solids content (%), *M1* is the starting mass, and *M2* is the final dough (g).

The TSC and MC values were not recorded for the species *A. mearnsii* because the commercial tannin of the species was used in the work. The Stiasny index was used, according to Guangcheng *et al.* (1991), with some adaptations to determine the CT of each species. In addition, 20 mL of crude extract were used, and 10 mL of distilled water was added. Subsequently, 4 mL of formaldehyde (37% w/w) and 2 mL of HCl were added. Each mixture was boiled under reflux for 35 min. Under these conditions, the tannins formed insoluble complexes that could be separated by simple filtration. The extract was filtered using a sintered glass crucible of porosity-2 and dried in an oven at 103 ± 2 °C for 24 h, after which the Stiasny index was estimated (Eq. 3), followed by the condensed tannins (Eq. 4),

$$SI\text{ (\%)}=\left(\frac{M2}{M1}\right)\times 100 \tag{3}$$

where *SI* is the Stiasny index, *M1* is the mass (g) of solids in 20 mL of extract, and *M2* is the dry weight (g) of the precipitated material. The condensed tannins amount was defined as,

$$\text{CT (\%)}=\frac{\text{TSC} \times \text{S}}{100} \tag{4}$$

where *S* is the Stiasny index and *TSC* is the total solids content (%).

Determination of Antimicrobial Activity

Isolated clinical and subclinical strains of *Streptococcus agalactiae*, *Staphylococcus aureus*, *Streptococcus uberis*, coagulase-negative *Staphylococcus* spp., and *Escherichia coli* from the bacterial bank of the Laboratory of Bacteriology of Veterinary Medicine, located at the Federal University of Lavras – UFLA, were used in this study (Table 1). These microorganisms were selected due to their more frequent association with inflammation in the bovine mammary gland, triggering bovine mastitis (Cheng and Han 2020).

Table 1. List of Bacteria, Clinical, and Subclinical Isolates, Used in the Antimicrobial Test

Microorganism	Reference Number
Staphylococcus aureus	1080; 1112; 1128
Streptococcus uberis	24A; 42
Negative-coagulase *Staphylococcus* spp.	740; 772; 796
Escherichia coli	11R; 20PJ; 124
Streptococcus agalactiae	1072; 1097; 1026

These bacteria were activated by transferring aliquots of the stock cultures to Brain Heart Infusion Agar (BHI), with incubation at 37 °C, for 24 h. To standardize the inoculum, after cultivation, the cell mass obtained on the plates was collected and transferred to tubes containing saline solution (0.9% m/v). Standardization was performed using the 0.5 tube on the McFarland scale (1.5×10^8 CFU/mL).

The antibacterial action of the different tannins was evaluated using the agar diffusion technique (CLSI 2020), with adaptations. Streaks of standardized cultures were seeded in Petri dishes (150×15 mm^2) containing tryptone soy agar (TSA - Kasvi®, São José do Pinhais, PR, Brazil) for *S. aureus*, negative-coagulase *Staphylococcus* spp., and *E. coli* strains; and blood agar containing 5% sheep blood for strains of *S. uberis* and *S. agalactiae*.

The treatments, consisting of different tannic extracts, were prepared from lyophilized tannins, which were diluted in distilled water at a concentration of 25 mg/mL. Aliquots of 0.5 µL (dose equivalent to 5.0 mg of extracts) were dispensed into standardized wells (6.0 mm in diameter) drilled in the culture medium. The positive control was performed by deposition of ampicillin disk (10 mcg) in the middle of the plate. As a negative control, distilled water was used, where there was no formation of inhibition halo. The petri dishes were incubated at 37 °C for 24 h, and the inhibition halos formed were measured with the aid of a millimeter ruler. The plates were divided into 6 areas, one for the negative control (distilled water) and five for the tannin-rich bark extracts under study.

Statistical Analysis

A completely randomized design (CRD) was applied to verify the efficiency of tannin-rich extracts as antimicrobial agents and to determine which one presented the best result among the different species of bacteria under study. Residual normality was determined by the Shapiro-Wilk test, and the homogeneity of variance was determined by Bartlett, both at 95% probability.

After confirming normality and homogeneity, analysis of variance (ANOVA) was performed and, when different from each other, they were subjected to Tukey's test, at 5% probability value. Data were analyzed using the statistical software R (The R Foundation for Statistical Computing, version 4.2.1, Vienna, Austria).

RESULTS AND DISCUSSION

Characterization of the Tannin-Rich Extract

Table 2 shows the values of SI, CT, TSC, total phenols (TF) present in the tannin-rich extracts, as well as the moisture content (MC) of the bark. For TSC, the highest concentration was found for the species *M. tenuiflora* (2.67%), whereas the species *M. caesalpiniifolia* was the one with the lowest concentration (1.04%). The concentration of condensed tannins in the bark of the species under study was higher for *M. tenuiflora* and *A. occidentale* (37.31% and 37.27%, respectively). The species *S. adstringens*, *A. mearnsii*, and *M. caesalpiniifolia* afforded a CT value ranging from 18% to 22%.

Table 2. Moisture Content, Condensed Tannins, Total Solids Content, Stiasny Index, and Total Phenols of Tannin-Rich Extracts

	Parameters				
Species	**TSC (%)**	**SI (%)**	**CT (%)**	**MC (%)**	**TF (mg GAE/g)**
Mimosa tenuiflora	2.67	65.84	37.31	12.77	141.21
Mimosa caesalpiniifolia	1.04	93.17	18.99	10.66	77.11
Anacardium occidentale	1.96	94.88	37.27	16.43	94.96
Stryphnodendron adstringens	1.6	89.47	22.13	14.25	93.41
Acacia mearnsii	-	85.68	21.42	-	-

The determination of the content of CT (components of easy extraction when compared with hydrolysable tannins and with greater commercial interest) is important to indicate the potential of the woody species regarding the application of its extracts in different industrial sectors. According to Paes *et al.* (2006), for a species to be considered as a potential tannin producer, its CT value must be greater than 11%. Based on this statement, all evaluated species presented values that indicate their potential for commercial exploitation, with CT percentages above 18%.

The TSC parameter, which informs the amount of total extractives present in the sample, was considerably lower than those found in the literature (Sartori *et al.* 2018). These positive variations in the concentration of tannins and the low levels of TSC may be related to the extraction method; while other studies used boiling water, the authors used an ethanol-acetone mixture. The use of organic solvents, such as ethanol and acetone, favors the extraction of material with less solid residues and higher yields of tannins (Naima *et al.* 2015).

The percentages for SI were higher for the species *M. caesalpiniifolia* (93%) and *A. occidentale* (94%). The Stiasny index provides an estimate of the amount of total polyphenols in the extracts from the reaction with formaldehyde, an important criterion to determine the quality of tannic extracts (Guangcheng *et al.* 1991). Ideally—for application in different industrial segments, such as pharmaceuticals—the extracts should have a higher SI value, because this parameter is directly linked to the purity of the material and, consequently, to the absence of other undesirable substances, such as sugars and gums. Regarding the concentration of TF present in the bark extract, *Mimosa tenuiflora* was the only species that presented a value greater than 100 mg GAE/g.

These variations between the values for the different parameters used in this study can be justified by the difference between the tree species, bark collection period, solvents used in the extraction method, and soil and climatic conditions of the trees' original environment (Naima *et al.* 2015; Souza *et al.* 2021).

Antimicrobial Activity

Among the bacteria tested, *S. uberis* was the most sensitive to the tannin-rich extracts studied, with an average inhibition halo around 12 mm, followed by *S. aureus* (10.3 mm) and negative-coagulase *Staphylococcus* spp. (10 mm), in which no significant difference was observed between the sensitivity of bacteria to the use of extracts, and the *S. agalactiae* (8.3 mm) was the least sensitive among them. *E. coli* was the most resistant species among all those studied, being inhibited only by tannic substances extracted from the bark of the species *A. mearnsii*, with an average inhibition halo around 9 mm (Fig.1). The greatest resistance of *E. coli* strains to antimicrobials can be observed for the antibiotic used as a positive control, ampicillin, in which the average inhibition halo was 5.33 ± 0.44 mm. As for *S. uberis*, *S. aureus*, negative-coagulase *Staphylococcus* spp., and *S. agalactiae*, their average inhibition halo were respectively, 32, 29.33 ± 1.15, 27.33 ± 2.3, and 21.33 ± 5.77 mm.

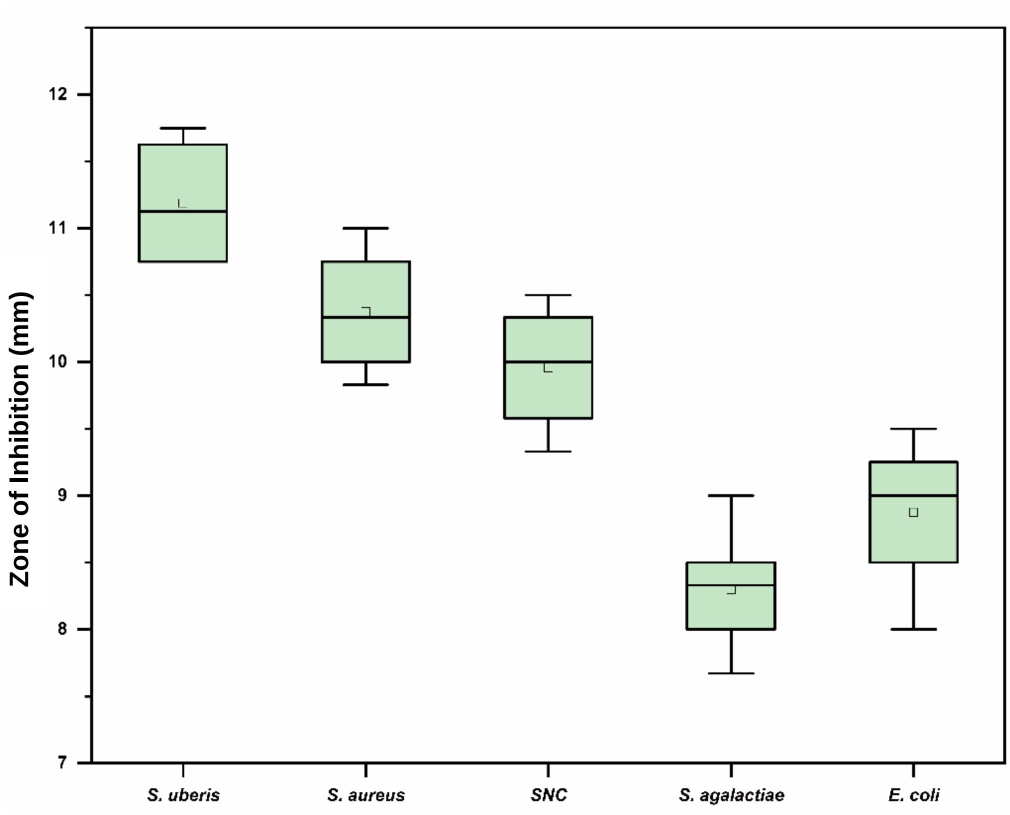

Fig. 1. Efficiency of tannins against bacteria isolated from bovine mastitis

The evaluated tannins showed good efficiency as antimicrobial agents against bacteria associated with bovine mastitis. This is because these substances present mechanisms that favor the interaction with microorganisms, inhibiting, in most cases, their development. Such actions can be justified due to tannins reacting with proteins irreversibly, complexing themselves within bacterial membranes, becoming a bactericidal agent (Pizzi 2019). They also function as chelating molecules, because metal, especially iron, is necessary for microbial growth (Lorenço *et al.* 2021).

Figure 2 shows the results of the antimicrobial action of different extracts rich in tannins on the studied bacteria. For *S. agalactiae*, the mean diameters of the halos for this strain showed no significant difference, with an average of 8.3 mm. For *S. aureus*, the extracts of *A. mearnsii* and *M. tenuiflora* showed better results when compared with the others; they presented a mean inhibition halo of 11.2 and 12.6 mm, respectively. The *A. mearnsii* extract showed significantly higher inhibitory activity against both negative-coagulase *Staphylococcus* spp. and *S. uberis* when compared with *S. adstringens*, *M. tenuiflora*, *M. caesalpiniifolia*, and *A. occidentale*. The inhibitory halo promoted by *A. mearnsii* on *S. uberis* was 14.5 mm, approximately 3 mm higher than the other species.

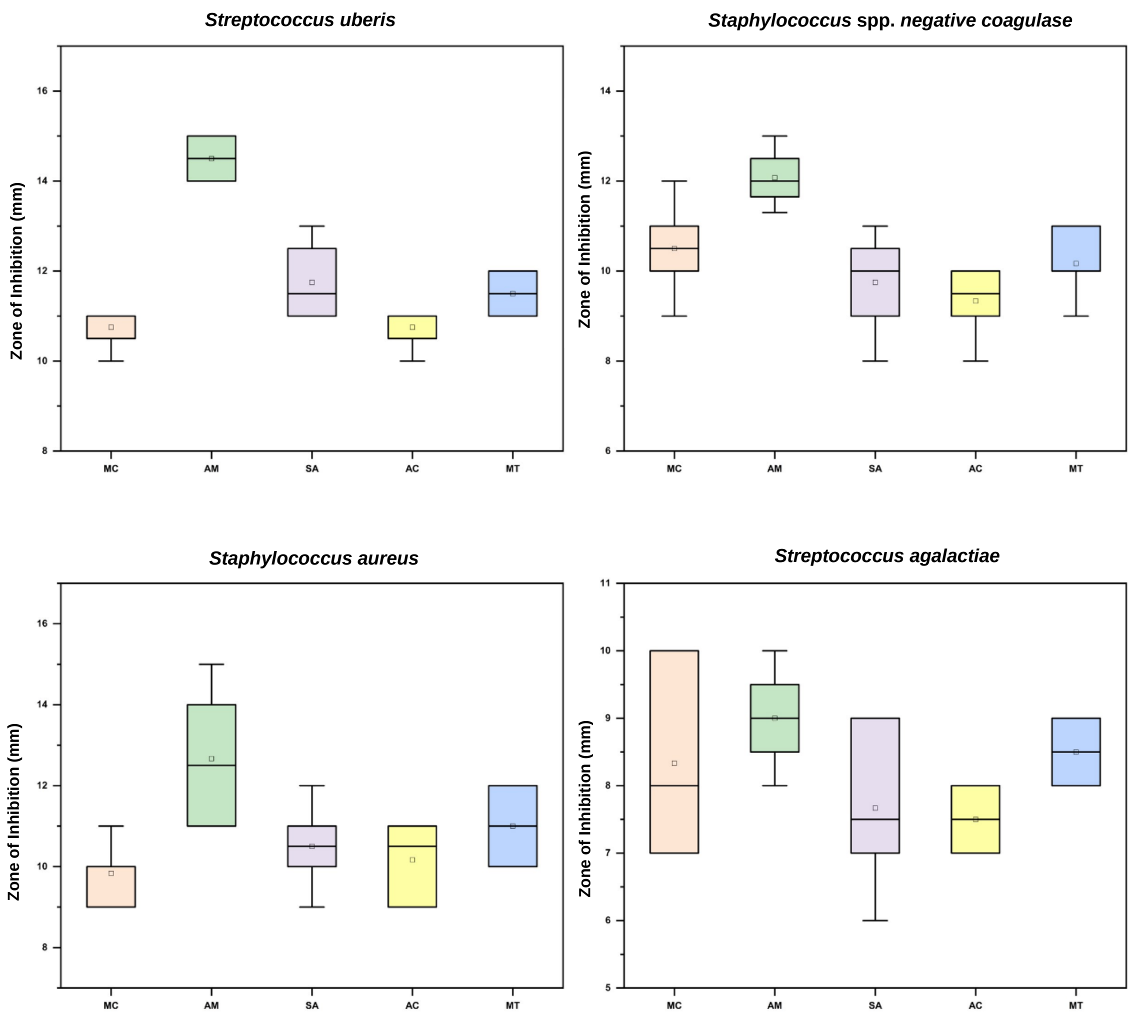

Fig. 2. Comparison of the antimicrobial activities of tannins against species of bacteria associated with bovine mastitis; where: MC = *M. caesalpiniifolia*; MT= *M. tenuiflora*; AM = *A. mearnsii*; AC = *A. occidentale*; AS = *S. adstringens*

The tannin-rich extracts of *A. mearnsii* stood out from the other samples in the study, being the only extracts capable of inhibiting *E. coli* development. *E. coli*, a Gram-negative bacteria, have become resistant to antibiotics designed to treat diseases caused by it, such that it is considered one of the main veterinary problems today (WHO 2016; Poirel *et al.* 2018). According to Molino *et al.* (2020), Gram-positive bacteria are more sensitive to tannin molecules. Therefore, this greater sensitivity may explain why bacteria of the

Staphylococcus and *Streptococcus* genera were more sensitive to tannins from the bark of the plants evaluated in this study.

Antimicrobial resistance is a key factor in the establishment and spread of bacterial clones in a herd. Thus, the tannin-rich extracts of *S. adstringens*, *M. tenuiflora*, *M. caesalpiniifolia*, *A. occidentale*, and *A. mearnsii* are potential substances capable of controlling infections caused by *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus uberis*, the last two being considered the main pathogens found most frequently in dairy herds worldwide.

The tannins present in the bark of these species, in addition to being antimicrobial (as verified in this research), can still act as antioxidant agents because of the presence of hydroxyl groups (OH) in their molecular structure (Tuyen *et al.* 2017; Pizzi 2019). The OHs can interact with free radicals, minimizing oxidative stress. Dairy cows, when they are in the transition phase from late pregnancy to early lactation, are susceptible to oxidative stress, which facilitates the emergence of infections, such as bovine mastitis (Abuelo *et al.* 2015). With this, tannins can help not only to control the disease, but also to prevent it.

CONCLUSIONS

The results found in this study are promising and important to serve as a basis for the development of new medicines for veterinary use, which can contribute to the use of compounds from natural and renewable sources with the high efficiency necessary for their medicinal application.

1. High values of condensed tannins were found in bark extracts of *S. adstringens*, *M. tenuiflora*, *M. caesalpiniifolia*, *A. occidentale*, and *A. mearnsii*.
2. The tannin-rich extracts from the bark of the investigated species proved to be excellent antimicrobial agents, capable of inhibiting the development of isolated strains of bovine mastitis, especially the tannin-rich extract of *A. mearnsii*.

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