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VETERINARY MICROBIOLOGY - RESEARCH PAPER

Salvinia auriculata: chemical profile and biological activity against *Staphylococcus aureus* isolated from bovine mastitis

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Abstract

The aquatic plant *Salvinia auriculata* has been shown to possess promising properties for the treatment of *Staphylococcus aureus* bovine mastitis. The disease affects cattle health and compromises dairy cattle productivity, resulting in reduced milk production and higher mortality rates. The aim of this study was to evaluate the antimicrobial activity, antibiofilm activity, and toxicity of *S. auriculata* root extracts using bovine mammary epithelial cells (MAC-T); determine the chemical composition of the most active extract; and develop an *S. auriculata* antiseptic solution for pre- and post-milking teat disinfection. Plants were collected during the four seasons of the year. The most active hexane extract was subjected to bioguided fractionation, which resulted in the isolation of six known compounds, stigmast-22-ene-3,6-dione, stigmasterol, friedelinol, β-sitosterol, octadecyl alcohol, and octadecanoic acid. The antimicrobial and antibiofilm activities of the most active extract and isolated compounds were determined against nine *S. aureus* strains isolated from cows with mastitis. The efficacy of the *S. auriculata* teat dip formulation was tested using an excised teat model (ex vivo), and promising results were obtained. The *S. auriculata* extract formulation proved to be as effective as commercial antimicrobials in reducing log counts in excised teats.

Keywords *Salvinia auriculata* · Antimicrobial activity · Antiseptic formulation · Stigmast-22-ene-3,6-dione · Phytosterol

Introduction

The dairy industry is one of the most important agribusiness sectors in the world, which is a result of the importance of milk and dairy products to the human diet [1]. A condition that contributes negatively to milk production is bovine mastitis. Inflammation of the mammary gland compromises cattle health and affects dairy cattle performance, resulting in reduced milk production and higher mortality rates. The high incidence and the economic losses associated with bovine mastitis, particularly because of the impact on milk production and quality, make it the most costly disease in dairy cattle [2].

Treatment of bovine mammary infections is based on the use of antibiotics, but some pathogens have become resistant to most currently available drugs, which limits treatment

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options. The emergence of resistant strains is a consequence of the abusive use of antibiotics. Nunes and co-workers [3] showed that Gram-positive bacterium *Staphylococcus aureus* isolated from Portuguese cattle exhibited high levels of resistance in vitro to penicillin. Machado and co-workers [4] also reported high levels of resistance to several antibiotics, including penicillin (93.5%), sulphonamide (88.9%), novobiocin (88.6%), and ampicillin (85.3%), in coagulase-negative staphylococci isolated from cattle in different Brazilian regions. The rapid development of resistance to antibacterial therapy has motivated the search for new drugs capable of controlling bovine mastitis in an effective and safe manner.

Plant-based medicines have been increasingly recognised by the scientific community as an alternative to conventional drugs against pathogenic bacteria. In recent decades, the research on antimicrobial properties of plants has been intensified. Plants are regarded as potential sources of pharmacologically active compounds that might contribute to the discovery of novel antibiotics from either their pure constituents or active extracts [5]. Plants living in nutrient-rich environments with high bacterial counts attract the interest of researchers because of their putative antimicrobial properties [6–8]. Aquatic plants appear to be especially valuable as sources of new antimicrobial agents.

Salvinia auriculate which belongs to the family Salviniaceae is a very common aquatic plant in tropical environments. It has broad geographic distribution but does not occur in cold climate regions [9]. Native to South America, the species is commonly found in Mexico, the Greater Antilles, Trinidad and Tobago, Colombia, Venezuela, Ecuador, Peru, Bolivia, Paraguay, Argentina, Chile, and Brazil. The plant is used in bioreactors for the removal of aquatic pollutants, such as heavy metals and dyes [10]. Recently, *S. auriculata* have also aroused the interest of farmers, who have utilised the plant as cattle feed because of its high biomass production [11].

Our research group has been investigating the antimicrobial and antibiofilm effects of *S. auriculata* [12, 13]. In the present study, we aimed to verify the best time of the year to collect the roots of the *Salvinia auriculata* plant, to isolate the active compounds responsible for its biological activities, and to develop an antiseptic formulation of the most active extract to be used as a pre- and post-milking teat disinfectant in conventional and organic dairy farms.

Materials and methods

Plant material

S. auriculata plants were collected from a pond in Recanto das Cigarras (20° 45′ 27″ S 45° 51′ 46″ W), Federal University of Viçosa, Minas Gerais, Brazil, in March (autumn), June (winter), September (spring), and December (summer) 2013. An authenticated voucher (VIC 32.122) was deposited in the university herbarium.

Extraction of plant material

The material was exhaustively washed with water, and roots were separated and dried for 24 h at 40 °C in an air-circulating oven after which they were ground into a fine powder. Then, 200 g of root powder collected in each season was extracted for 3 days with *n*-hexane. This procedure was repeated at least five times. Extraction was performed under ultrasonic irradiation, which makes it possible to obtain higher extraction yields in shorter times [14]. Extracts were concentrated under reduced pressure and stored at 4 °C.

Microorganisms

The bacterial strains used in this study were isolated from cows with mastitis. Strains were kindly provided by the Embrapa Dairy Cattle Milk Microbiology Laboratory (Juiz de Fora, Minas Gerais, Brazil). Six *S. aureus* strains (3008, 3828, 4075, 4125, 4182, and 4347) and one *S. aureus* ATCC 29,213 were used to investigate the antimicrobial activity of extracts, fractions, and isolated compounds. Bacteria were routinely cultured on brain heart infusion (BHI) agar at 37 °C for 16 h prior to experiments. Cell concentration was adjusted to 10^6 colony-forming units (CFU) mL^{-1} (optical density of 600 nm). Stock cultures were maintained on BHI agar supplemented with 10% glycerol at – 80 °C.

Gas chromatography–mass spectrometry analysis of the spring and summer hexane extract of roots of *Salvinia auriculata.*

The chromatographic profile of extract was determined on a Shimadzu GCMS-QP5050A gas chromatograph–mass spectrometer. Gas chromatography-mass spectrometry (GC–MS) analysis was carried out on a QP2010 Ultra Shimadzu system, employing the following conditions: column fused silica capillary column Rtx-5MS (30 m; 0.25 mm ID; 0.25 film µm). Helium (99.999%) was used as a carrier gas at a constant flow of 1 mL/min, and an injection volume of 1.0 µL was employed (split ratio of 10:1) at an injector temperature of 290 °C and an ion source temperature of 200 °C. The oven temperature was programmed at 80 °C for 5 min, then increased to 285 °C at a 4 °C rate/min and kept at this temperature for 40 min. Mass spectra were taken at 70 eV, a scan interval of 0.5 s, and fragments from 35 to 700 Da. The MS transfer line temperature was 290 °C. Compounds were identified using the Wiley 7 and National Institute of Standards and Technology (NIST) libraries [15].

Bio-guided isolation of secondary metabolites

The bioactive spring hexane extract of roots 2 g (was subjected to column chromatography on silica gel (70–230 mesh) using hexane as mobile phase. Eluent polarity was gradually increased with CH_2Cl_2 to yield 58 fractions, which were subsequently submitted to biological assays against *S. aureus* strain 3828, used as a reference microorganism for bioguided isolation. Active fractions were collected and submitted to repeated purification steps by preparative thin-layer chromatography. Elution with an 8:2 mixture of petroleum ether/ethyl acetate allowed the isolation of compounds **1** (10 mg), **2** (15.0 mg), **3** (8 mg), **4** (12.0 mg), **5** (10.0 mg), and **6** (8.0 mg). Compounds were identified by comparison of their ^{1}H- and ^{13}C-NMR spectra with literature data [13, 16–18].

Hole-plate diffusion method (in vitro antibacterial assay)

The hole-plate diffusion assay was performed to evaluate the antibacterial activity of *S. auriculata* root extracts, fractions, and isolated compounds. Bacteria were cultivated overnight, and a 10^6 CFU mL^{-1} suspension was spread on Müeller–Hinton agar (HiMedia). Holes of approximately 5 × 3 mm were made in the agar and filled with 30 µL of extract (50 mg mL^{-1}), fractions (10 mg mL^{-1}), or compounds **1**, **2**, **3**, **4**, **5**, and **6** (10 µg mL^{-1}). Extract, fractions, and compounds were solved in DMSO. After incubation at 37 °C for 24 h, inhibition zones were measured in millimetres and compared with those of controls. Ciclopirox olamine (Uci-Farma) was used as a positive control because of its antibacterial properties [19]. Dimethyl sulfoxide (DMSO) was used as a negative control. Experiments were performed twice in triplicate.

Determination of minimum inhibitory concentration

The inhibitory activity of extracts and isolated compounds on bacterial growth was determined by the microdilution method [20]. Bacteria were plated onto BHI agar (HiMedia) and pre-incubated at 37 °C for 24 h. Isolated colonies were then inoculated into Müeller–Hinton broth (HiMedia) and incubated at 37 °C under shaking (180 rpm) until the exponential phase. The culture was subsequently diluted to an optical density of 0.5 on the McFarland scale (OD_{620} = 0.10). Microplate wells were filled with 100 µL of Müeller–Hinton broth containing extract concentrations of 0.01 to 10 mg mL^{-1} and 100 µL of a 10^6 CFU mL^{-1} bacterial suspension. A negative control was prepared by adding 100 µL of bacterial suspension and 100 µL of Müeller-Hinton broth containing the highest concentration of DMSO used in the extracts. After 24 h of incubation at 37 °C, 4 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and the plate was incubated for an additional 2 h at 37 °C. Changes in the colour of the medium from yellow to pink-violet were considered to indicate bacterial growth. Experiments were performed twice in triplicate.

Determination of biofilm inhibitory concentration

The biofilm inhibitory effect of isolated compounds 1, 4, and 6 was determined by the broth microdilution method. First, 100 µL of BHI broth supplemented with 0.25% glucose (BHIg) and 100 µL of a bacterial suspension adjusted to 0.5 McFarland turbidity were added to the wells of a 96-well microplate. The plate was incubated at 37 °C for 24 h. Immediately after, absorbance was read at 600 nm on an ELISA reader to confirm bacterial growth in the culture medium. The supernatant was carefully removed from the wells, and 200 µL of BHI broth containing the isolated compounds at 4 minimum inhibitory concentration (MIC), 2 MIC, MIC, ½ MIC, and ¼ MIC was added. The plate was incubated for further 6 h, the supernatant was withdrawn, and wells were washed three times with 200 µL of phosphate-buffered saline (PBS) pH 7.4. Subsequently, bacteria were stained with 200 µL of 0.1% crystal violet solution for 30 min. Excess crystal violet was removed by washing three times with 200 µL of distilled water. Finally, 150 µL of 95% ethanol was added to the wells. After 45 min, plates were shaken for 10 s and absorbance readings were taken at 560 nm. The negative control consisted of BHIg and bacterial suspension. The positive control was 10 µL of ciclopirox olamine, BHIg, and bacterial suspension. Biofilm inhibition was determined by comparing the optical density of test samples with that of the controls. Experiments were performed in triplicate.

Effect on disrupt preformed biofilms

The most active Spring hexane extract as well as isolated compounds 1, 4, and 6 and herbal formulation were evaluated for their ability to disrupt preformed biofilms according to the method described by Klein and co-workers [21], with some modifications. To a 96-well microplate were added 100 µL of BHI broth supplemented with 0.25% glucose and 100 µL of a bacterial suspension adjusted to a final concentration of 10^6 CFU mL^{-1}. The plate was incubated at 37 °C for 24 h, and absorbance was read at 600 nm on a microplate reader. The supernatant was carefully removed from the wells, and 200 µL of BHI broth containing herbal formulation or extract at different concentrations (2, 1, 1/2, 1/4, and 1/8 × MIC) was added. The positive control was 200 µL of BHI broth. The negative control was 0.1% Tween 80 and vehicle for experiments using extract and formulation,

respectively. Cefoperazone (0.12 mg mL^{-1}) was also tested for comparison. After 18 h of incubation at 37 °C, biofilms were quantified by the crystal violet assay [22]. The microplate was shaken for 10 s on a microplate reader, and absorbance was quantified at 560 nm. Biofilm disruption was determined by comparing the optical density of test and control wells. Experiments were performed in triplicate. Data were subjected to ANOVA, followed by Tukey's test ($p \leq 0.05$) for pairwise comparisons between treatments.

Cytotoxicity assay

The cytotoxic activity of the active spring hexane extract of roots was evaluated by determining cell viability by the MTT colourimetric assay. MAC-T cells, cultured at 37 °C in a 5% CO_2 humidified atmosphere, were used. Cells (3.5×10^4 cells $well^{-1}$) were distributed in 96-well microplates and incubated at 37 °C in a 5% CO_2 atmosphere for 12 h to allow cell adhesion, growth recovery, and cell layer formation. Subsequently, cells were treated with extract solutions at different concentrations and incubated again for 72 h. Cells were monitored daily by light microscopy. After 72 h, the medium was removed, cells were washed twice with PBS pH 7.2, and 50 µL of MTT solution (1.0 mg mL^{-1}) was added to each well. Plates were incubated at 37 °C in a 5% CO_2 humidified atmosphere for 4 h. Subsequently, the MTT solution was removed, and the salt formed was solubilised by the addition of DMSO (100 µL $well^{-1}$) and by stirring on a shaker for 15 min at 76 rpm and 37 °C. Microplates were incubated for 1 h at 37 °C in a 5% CO_2 humidified atmosphere, and absorbance was read at 492 nm.

Cell viability was calculated for each concentration tested using the following equation: %Viable cells = mean optical density of treated cells/mean optical density of the control × 100.

The 50% and 10% cytotoxic concentrations (CC_{50} and CC_{10}) correspond to extract concentrations at which optical density was reduced respectively to 50% and 10% of that of the control. CC_{50} and CC_{10} were calculated by linear regression analysis of the dose–response curve. We determined the CC_{10} for DMSO to ensure that the solvent concentration used did not interfere with the cytotoxic activity of extracts.

Antiseptic formulation for pre- and post-milking teat disinfection

An antiseptic pharmaceutical formulation for pre- and post-milking teat disinfection was prepared using active Spring hexane extract of roots of *S. auriculata* and the excipients propylene glycol, methocel, paraben solution, and glycerin (q.s.).

Determination of the antiseptic efficacy by the excised teat model

Teats were excised from slaughtered dairy cows (dairy cows that were used for human consumption). Excess skin and tissue were removed, teats were washed with detergent under hot water, rinsed with water, dried, and dipped in 70% ethanol. Samples with rough skin, chaps, or abrasions were discarded. Washed samples were placed in plastic bags containing water/glycerine (8:2) solution and stored at -80 °C for further use. The *S. aureus* strains 3828, 4125, and 4182 were used in these assays.

Frozen teats were thawed in warm water, dipped in 70% ethanol, dried with a paper towel, and suspended by metal clips. Teats were contaminated with 5 mL of a 10^6 CFU mL^{-1} bacterial suspension to a depth of approximately 15 mm and allowed to drain. After 5 min, teats were immersed in one of the following solutions: glycerine (control), test formulation, pre-milking disinfectant of known efficacy (Agrisept MC TABS, Intervet), and post-milking disinfectant of known efficacy (Lat Plus Qualimilk, Chemical Start). After 10 min, teats were rinsed with 5 mL of physiological saline to collect microorganisms, and 0.10 mL of this solution was plated on BHI agar. The negative control wash solution was diluted to 10^1 CFU mL^{-1} to facilitate counting. Wash solutions containing the test formulation or positive controls were diluted to 10^4 CFU mL^{-1} prior to plating to obtain plates with countable colonies. Plates were incubated at 37 °C for 24 h, and colonies were counted. The geometric mean of colonies was determined, multiplied by 50 and by the number of teats to correct for the total wash volume, and multiplied by the dilution factor to obtain the total CFU recovered from each teat. Total CFU counts were converted into log values, which were subtracted from the log value of the negative control to obtain the log reduction. In cases where no microorganisms were recovered from the teats, zero was replaced by 0.01 to allow the calculation of mean counts.

Statistical analysis

Each experiment was performed at least in three. The data were subjected to Tukey's test for comparison of means at $P \leq 0.05$.

Results

In vitro antibacterial assay

The antimicrobial activity of hexane extracts of roots of *S. auriculata* collected in the autumn, winter, spring, and summer were evaluated by antimicrobial activity and shown in Fig. 1. The Spring hexane extract of *S. auriculata* roots was the most active.

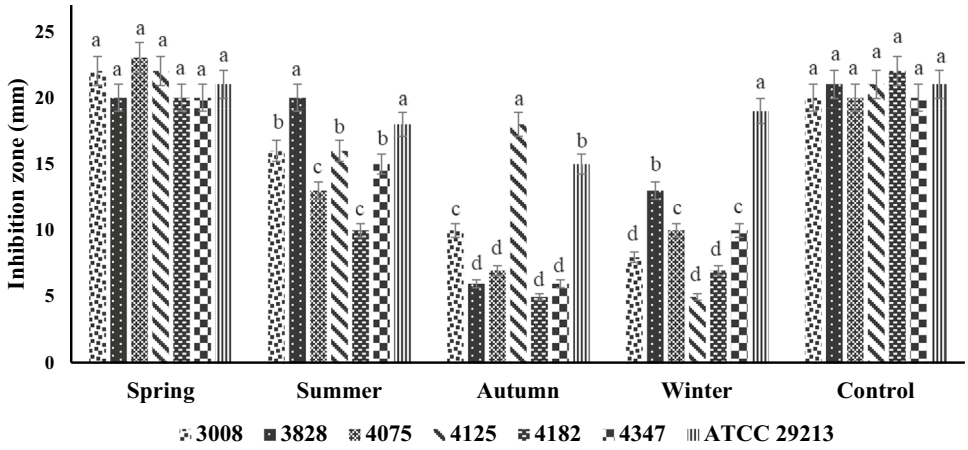

Fig. 1 Inhibition zones in mm of hexane extracts of *S. auriculata* roots collected in different seasons against bacteria *Staphylococcus aureus* strains (3008, 3828, 4075, 4125, 4182, 4347, ATCC 29,213) and positive control (ciclopirox olamine antibiotic). Different letters between two bars indicate significant differences at $P < 0.05$ by Tukey's test

Table 1 shows the minimum inhibitory concentration (MIC) values of hexane extracts of roots collected in the four seasons. The most active Spring hexane extract was evaluated for its ability to inhibit the formation of biofilms produced by *S. aureus* strains 3828, 4125, and 4182 (Fig. 2). These strains were chosen because they are strong biofilm producers. The Spring hexane extract of *S. auriculata* roots disrupted biofilms of all *S. aureus* isolates at MIC, 2 × MIC, and 4 × MIC showing reduction ranged from 50 to 95%

Bioguide isolation

In order to determine the compounds responsible for the biological activity of the most active Spring hexane extract, it was submitted the phytochemical analysis led to the isolation of stigmast-22-ene-3,6-dione (**1**), stigmasterol (**2**), friedelinol (**3**), β-sitosterol (**4**), octadecyl alcohol (**5**), and octadecanoic acid (**6**) (Fig. 3).

All the isolated compounds were tested against *S. aureus* strains. However, only compounds **1**, **4**, and **6** showed activity as shown in Fig. 4.

Table 1 Minimum inhibitory concentration (MIC) of hexane extracts of *Salvinia auriculata* roots collected in different seasons against *Staphylococcus aureus* strains

S. aureus strain	MIC (mg mL^{-1})				
	Season				Control*
	Spring	Summer	Autumn	Winter	
3008	0.04	0.8	2.50	0.5	0.05
3828	0.08	0.63	2.50	1.25	0.05
4075	0.04	1.25	2.50	1.25	0.05
4125	0.04	0.31	2.50	2.50	0.05
4182	0.04	0.73	2.50	1.67	0.05
4347	0.05	0.63	2.50	1.25	0.05
ATCC 29,213	0.08	0.70	0.70	0.07	0.05

* Ciclopirox olamine antibiotic

Figure 5 shows the reduction in preformed biofilm by compounds 1, 4, and 6. Compound 1 disrupted biofilms with reduction ranged from 80 to 100% at concentrations MIC, 2 × MIC and 4 × MIC. Compound 4 showed reduction ranged from 60 to 90% at concentrations 2 × MIC and 4 × MIC and compound 6 showed reduction ranged from 55 to 80% at concentrations 2 × MIC and 4 × MIC.

The Minimum inhibitory concentration (MIC) and biofilm inhibitory concentration (BIC) values of active compounds were determined. Compound **1** was as active as the control, and compound **4** was the second most active (Table 2).

GC–MS analysis

As the hexane extracts of roots collected in the spring and summer showed the highest activity against *S. aureus* strains, they were analysed for differences in secondary metabolite composition using GC–MS (Table 3).

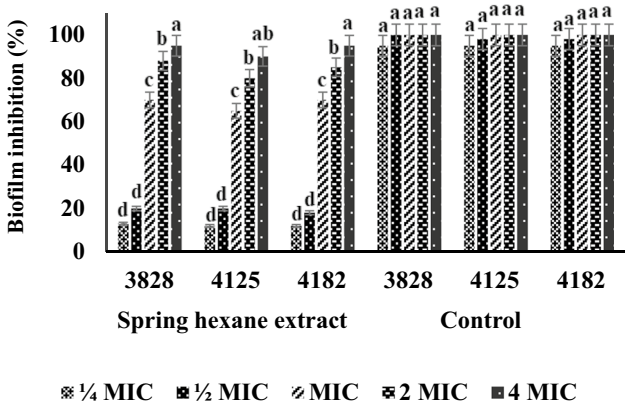

Fig. 2 Percent inhibition of Spring hexane extract of *S. auriculata* roots on the preformed biofilm of *S. aureus* strains. Control: ciclopirox olamine antibiotic. Different letters between two bars indicate significant differences at $P < 0.05$ by Tukey's test

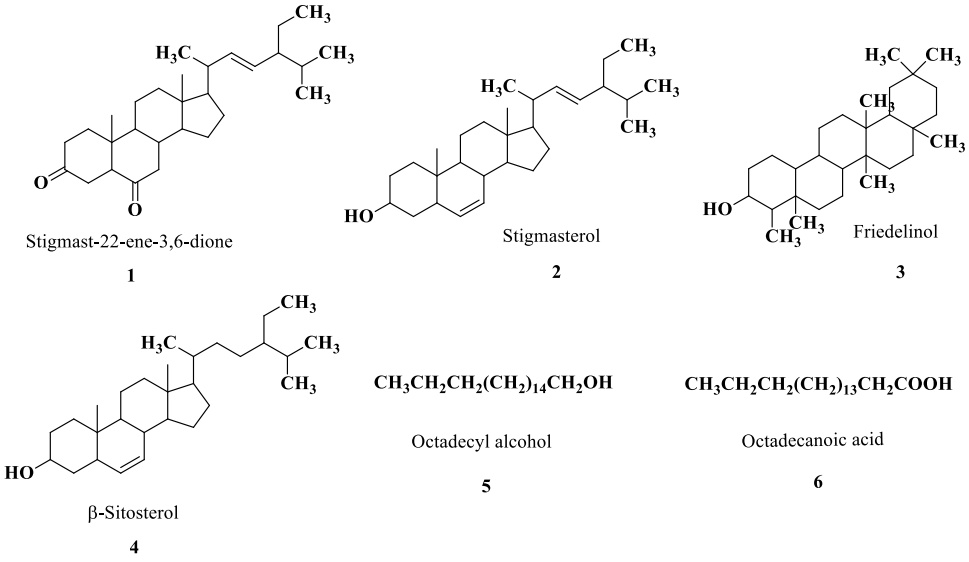

Fig. 3 Compounds isolated from the active fractions of Spring hexane extract of *Salvinia auriculata* roots

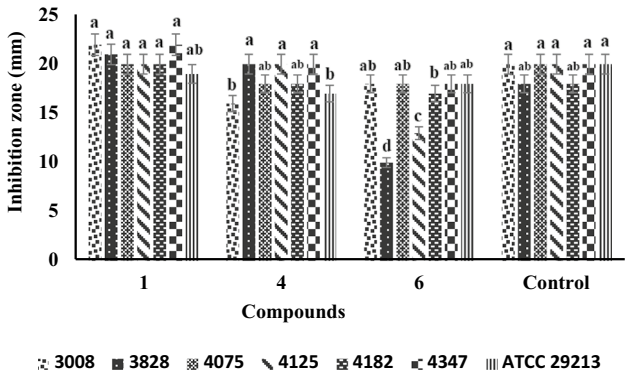

Fig. 4 Inhibition zones in mm of isolated compounds **1**, **4,** and **6** from Spring hexane extract of *S. auriculata* roots against bacteria *Staphylococcus aureus* strains (3008, 3828, 4075, 4125, 4182, 4347, ATCC 29,213) and positive control (ciclopirox olamine antibiotic) Different letters between two bars indicate significant differences at $P < 0.05$ by Tukey's test

Cytotoxicity assay of the most active extract

The cytotoxicity of the most active Spring hexane extract was evaluated towards MAC-T cells to assess its safety and possible use in the control of bovine mastitis in small or organic farms (Table 4).

Ex vivo *model assay*

The excised teat model (ex vivo) was used to evaluate the efficacy of the antiseptic formulation prepared using the most active Spring hexane extract (Table 5).

Figure 6 shows the reduction in preformed biofilm by the Spring hexane extract-based formulation. The formulation disrupted biofilms of all *S. aureus* isolates, with reduction ranged from 90 to 100% at the concentration MIC, 2 × MIC, and 4 × MIC.

Discussion

In the present study, the antimicrobial activity of *S. auriculata* roots collected in the autumn, winter, spring, and summer were assessed to determine the season in which roots have maximum antimicrobial activity and thus isolate the active compounds. The hexane extract of roots collected in the spring had the highest activity, producing inhibition zones of 18–23 mm, followed by the hexane extract of roots collected in the summer producing inhibition zones of 10–20 mm (Fig. 1). The hexane extracts that were obtained in autumn and winter showed low activity and compared to extracts obtained in spring and summer. A possible explanation is a decrease in the production of secondary metabolites during these seasons. The hexane Spring extract and hexane Summer extract of roots showed a pronounced activity against *S. aureus*, comparable to that of the control used (ciclopirox olamine antibiotic). This result corroborates the findings of Rossi and co-workers [12], who evaluated the antibacterial activity of *S. auriculata* leaf and root extracts using hexane, dichloromethane, ethyl acetate, and ethanol as extractive solvents. The authors reported that only the hexane extract of the roots showed antibacterial activity against all *S. aureus* strains tested.

The minimum inhibitory concentration of the extracts obtained in the four seasons is shown in Table 1. Hexane extracts of roots obtained in the spring (MIC of 0.04 to 0.08 mg mL^{-1}) were more active than hexane extracts of roots collected in the summer, autumn, and winter and

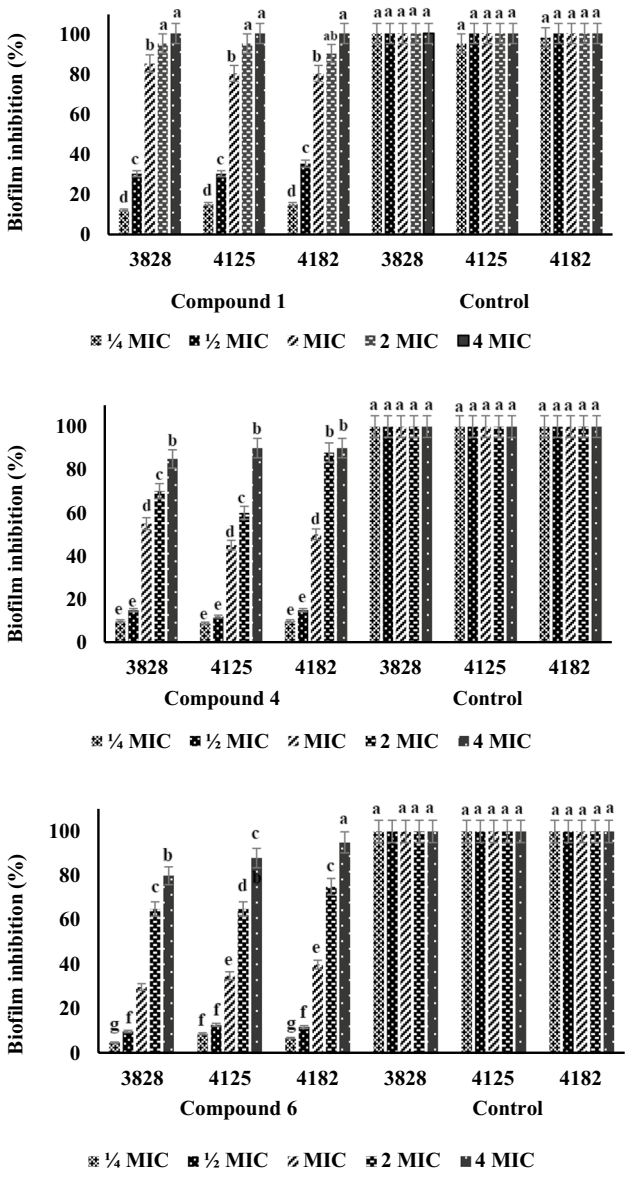

Fig. 5 Percent inhibition of compounds isolated from Spring hexane of *S. auriculata* roots on the preformed biofilm of *S. aureus* strains. Control: ciclopirox olamine antibiotic. Different letters between two bars indicate significant differences at $P < 0.05$ by Tukey's test

other plant extracts with antimicrobial activity reported in the literature [23–25]. As proposed by Aligiannis and coworkers [26], extracts can be classified by their MIC values as having strong (0.05 to 0.5 mg mL^{-1}), moderate (0.6 to 1.5 mg mL^{-1}), or weak (> 1.5 mg mL^{-1}) activities. According to this classification, the hexane extract from Spring has a strong antimicrobial activity.

Figure 2 shows the ability of the Spring hexane extract to inhibit the formation of biofilms produced by *S. aureus* strains. It was able to inhibit biofilm formation by the three strains tested by almost 82%, which shows its potential as a biofilm inhibitor. Bacterial biofilms are composed of communities of microorganisms and are often associated with chronic infections. Some antibiotics are not capable of penetrating the biofilm matrix and thus fail to eradicate the infection. The concomitant use of antimicrobial treatment and sanitisation is of strategic importance for the control and prevention of diseases such as bovine mastitis. This strategy can reduce or prevent microbial adhesion and destabilise the biofilm extracellular matrix [27].

Once it was identified that the most active extract was the one obtained in the spring, it was necessary to carry out a biomonitoring study of the extract in order to facilitate the isolation of the active compounds, as shown in Fig. 3. The compounds isolated were identified by comparing their spectral data with literature values [16, 23, 28–31]. Compounds **4**, **5**, and **6** were isolated from *S. auriculata* for the first time, whereas **1**, **2**, and **3** had been isolated previously [13]. All isolated compounds were tested against *S. aureus* strains. However, only compounds **1**, **4**, and **6** showed activity (Fig. 4). Compound **1** (stigmast-22-ene-3,6-dione) was the most active. It produced an inhibition zone of approximately 22.0 mm, which confirms its high antimicrobial activity against *S. aureus*. β-Sitosterol **4** and octadecanoic acid **6** also produced large inhibition zones (20.0 mm and 18 mm, respectively); these compounds are known to exhibit antimicrobial activity [32]. The results suggest that compounds stigmast-22-ene-3,6-dione **1** is responsible for the high activity of Spring hexane extract. β-Sitosterol **4** and octadecanoic acid **6** are also responsible for the antimicrobial activity of hexane extracts of *S. auriculata* roots in the

Table 2 MIC and BIC values of bioactive compounds isolated from Spring hexane extracts of *S. auriculata* roots against *Staphylococcus aureus* strains

Compound	MIC (mg mL^{-1})			BIC (mg mL^{-1})		
	3008	3828	4075	3008	3828	4075
Stigmast-22-ene-3,6-dione (**1**)	0.01	0.02	0.04	0.02	0.02	0.02
β-Sitosterol (**4**)	0.02	0.04	0.01	0.09	0.08	0.09
Octadecanoic acid (**6**)	0.08	0.10	0.10	0.50	0.30	0.50
Ciclopirox olamine antibiotic*	0.05	0.05	0.05	0.025	0.025	0.025

* Control

Table 3 Major compounds identified by GC/MS analysis of hexane extracts of *S. auriculata* roots collected in the spring and summer

Hexane extract of roots collected in the					
Spring			Summer		
RT (min)	Compound	Peak area (%)	RT (min)	Compound	Peak area (%)
12.32	**Octadecanoic acid**	**94**	**12.32**	**Octadecanoic acid**	**93**
12.77	Octadecyl alcohol	86		nf	
18.01	Campestenone	80	17.82	Campestenone	80
18.82	Campesterol	80	18.82	Campesterol	80
19.16	Stigmasterol	87	19.13	Stigmasterol	80
19.74	**β-Sitosterol**	**80**	**19.69**	**β-sitosterol**	**80**
19.88	Lupeol	86	19.85	Sitostanol	80
20.62	**Stigmast-22-ene-3,6-dione**	**78**		nf	
21.35	Stigmast-4-en-3-one	85	21.34	Stigmast-4-en-3-one	87
22.36	Lupanol	80	22.30	Lupanol	80
23.03	Stigmasterone	85		nf	
23.40	Stigmastane-3,6-dione	82	23.80	Stigmastane-3,6-dione	82

RT, retention time; *nf* not found

Table 4 Cytotoxic activity of the most active extract towards bovine mammary epithelial cells (MAC-T)

Cytotoxic concentration (μg mL^{-1})	
CC_{10} [a]	87.12
CC_{50} [b]	562.6

[a] Extract concentration that caused a 10% reduction in cell viability

[b] Extract concentration that caused a 50% reduction in cell viability

Table 5 Antimicrobial efficacy of the formulation obtained from most active Spring hexane extract of *S. auriculata* roots against *Staphylococcus aureus* strains isolated from cows with mastitis

Commercial antimicrobial and antiseptic formulation	*S. aureus* strains		
	3828	4125	4182
	Logarithm reduction*		
Agrisept® MC TABS, Intervet (Pre-milking)	4.17	4.15	4.19
Lat Plus Qualimilk, Start Química (Post-milking)	4.12	4.23	4.12
Antiseptic formulation produced with the extract Spring hexane extract	3.95	3.97	3.89

* Log count of the control = 8.30

two seasons. These compounds were also evaluated for their ability to inhibit the formation of biofilms produced by *S. aureus.* The results showed they were able to inhibit biofilm formation by the three strains tested by almost 92%, 81%, and 78% for compounds **1**, **4**, and **6**, respectively.

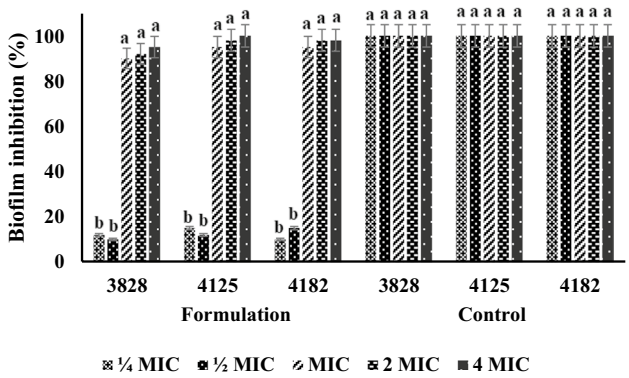

Fig. 6 Percent inhibition in the preformed biofilm of *Staphylococcus aureus* strains. by a formulation containing *S. auriculata* Spring hexane extract. Control: ciclopirox olamine antibiotic. Different letters between two bars indicate significant differences at $P < 0.05$ by Tukey's test

Corroborating the results obtained for the active Spring hexane extract which shows its potential as a biofilm inhibitor. Isolated compounds or plant extracts with potential antibiofilm activity can act by blocking bacterial cell adhesion to a surface or by disrupting bacterial cell communication [33]. Probably, this could be the effect attributed to the isolated compounds and the Spring hexane extract of *S. auriculata* roots (Fig. 5).

MIC is the lowest compound concentration that produced antibacterial effects, whereas BIC is the lowest compound concentration that inhibited biofilm formation. As shown in Table 5, compound **1** was as active as the control, and compound **4** was the second most active. Our results are consistent with the MIC [34, 35] and BIC [36, 37] values reported in the literature.

In order to compare the hexane extracts obtained in the Spring and Summer since both showed biological activity. They were submitted to GC–MS analysis. In general, the same compounds were found in both extracts, except for stigmast-22-ene-3,6-dione (**1**), which was found only in the Spring hexane extract. A plausible explanation found for the Spring hexane extract to be the most active is probably due to the presence of compound **1** in the extract, as it, in isolation, presented the highest activity (zone of inhibition from 18 to 23 mm) (Table 3).

Considering the interesting biological activity of the plant *S. auriculata* and its potential for use to treat bovine mastitis, we formulated a pre- and post-milking teat disinfection using the Spring hexane extract. For this purpose, the cytotoxicity of this active extract was evaluated towards MAC-T cells to assess its safety (Table 4). According to Dolabela [38], extracts, fractions, or substances can be classified by their cytotoxicity toward Vero cells (normal cells) as measured by the MTT assay: $CC_{50} < 100$ μg mL^{-1} indicates highly toxic substances; 100 μg $mL^{-1} < CC_{50} < 500$ μg mL^{-1}, moderately toxic substances; and $CC_{50} > 500$ μg mL^{-1}, slightly toxic substances. In the present study, the CC_{50} of the most active extract was 562.6 μg mL^{-1}. This result shows that hexane extracts can be considered safe for topical and intramammary use and that they would not cause inflammation as a result of epithelial cell death. Thus, this extract can be considered a safe herbal antiseptic. The in vitro cytotoxicity model using MAC-T cells is reliable and can be used to investigate functions of the mammary gland and mediators of inflammatory processes, especially when there is no literature data on the use of a specific plant or active compound for pre- and post-milking teat disinfection [39].

The efficacy of the antiseptic formulation was evaluated using excised teat model (ex vivo) and comparing it with commercial formulations as shown in Table 5. The formulation of an effective teat dip should achieve a 3- or, preferably, a 4- or 5-log reduction (LR) in bacterial count in excised teats [40]. The commercial antimicrobials Agrisept® and Lat Plus Qualimilk (for pre- and post-milking, respectively) caused a greater than 4.0-log reduction, whereas the antiseptic formulation caused a log reduction of approximately 4.0. This finding shows that the formulation was as effective as the commercial antimicrobials. The formulation was also evaluated for its ability to inhibit the formation of biofilms produced by *S. aureus* by inhibiting biofilm formation by the three strains tested by almost 96% (Fig. 6). This result of together with the ex vivo model test confirm the efficacy of the active spring hexane extract against *S. aureus* isolated from bovine mastitis.

Lima and co-workers [13] prepared antiseptic soap using active hexane extracts of *S. auriculata* roots and reported that the formulation inhibited microbial growth in vitro.

Conclusions

In the present study, *S. auriculata* root extracts showed high antimicrobial and antibiofilm activities, particularly Spring extracts. Antimicrobial activity was associated with the presence of three compounds isolated from the most active spring hexane extract: stigmast-22-ene-3,6-dione, β-sitosterol, and octadecanoic acid. In addition, the most active spring hexane extract showed low toxicity towards MAC-T cells, which suggests that it can be used to produce a safe teat dip for pre- and post-milking disinfection. Therefore, the formulation may prove to be a potential alternative for ecological livestock production systems because it does not pollute or degrade the environment.

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Author contribution G.A. Purgato, S. Lima, and J.V.P.B. Baeta performed the data acquisition and interpretation of data. M.A.N. Diaz, V.R. Pizziolo, G.N. Souza, and G. Diaz-Muñoz performed data analysis, interpretation of data and elaborated the manuscript. All authors performed a critical review and approved the final approval of the version to be published.

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Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Plant study This plant was registered in SisGen in compliance with Law no. 13,123/2015 and its regulations. Registration number A99795C.

Animal study The teat experiments were carried out with dairy cows used for human consumption and provided by a slaughterhouse certified by the Department of Inspection of Products of Animal Origin (DIPOA). We declare that no animals were killed to be used in this research.

Conflict of interest The authors declare no competing interests.

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