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Evaluation of the toxicity of bioactive compounds against mastitis-causing pathogens in bovine mammary epithelial cells

Avaliação da toxicidade de compostos bioativos contra patógenos causadores de mastite em células epiteliais mamárias bovinas

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Abstract: Bovine mastitis represents a significant economic challenge in dairy production and is responsible for the extensive use of antimicrobials, accounting for approximately 60% of all antibiotics administered to dairy cows. This contributes to the selective pressure for antimicrobial-resistant bacteria. However, the use of alternative therapeutic compounds has emerged as a promising strategy. The aim of this study was to investigate the cytotoxicity of nisin and three plant-derived essential oils from *Melaleuca alternifolia*, *Syzygium aromaticum*, and *Citrus sinensis* in immortalised bovine mammary epithelial cells (MAC-T cells), using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Furthermore, antimicrobial activity was assessed by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against the bovine major mastitis pathogens. The results demonstrated that, although the plant-derived essential oils exhibited antimicrobial activity against the major mastitis pathogens, their clinical application would be limited due to the high cytotoxicity observed at effective antimicrobial concentrations. In contrast, nisin showed notable antimicrobial efficacy combined with *in vitro* safety, preserving the viability of mammary epithelial cells at all tested concentrations. This highlights the potential of this bacteriocin for the treatment of intramammary infections in dairy cattle. Future research involving the antimicrobial application of the essential oils analysed in this study should focus on identifying the specific molecules responsible for the antimicrobial activity, with the aim of developing effective and less cytotoxic formulations. Therefore, cytotoxicity assessment is essential to ensure the safe clinical use of alternative antimicrobials.

Key-words: alternative antimicrobials; bacteriocin; intramamamary infection; cytotoxicity.

Resumo: A mastite bovina representa um desafio econômico significativo na produção leiteira, sendo responsável pelo uso extensivo de antimicrobianos, que representam aproximadamente 60% de todos os antibióticos administrados em vacas leiteiras, aumentado a pressão seletiva de bactérias resistentes aos antimicrobianos. Todavia, o uso de compostos terapêuticos alternativos tem se mostrado uma opção promissora. O objetivo deste estudo foi investigar a citotoxicidade da nisina e de três óleos essenciais de origem vegetal oriundos da *Melaleuca alternifólia*, *Syzygium aromaticum* e *Citrus sinensisi*, em células epiteliais mamárias bovinas imortalizadas (células MAC-T), por meio do ensaio de redução de dimetiltiazol-2-il e 2,5-difeniltetrazólio brometo (MTT). Ademais, a atividade antimicrobiana foi avaliada pela determinação da concentração inibitória mínima (CIM) e concentração bactericida mínima (CBM) contra os principais patógenos da mastite bovina. Os resultados demonstraram que, embora os óleos essenciais de origem vegetal tenham apresentado atividade antimicrobiana contra os patógenos principais da mastite bovina, sua aplicação clínica seria inviabilizada pela elevada citotoxicidade observada em concentrações antimicrobianas eficazes. Por sua vez, a nisina destacou-se por apresentar eficácia antimicrobiana associada a segurança *in vitro*, preservando a viabilidade das células epiteliais mamárias em todas as concentrações testadas, evidenciando assim o potencial dessa bacteriocina no tratamento de infecções intramamárias em bovinos leiteiros. Futuros estudos, que envolvam a utilização antimicrobiana dos óleos essenciais de origem vegetal aqui analisados, deverão focar na identificação das moléculas específicas responsáveis pela atividade antimicrobiana, visando desenvolver formulações eficazes e menos agressivas. Portanto, a avaliação da citotoxicidade torna-se fundamental para o uso clínico seguro de antimicrobianos alternativos.

Palavras-chave: antimicrobianos alternativos; bacteriocina; citotoxicidade; infecção intramamária.

1. Introduction

Bovine mastitis, an inflammation of the mammary glands, is recognized as one of the main obstacles to productive efficiency in dairy farming and carries significant economic consequences [1]. This condition reduces milk yield and quality, increases the premature culling of animals, and raises treatment and veterinary care costs, directly compromising the sustainability of production and the competitiveness of the sector [1]. Studies indicate that approximately 60% of antimicrobials administered to adult dairy cattle are used to treat and prevent mastitis [2]. The frequent and inappropriate use of these drugs contributes to the emergence of resistant bacteria [2]. Global concerns regarding antimicrobial resistance have therefore driven the search for safe and effective therapeutic alternatives for mastitis control [2, 3].

In this context, the use of alternative antimicrobials has emerged as a promising approach to reduce dependence on conventional antibiotics and mitigate the negative consequences of antimicrobial resistance [2, 3, 4]. The plant-derived essential oils evaluated in this study, melaleuca (*Melaleuca alternifolia*), clove (*Syzygium aromaticum*), and orange (*Citrus sinensis*), are known for their antimicrobial and anti-inflammatory properties and are widely studied in scientific research [5, 6, 7, 8, 9, 10]. Nisin, produced by *Lactococcus lactis* and commonly used as a food preservative, has more recently been explored for therapeutic purposes owing to its strong antimicrobial activity and favorable safety profile [11, 12, 13, 14].

Although several authors have investigated the antimicrobial activity of alternative therapeutic compounds against mastitis-causing pathogens, their safety in bovine mammary epithelial cells has received relatively little attention [3, 10]. Therefore, the aim of this study was to evaluate the viability of bovine mammary epithelial cells after treatment with different concentrations of these four compounds and to assess their cytotoxic potential. Determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against the main bovine mastitis pathogens represents a fundamental first step in defining the concentrations used to assess compound cytotoxicity in mammary epithelial cells.

2. Material and methods

2.1 Experimental design

Initially, the antimicrobial activities of nisin and essential oils of plant origin against the six main pathogens of bovine mastitis were evaluated by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Mammary epithelial cells were treated with the same concentrations of the compounds to determine cytotoxicity in mammary epithelial cells.

2.2 Selection of antimicrobial compounds

The compounds used in this study were selected based on their antimicrobial potential [6, 8, 14]. The bacteriocin nisin (code N5764) was purchased commercially from Sigma-Aldrich® (Sigma-Aldrich Brasil Ltda, São Paulo, SP, Brazil). The essential oils, in turn, were extracted by the researchers in compliance with applicable technical standards [15], using the hydrodistillation technique with a Clevenger-type graduated apparatus, with the exception of melaleuca (*Melaleuca alternifolia*). Due to the unavailability of this plant in the study region, melaleuca oil was purchased commercially from Lazlo (Lazlo Aromatherapy®, Belo Horizonte, MG, Brazil), where it was extracted from the leaves using the steam distillation method [16].

The essential oil of orange (*Citrus sinensis*) was extracted from leaves harvested between March and April 2018 (late summer/early autumn), whereas the essential oil of clove (*Syzygium aromaticum*) was extracted from flower buds and purchased commercially from a health food store. To ensure standardization of the concentrations tested and to accurately evaluate the intrinsic activity of each compound without interference from secondary components present in crude extracts, the compounds were used in pure form. All essential oils were purified as described by Silva. [17].

2.3 Selection of pathogens

The pathogens used in this study were *Streptococcus agalactiae*, *Staphylococcus aureus*, *Streptococcus uberis*, *Escherichia coli*, *Klebsiella* sp., and *Prototheca* sp., as they are considered the main causative agents of bovine mastitis [1, 18, 19]. This is further supported by evidence that some of these pathogens, such as *S. aureus*, *Klebsiella* sp. [4, 19], and *Prototheca* sp. [20], show little or no response to conventional antimicrobial therapy. The microorganisms were isolated from cases of subclinical mastitis (*S. agalactiae, S. aureus, S. uberis*, and *Prototheca* sp.) and clinical mastitis (*E. coli* and *Klebsiella* sp.) in previous research conducted by our group. They were preserved in Brain Heart Infusion (BHI) broth with 20% sterile glycerin and stored at –80 °C until use. Microbial inocula were prepared as previously described by our research group [19].

2.4 Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of each compound was defined as the lowest concentration capable of inhibiting growth of the evaluated microorganism. For this purpose, cell viability was measured using the resazurin assay (Sigma-Aldrich®, São Paulo, Brazil), as described by Araujo and Longo [21], following the guidelines of BrCAST - Brazilian Committee on Antimicrobial Susceptibility Testing [22].

2.5 Minimum Bactericidal Concentration (MBC)

Minimum Bactericidal Concentration (MBC) was defined as the lowest concentration capable of killing the assessed microorganisms. After measuring the MBC, aliquots were taken from the wells in which there was no visible microbial growth, cultured on plates containing Mueller Hinton Agar or Sabouraud Dextrose for *Prototheca* sp, according to the methodology described by Silva *et al.* [10], following the BrCAST guidelines [22], and if there was no microbial growth, the MIC was confirmed.

2.6 Cytotoxicity test

A clonal line of bovine mammary epithelial cells originating from primary alveolar cells (MAC-T) was imported and deposited by our team at the Rio de Janeiro Cell Bank (BCRJ). The MAC-T cells were cultured in Dulbecco's Modified Eagle's Medium (cat. no. D5796, Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (cat. no. F9665, Sigma-Aldrich®, St. Louis, MO, USA), 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL fungizone (cat. no. 15240-062, Life Technologies®, Paisley, UK), 5 µg/mL insulin, and 1 µg/mL hydrocortisone, as previously described by our research group [19].

MAC-T cells were seeded in 96-well plates at a density of 5×10^5 cells/well and supplemented with the minimum bacteriostatic and bactericidal concentrations of the respective compounds (nisin and essential oils of tea tree, orange, and clove) determined by MIC and MBC assays. Each assay was performed in quintuplicate. After supplementation, the plates were incubated at 37 °C with 5% CO_2 for 16 h.

Cytotoxicity was assessed by measuring the mitochondrial activity of MAC-T cells after treatment with different concentrations of the compounds using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay, as described by Supino [23]. This method measures the intracellular conversion of MTT into insoluble formazan crystals (blue or purple), which are quantified spectrophotometrically at 562 nm, reflecting cell viability after compound exposure and, consequently, the cytotoxicity of each concentration.

3. Results and discussion

The results of this study reinforce the known microbicidal capacity of the essential compounds studied against mastitis-causing pathogens (Table 1). The findings for clove oil are consistent with those of previous studies that attributed this activity to eugenol, its main component [7]. Orange bloom oil was most effective against *S. aureus* and *Prototheca* sp., but showed reduced performance against gram-negative bacteria (Table 1), requiring higher concentrations. Orange tree oil showed the worst results, with high MICs and MBCs against *E. coli* and *Klebsiella* sp., which may be related to the lower penetration of its active ingredients in these microorganisms [7, 9].

Literature suggests that the antimicrobial action of plant-based essential oils results from a combination of molecules that act synergistically. These molecules vary depending on their botanical origin, cultivation conditions, and extraction methods. However, it is important to consider that the complexity of the chemical composition of these extracts can present both benefits and limitations, hindering standardization, reproducibility, and understanding of specific mechanisms of action [3].

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), in percentage, of different alternative antimicrobials against pathogens causing bovine mastitis.

	MIC Clove	MBC Clove	MIC Tea.	MBC Tea.	MIC Ora.	MBC Ora.	MIC Nis.	MBC Nis.
S. agalactiae	0,0097	0,0097	0,3125	1,2500	0,1563	0,6250	0,0039	0,1250
S. aureus	0,3125	1,2500	0,3125	0,6250	0,6250	1,2500	0,0156	0,0625
S. uberis	1,2500	5,0000	2,5000	10,0000	0,6250	5,0000	4,0000	4,0000
Prototheca sp.	0,3125	0,6250	0,3125	0,6250	0,3125	0,6250	0,0312	2,0000
Klebsiella sp.	1,2500	5,0000	10,0000	10,0000	10,0000	10,0000	4,0000	4,0000
E. coli	0,0190	0,0190	0,6250	2,5000	5,0000	10,0000	2,0000	4,0000

Note: MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; Tea. = tea tree; Ora. = orange tree; Nis. = nisin. Values in %.

Although they exhibited antimicrobial activity against the pathogens evaluated (Table 1), essential oils of plant origin (clove, tea tree, and orange) also showed high cytotoxicity at all concentrations tested (Figure 1), suggesting that future studies involving the antimicrobial use of such oils should investigate the isolation and characterization of specific molecules, allowing their synergistic effects to be explored to maximize antimicrobial efficacy and minimize cytotoxicity through adjustments in the extraction method or combination of components, aiming to create less aggressive formulations [3]. The results of the present study do not support the in vivo clinical application of such pure plant-derived essential oils, as used in this study, due to their cytotoxicity in bovine mammary cells used in the present study (Table 1).

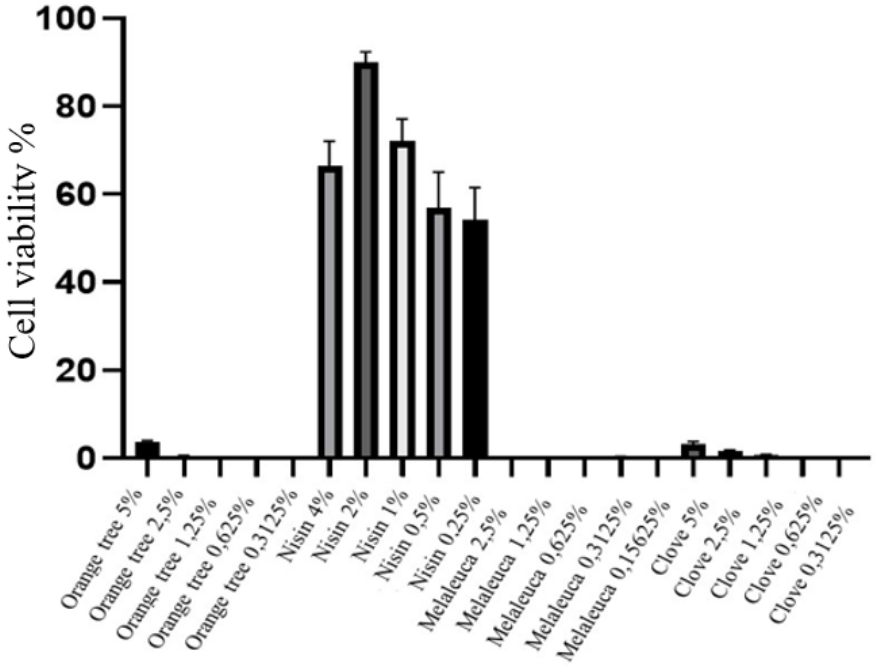

Figure 1. Cell viability (%) of the MAC-T bovine mammary epithelial cell line treated with different concentrations of alternative antimicrobial compounds – essential oils of *Melaleuca alternifólia*, *Syzygium aromaticum*, *Citrus sinensis* and the bacteriocin nisin — measured using dimethylthiazol-2-yl and 2,5-diphenyltetrazolim bromide (MTT) reduction assay.

In addition, nisin showed particularly promising potential for controlling the tested pathogens, indicating that it is the most effective alternative antimicrobial among the compounds evaluated based on the MIC and MBC results (Table 1). Similarly, Cao *et al.* [14] reported the efficacy of nisin in the treatment of clinical mastitis, emphasizing its broad-spectrum activity against gram-positive bacteria. In addition,

in contrast to the other compounds evaluated, nisin was the only compound that demonstrated an acceptable safety profile in relation to cytotoxicity (Figure 1) and was the only compound that presented antimicrobial efficacy associated with the maintenance of cell viability.

Interestingly, nisin showed a peculiar pattern in relation to cell viability rates, which were higher when cells were exposed to an intermediate concentration of 2% compared to concentrations of 4% (highest), 1%, and 0.5% (lowest). This observation may be related to the findings of Namjoo [24], who described the anti-apoptotic and cell proliferation-inducing effects of nisin in mesenchymal stem cells (MSCs) in a dose-dependent manner. Previous studies have also identified nisin as an agent that promotes cell proliferation and increases cell lifespan [25, 26]. This finding may be due to the method used to measure cell viability, the MTT reduction assay, which quantifies the conversion of MTT to formazan crystals, reflecting mitochondrial activity and cell viability [23]. Consequently, possible cell proliferation induced by nisin would result in an increase in cell viability rate at measurement. In addition to the aforementioned cytoprotective and cytoproliferative effects, Gao *et al.* [26] reported that nisin could modulate the oral microbiome, promote health, and stimulate a new proliferative phenotype in reparative connective tissue cells when associated with the treatment of periodontal disease.

4. Conclusion

The results obtained in this study showed that although they presented relevant antimicrobial effects against some of the main pathogens of bovine mastitis, plant extracts (essential oils of *Melaleuca alternifolia*, *Syzygium aromaticum*, and *Citrus sinensis*) were highly cytotoxic to mammary epithelial cells, making in vivo therapy unfeasible. Only nisin, a compound of bacterial origin, demonstrated efficacy in microbial control and maintenance of cell viability. These results demonstrate the applicability and favorable cost-benefit ratio of nisin for the treatment of intramammary infections in dairy cattle. Future studies on the antimicrobial use of plant-derived essential oils should focus on identifying specific molecules to develop purer, safer, and more effective formulations.

Conflicts of interest statement

The authors declare that there is no conflict of interest.

Data availability statement

The complete data set supporting the results of this study is available upon request to the corresponding author.

Author contributions

Conceptualization: Santa Catarina, A. Methodology: Santa Catarina, A.; Benvegnú, D M; Starikoff, K R; Blagitz, M G; Souza, F N and Ferronatto, J A. Investigation: Santa Catarina, A; Benvegnú, D M; Starikoff, K R and Ferronatto, J A. Supervision: Blagitz, M G; Benvegnú, D M; Souza, F N; Della Libera, A M M P; Casimiro da Costa, L B SB and Leal, M L R. Writing: Santa Catarina, A.; Oliveira, R S L; Silva, P T F; Silva, K P C and Souza, F N. Review and editing: Souza, F N and Blagitz, M G.

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