IMPACTO CIENTÍFICO EDITORA

IN VITRO EVALUATION OF STAR ANISE (ILLICIUM VERUM) AGAINST PATHOGENIC STRAINS OF STAPHYLOCOCCUS AUREUS ISOLATED FROM CLINICAL CASES OF BOVINE MASTITIS

AVALIAÇÃO IN VITRO DE ANIS ESTRELADO (ILLICIUM VERUM) FRENTE A CEPAS PATOGÊNICAS DE STAPHYLOCOCCUS AUREUS ISOLADAS DE CASOS CLÍNICOS DE MASTITE BOVINA

EVALUACIÓN IN VITRO DEL ANÍS ESTRELLADO (ILLICIUM VERUM) FRENTE A CEPAS PATÓGENAS DE STAPHYLOCOCCUS AUREUS AISLADAS DE CASOS CLÍNICOS DE MASTITIS BOVINA

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ABSTRACT

Bovine mastitis is a common and economically damaging disease in dairy farming. This mammary gland infection can impact the reproductive performance of farm animals. Among the agents that cause mastitis, bacteria are responsible for approximately 90% of cases, with Staphylococcus aureus standing out, which is present in dairy herds with a prevalence of 30 to 85%. For bacterial control, various strategies and antimicrobials are tested and developed

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for applicability and resolution of the problem. However, certain challenges exist, such as bacterial resistance, toxicity (when applied to feed or animal skin), and high financial costs, which minimize efficacy and often render it unviable. In this context, alternative substances of natural origin are sought. Given this scenario, the objective of this study was to investigate star anise essential oil (Illicium verum) as an antibacterial agent against mastitis-causing S. aureus isolated from dairy herds in western Santa Catarina. The essential oil was purchased commercially and tested against four strains of S. aureus isolated from milk samples of cows with clinical and subclinical mastitis. Disk diffusion and planktonic cell quantification (CFU/mL) tests were performed to test antibacterial activity. Although the results of the disk diffusion tests did not demonstrate significant inhibitory zones, there was a reduction in colony-forming units, with two bacterial strains being reduced by 100%. The results demonstrate that the use of essential oil is promising in controlling mastitis caused by S. aureus.

Keywords: Essential Oil. Bacteria. Control.

RESUMO

A mastite bovina é uma doença frequente e economicamente prejudicial na pecuária leiteira. Esta infecção na glândula mamária pode impactar no desempenho reprodutivo dos animais da fazenda. Dentre os agentes que causam mastite, as bactérias são responsáveis por aproximadamente 90% dos casos, destacando-se Staphylococcus aureus, que está presente nos rebanhos leiteiros com prevalência de 30 a 85%. Para o controle bacteriano, diversas estratégias e antimicrobianos são testados e desenvolvidos para a aplicabilidade e resolução do problema. Entretanto, há certas dificuldades, tais como a resistência bacteriana, toxicidade (no caso de aplicabilidade em rações ou na pele do animal), alto valor financeiro, que minimizam a eficiência e muitas vezes tornam-se inviáveis. Nesse contexto, buscam-se substâncias alternativas, de origem natural. Perante a esse cenário, o objetivo do presente estudo foi pesquisar o óleo essencial de anis estrelado (Illicium verum) como antibacteriano frente S. aureus causadores de mastite, isolados em rebanhos leiteiros na região do oeste catarinense. O óleo essencial foi adquirido comercialmente e testado frente a 4 cepas de S. aureus isolados a partir de amostras de leite de vacas com mastite clínica e subclínica. Para os testes de atividade antibacteriana foram realizados os testes de Disco-difusão e de Quantificação de células planctônicas (UFC/mL). Embora os resultados dos testes de disco-difusão não tenham demonstrado halos inibitórios significativos, houve redução de unidades formadoras de colônias, sendo que duas cepas bacterianas foram reduzidas 100%. Os resultados obtidos demonstram que a utilização do óleo essencial é promissora no controle de mastite causada por S. aureus.

Palavras-chave: Óleo Essencial. Bactérias. Controle.

RESUMEN

La mastitis bovina es una enfermedad común y económicamente perjudicial en la producción lechera. Esta infección de la glándula mamaria puede afectar el rendimiento reproductivo de los animales de granja. Entre los agentes causantes de mastitis, las bacterias son responsables de aproximadamente el 90% de los casos, destacando el Staphylococcus aureus, presente en hatos lecheros con una prevalencia del 30 al 85%. Se han probado y desarrollado diversas estrategias y antimicrobianos para controlar las bacterias y abordar el problema. Sin embargo, existen ciertos desafíos, como la resistencia bacteriana, la toxicidad (al aplicarse al alimento o en la piel del animal) y los altos costos financieros, que minimizan su eficacia y, a menudo, los hacen inviables. En este contexto, se buscan sustancias alternativas de origen natural. Ante este escenario, el objetivo de este estudio fue investigar

el aceite esencial de anís estrellado (Illicium verum) como agente antibacteriano contra S. aureus causante de mastitis, aislado de hatos lecheros del oeste de Santa Catarina. El aceite esencial se adquirió comercialmente y se probó contra cuatro cepas de S. aureus aisladas de muestras de leche de vacas con mastitis clínica y subclínica. Se realizaron pruebas de difusión en disco y cuantificación de células planctónicas (UFC/mL) para evaluar su actividad antibacteriana. Si bien los resultados de las pruebas de difusión en disco no mostraron zonas inhibitorias significativas, se observó una reducción de las unidades formadoras de colonias, con una reducción del 100% en dos cepas bacterianas. Los resultados demuestran que el uso del aceite esencial es prometedor para el control de la mastitis causada por S. aureus.

Palabras clave*:* Aceite esencial. Bacterias. Control.

1 INTRODUCTION

Milk is a product widely consumed and produced in the world. According to the United States Department of Agriculture (USDA, 2025), Brazil was the sixth largest global producer between January and December 2024, accounting for about 4.2% of world production. According to the Brazilian Institute of Geography and Statistics (IBGE, 2025a), the national production in 2023 was estimated at 35.37 billion liters of milk, with Santa Catarina being the fourth largest state producer (IBGE, 2025b).

Among the diseases that affect the dairy herd, mastitis stands out for its significant negative impact on zootechnical indices and on the profitability of the activity, due to the reduction in the quality and volume of milk, in addition to the costs associated with the treatment and disposal of the product. It is an inflammatory process of the mammary gland, of multifactorial etiology, which can manifest itself in two forms: the clinical, characterized by evident signs such as edema, hyperemia, pain and changes in the characteristics of the milk; and the subclinical, which has no visible symptoms, but already compromises milk production and quality (Massote et al., 2019).

Staphylococcus aureus (*S. aureus*) stands out as one of the main etiological agents of bovine mastitis, and is often difficult to treat due to its ability to develop antimicrobial resistance (Gordon et al., 2021). In addition, there is evidence that this microorganism has considerable ability to form biofilms, a characteristic that is directly related to the severity of the disease (Di Domenico et al., 2019).

In view of this scenario, the importance of adopting innovative therapeutic strategies for the control and treatment of mastitis, especially in high-producing cows, is highlighted, with the objective of reducing productive and economic losses in the dairy chain, based on antibacterial activity against *Staphylococcus aureus*.

Plants have several secondary metabolic pathways responsible for the formation of compounds that have antibacterial, repellent, antifungal, antioxidant, and even anti-inflammatory properties (SZCZEPANSKI AND LIPSKI, 2014; MILLEZI et al., 2016; DE JESUS, et al., 202; SPERANDIO, et al., 2023). The set of secondary compounds in plants is the result of the balance between the formation and elimination of these metabolites during plant growth, among them are essential oils (EO). Recently, there has been considerable interest in the study of plant materials as sources of new compounds for transformation into antiseptic agents. In this perspective, more and more research has been developed in order to investigate new natural sanitizers with antimicrobial action or, if possible, molecules that modulate the activity of commercial antibacterials (BELLAVER, et al. 2022; DAL BELLO et al., 2023).

One of the plant species frequently mentioned in the scientific literature due to its broad therapeutic properties is *Illicium verum*, popularly known as star anise, badiana, or badiana-da-china (Brasil, 2019). The technological prospecting carried out by Silva *et al.* (2020), demonstrated that among the various pharmacological activities attributed to the star anise EO, the antibacterial, antifungal, anti-inflammatory, antioxidant, and antiviral activity stand out. Such antifungal and antibacterial properties are attributed to anethole, which is the majority compound of star anise essential oil, also present in the plant extract (Silva et al., 2020).

From this perspective, the objective of the present study was to evaluate the bacterial activity of star anise essential oil against strains of *S. aureus* isolated from milk of cows with mastitis, in the western region of Santa Catarina.

2 MATERIAL AND METHODS

Places where the experiments were conducted

The isolation of the strains was carried out at the Veterinary Microbiology Laboratory of the Federal Institute of Santa Catarina, Campus Concórdia. The other microbiological analyses were carried out at the Biology and Microbiology and Phytopathology Laboratories of the Federal Institute of Santa Catarina, Camboriú Campus.

Obtaining the EO and sanitizing solution

The essential oil was obtained commercially from the company Ferquima (Vargem Grande, SP). The essential oil-based solution followed the protocol described by Millezi *et al.* (2016), being composed of 0.9% saline water, 2% PA ethyl alcohol and the rate corresponding to 0.1%, 0.2% and 0.4%.

Bacterial strains

The bacterial strains were from the isolation of clinical cases of mastitis in cattle from the western region of Santa Catarina, and were isolated at the Veterinary Microbiology Laboratory of the Federal Institute of Santa Catarina, Campus Concórdia. Four bacterial strains were used, being *Staphylococcus aureus* 34/22, 62/22, 153/23 and 190/23.

Milk samples

Milk samples were collected from clinical cases of bovine mastitis in lactating dairy herds. The diagnosis of mastitis was based on macroscopic changes in the milk (presence of pus, dessora, lumps and/or blood streaks), evaluated by means of the dark-bottomed screened mug test (Tamis); in addition to the presence of inflammatory signs in the mammary gland (pain, hyperemia, sensitivity) and/or systemic signs (MEGID et al., 2016). Milk samples were collected by manual milking, aseptically, by rubbing the ostium of the teats with cotton

soaked in 1% glycerinated iodine solution, and transported under refrigeration (4–8 °C) for microbiological culture.

Microbiological culture and identification of *Staphylococcus aureus*

Milk samples were seeded on 5% bovine blood agar and incubated at 37°C in aerobiosis for 72 hours, with readings every 24 hours. The isolated lines were identified based on morpho-dyeing, biochemical and culture characteristics. *S. aureus* was characterized as Gram-positive, facultative anaerobic (fermenting), catalase-positive, oxidase-negative and immobile bacteria, multiplying between 15 and 45 °C and tolerant to up to 15% NaCl. It is coagulase-positive, as are most pathogenic strains of staphylococci (*S. intermedius* and *S. hyicus*), ferments mannitol, maltose, and is resistant to polymyxin B (300 IU) (QUINN et al., 2011). On microscopy, it was in the form of cocci (0.8–1.0 µm in diameter), grouped as "grape bunches", although isolated cells could occur. In solid medium, the colonies were circular-convex, 2–3 mm in diameter, white to golden-yellow in color, with a halo of hemolysis. Thus, colonies with these characteristics were reseeded for Gram staining. After microscopic observation and evaluation of hemolysis, the isolates were submitted to the catalase test. Those presumably identified as staphylococci were classified as *S. aureus* if they showed positivity in the coagulase test in the tube, fermentation of maltose and mannitol, as well as resistance to polymyxin B (SIMÕES et al., 2013).

Disk-diffusion analysis

The qualitative and preliminary disc-diffusion technique was used as a method to verify the antibacterial action (CLSI, 2013). The bacteria were inoculated in BHI broth and incubated at 28ºC for 96 hours. The inoculum standardization was based on the McFarland scale 0.5 ($1x10^8$ CFU/mL), followed by seeding on Mueller Hinton Agar (AMH) in 140 mm Petri dishes. The bacterium was spread with the aid of sterile swabs. The concentrations of EO tested were: 100%, 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.78% and 0.39%, using PA alcohol as diluent. 5 µL of each concentration were pipetted onto filter paper discs, which were positioned with sterile tweezers. Chloramphenicol was used as a positive control and alcohol PA as a negative control. The plates were incubated in B.O.D. at 37ºC for 18 and 24 hours. The measurement of the inhibition halos was performed with a digital caliper.

Planktonic cell quantification

The quantification of planktonic bacterial cells was performed according to Bellaver et al (2022). After 24 hours of incubation, 100 µL of the treated wells and the positive control were withdrawn for serial dilution. Aliquots of 10 µL were plated in TSA medium for CFU counting, using the microdrop technique. The plates were incubated at 37 ºC for 24 h in an incubator (Lucadema, Brazil), and the count was expressed in CFU/mL.

Data analysis

For the data from the Disc-diffusion test, the bacteria were classified according to Stanko et al. (2010): strongly inhibited (>15 mm), moderately inhibited (10–15 mm) and not inhibited (<10 mm). The data related to the quantification of planktonic cells were analyzed using the GraphPad software. Analysis of variance (ANOVA) was performed at a significance level of 5%, with comparison of the means by the Bonferroni test. All analyses were performed with three replications and in triplicate.

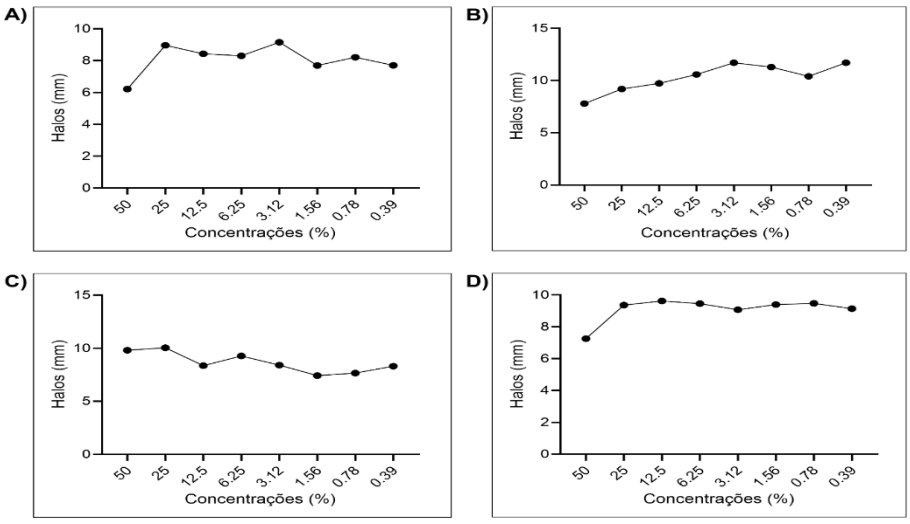
3 RESULTS AND DISCUSSION

Disk-diffusion analysis

For the four strains analyzed, the inhibition halos ranged from 6 to 12 mm (Figure 1), none of the strains was considered strongly inhibited (greater than 15 mm), according to the classification parameters of Stanko *et al.* (2010). Star anise essential oil did not promote intense inhibition of the bacterial strains tested by means of this methodology.

Figure 1

Diameters of the inhibition halos of bacterial strains from different concentrations of star anise essential oil. A) *S. aureus* 32/22, B) *S. aureus* 62/22, C) *S. aureus* 153/23 and D) *S. aureus* 190/23



Inhibition halos between 10 and 12 mm were observed for the *S. aureus* 62/22 strain (Figure 1B), being classified as moderately inhibited according to Stanko *et al.* (2010). Among the concentrations tested, the 3.12% was the one that resulted in the largest halo, being 12 mm. For the other strains, most of the concentrations tested presented halos below 10 mm, which were classified as not inhibited (Stanko *et al.*, 2010). However, for strain 153/23, at concentrations of 50% and 25%, respectively, and for strain 190/23, at concentrations of 12.5%, there was formation of 10mm halos, thus being classified as moderately inhibited.

These findings are in line with the literature, which recognizes that the effectiveness of essential oils depends on multiple factors. As highlighted by the research by Galgano et al. (2022), aspects such as the type of oil, its chemical composition, the concentration applied, and the specific characteristics of the bacterial strain directly influence the results obtained. Such variability was evidenced in the present analysis, indicating that even within the same bacterial species, different strains may respond differently to treatment with essential oil.

The study conducted by Alhaji *et al.* (2019), in which three crude extracts of *Illicium verum* (absolute methanol, 50% methanol, and aqueous extract) were analyzed for antibacterial activity using the disk-diffusion method on agar. The extracts were tested against four bacteria: *Staphylococcus aureus* ATCC 25923*, Listeria monocytogenes* ATCC 7644*, Escherichia coli* ATCC 25922 *and Salmonella arizona* ATCC 13314. The results showed that star anise extracts exhibited inhibitory effects against all bacteria tested, with absolute methanol extract showing the highest inhibitory activity against *S. aureus*, with an inhibition zone diameter of 14.7 mm.

In Noumi's research *et al.* (2023), the Mueller-Hinton agar disk-diffusion test was also used to determine the antibacterial effect of star anise and trans-anethole essential oil against bacteria: *Listeria monocytogenes* CECT 933*; Vibrio vulnificus* CECT 529*; Salmonella enterica* CECT 443*; Shigella flexeneri* CECT 4804*; Staphylococcus aureus* ATCC 6538*; Bacillus subtilis* CIP 5265*Escherichia coli* ATCC 35.218 *and Pseudomonas aeruginosa* PAO1. The results showed that the trans-anethole promoted a zone of 8.33 mm in front of *S. aureus*, while the star anise EO promoted an inhibition zone of 13.66 mm, indicating a significant antibacterial activity of the oil in its integral form.

Already in Benmalek's research *et al*. (2013), the results obtained by the disk-diffusion analysis demonstrate that the extracts of aglycones (flavones/flavonols) and anthocyanins of *I. verum* against *Staphylococcus aureus* ATCC 25923 and *Staphylococcus aureus* ATCC 43300, did not show significant inhibitory activity against the strains of *S. aureus*, showing that certain isolated compounds may not have the same antimicrobial potential as more complex or whole extracts.

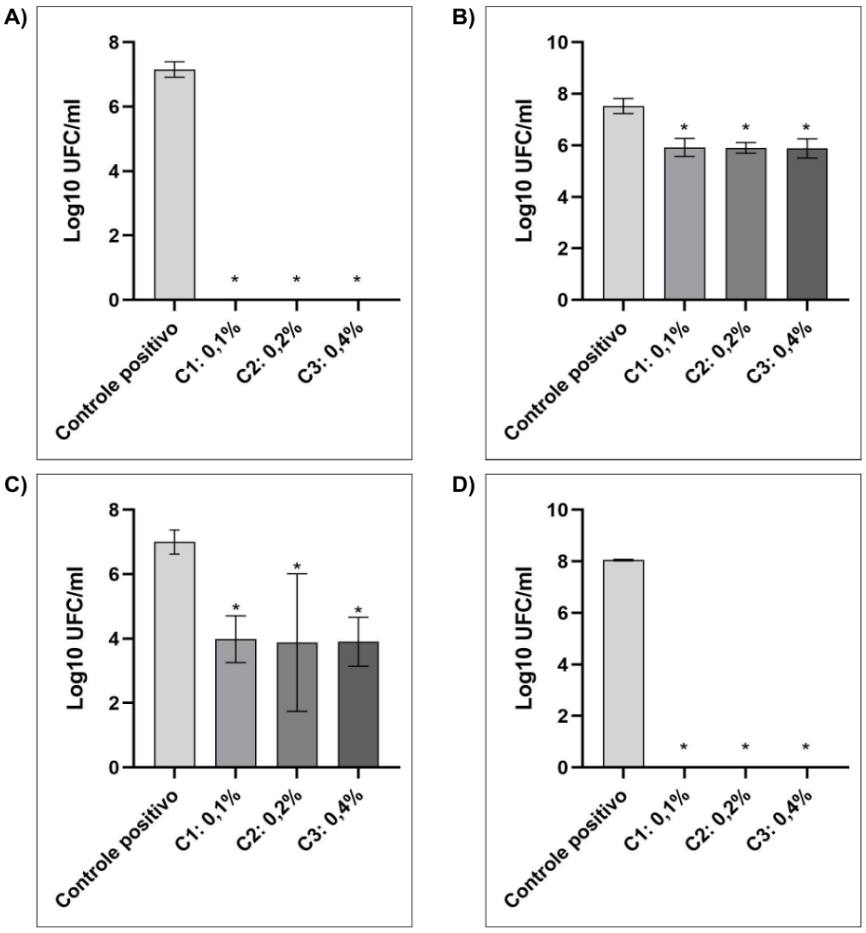
The results obtained by the present study demonstrate how the antibacterial effect against *S. aureus* was moderate and highly dependent on the concentration and strain tested. When compared to data in the literature, it is clear that the antimicrobial activity of *Illicium verum* can vary considerably according to the methodologies used, the types of strains and the compounds applied.

Planktonic cell quantification

The treatments used with star anise EO promoted satisfactory results, inhibiting bacterial growth (Figure 2).

Figure 2

*Quantification of Staphylococcus aureus colony-forming units (CFU) from different concentrations of star anise essential oil. A) S. aureus 32/22, B) S. aureus 62/22, C) S. aureus 153/23 and D) S. aureus 190/23. * indicates statistical difference according to the Bonferroni test at the level of 5% significance compared to the positive control (without treatment with essential oil)*



For the four bacterial strains analyzed, a significant reduction in the CFU count was observed after exposure to star anise essential oil (*Illicium verum)* at all concentrations tested (0.1%, 0.2% and 0.4%) when compared to the control group. The statistical analysis proved that the reductions observed in the experiment were not only a visual trend but an effective antimicrobial effect ($p < 0.05$).

For strains 32/22 and 190/23, there was complete inhibition of bacterial growth at all concentrations tested (0.1%, 0.2% and 0.4%), which indicates high sensitivity of these isolates even at the lowest concentration (Figures 2B and 2D). On the other hand, in the SA 62/22 and SA 153/23 strains, the application of the essential oil also promoted a significant reduction in bacterial growth compared to the positive control (Figure 2A and 2C). For the SA 62/22 strain, a decrease of 1.61 log cycles was observed at the concentration of 0.1%; 1.62 cycles log at 0.2% concentration; and 1.65 log cycles with 0.4% (Figure 2B). The SA 153/23 strain showed an even more significant reduction, with 3.00 log cycles at a concentration of 0.1%; 3.11 log cycles at 0.2%; and 3.10 log cycles at 0.4% (Figure 2C).

These results corroborate previous studies that demonstrated the antimicrobial action of star anise essential oil (EO) against strains of *S. aureus*, including methicillin-resistant isolates (MRSA). Patra *et al.* (2020) reported that *Illicium verum essential* oil had low MIC (0.70 µg/mL) and MBC (0.97 µg/mL) values against clinical MRSA isolates. Salem *et al.* (2021) showed that the application of EO not only inhibited formation, but also caused a significant detachment of the already established biofilm. These results corroborate his in vivo experiment with mice, in which highly virulent strains of *S. aureus* (USA300) were injected into the intradermal region, causing abscesses. After treatment with star anise extracts, the bacterial count showed a significant reduction.

According to Bai *et al.* (2015; 2018), one of the main compounds of star anise is shikimic acid, which has great potential as an antimicrobial agent, especially against *S. aureus,* possibly by compromising the permeability of the cell membrane after its binding to the proteins and lipids of this structure. Another component close to shikim and also present in *I. verum* is quinic acid which has two derivatives, two phenolic compounds called coumaroylquinic acid and feruloylquinic acid playing a large role as antimicrobial agents.

Zhang et al. (2025) investigated the antibacterial and antibiofilm activities of star anise and cinnamon essential oil against multidrug-resistant Salmonella Thompson. Through time-death curve assays, they observed a rapid reduction in colony forming unit (CFU) counts in the first hours, with an even more significant decrease until the sixth hour of treatment. These tests demonstrated the efficacy of using star anise as an antimicrobial agent in the control of the bacteria studied.

Complementing these findings, Yang et al. (2021) evaluated the phytochemical composition and antibacterial activity of extracts obtained from the leaves and twigs of *I. verum* against nine antibiotic-resistant clinical isolates, including *Staphylococcus aureus*. Also using the time-death curve analysis, the authors demonstrated that the extracts showed bactericidal activity that can last up to 24 hours. These results reinforce the potential of star anise as a promising natural alternative to conventional antibiotics, especially in combating resistant clinical pathogens.

In addition to this evidence, recent studies show the efficacy of the use of star anise for the reduction of potentially pathogenic microorganisms present in the oral cavity, where an experiment was carried out with 50 people using mouthwash based on *I. verum,* showing a significant reduction in the presence of several causative agents of periodontal diseases, such as *Staphylococcus aureus,* corroborating the findings of this study and reinforcing the relevance of star anise as a promising alternative for infection prevention and control (Assiry et al., 2021).

4 CONCLUSION

Star anise essential oil was potentially antibacterial against S*. aureus strains* isolated from clinical mastitis cases. Disk-diffusion analysis is a preliminary test that does not always demonstrate antibacterial activity efficiently, in this work, the quantification of viable cells demonstrated that there was a significant antibacterial effect.

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