ANTIBACTERIAL ACTIVITY OF CAATINGA BIOACTIVES- BASED NANOEMULSION ON Staphylococcus aureus: AN ALTERNATIVE IN THE TREATMENT OF MASTITIS

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ABSTRACT

Mastitis is the most important inflammatory disease of livestock in the world because it causes serious economic losses to the milk production chain. Among the treatment alternatives, products based on bioactives such as *Lippia origanoides* essential oil and *Syagrus coronata* fixed oil, stand out for their antimicrobial action favoring the sustainable and rational management of this problem. Therefore, the objective of this study is to develop a nanoemulsion for topical application, combining the essential oil of *L. origanoides* (EO-LO) with the fixed oil of *Syagrus coronata* (FO-SC) and evaluate its action against *S. aureus*. The nanoemulsions were obtained using the spontaneous emulsification method with physicochemical characterization (aspect, pH, density, conductivity, refractive index, average size, PdI, Zeta potential, active content, morphology by transmission electron microscopy) and permeation tests in Franz cell, antimicrobial activity against *Staphylococcus aureus* CCMB263. The nanoemulsions showed a hydrodynamic diameter <200 nm and a polydispersity index <0.2, increased antimicrobial action and excellent permeability (~80%) in vitro. Finally, it was found that the nanoemulsion was stable over the 30 days evaluated, with greater antimicrobial efficiency and potential for topical application in the treatment of mastitis.

Keywords: Mastitis. *Lippia origanoides*. *Syagrus coronata*. Veterinary use. Low energy.

1 INTRODUCTION

Mastitis is an inflammatory disease that affect the mammary glands of dairy cattle. It is considered the most important in herds due to the high incidence of clinical cases, subclinical infections and because it generates serious economic losses to the production chain of milk. This infection causes global annual losses of approximately US$35 billion, impacting producers, the dairy industry and consumers[1]. Currently, Brazil is the third largest producer of milk in the world, and the financial losses due to mastitis can reach R$6 billion/year[2]. Furthermore, there is also an important relevance for public health, due to the risk of not only transmitting etiological agents (psychotrophic and/or antimicrobial-resistant microorganisms) of traditional and emerging zoonoses, but also heat-resistant toxins to humans[3]. *Staphylococcus aureus*, microorganism with the highest incidence in mastitis, is associated with several virulence factors, including proteins involved in bacterial adherence, extracellular enzymes and toxins that promote tissue necrosis, and factors that interfere with the immune system[4].

To reduce economic losses and promote animal welfare, some strategies must be adopted considering environmental hygiene and pharmacological treatment. However, the indiscriminate use of broad-spectrum antimicrobials increases the risk of emergence and dissemination of antimicrobial resistance (AMR) among pathogens. Therefore, antimicrobial rational use is recommended[5]. The society has demanded alternatives treatment associated to medicinal plants or natural products. Essential oils (EO) or resin-oil or fixed oils can be a promising strategy. *Lippia origanoides* essential oil (EO-LO), rich in carvacrol, has excellent antimicrobial activity[6], but it is highly lipophilic. In general, those oils have low absorption and distribution which, as a result, lead to loss of bioavailability and efficacy. To overcome these impediments, several nanosystems have been proposed to further enhance this pharmacological action and stabilize the bioactive. Nanoemulsions are a flexible platform for bioactive administration across various therapeutic areas since they can encapsulate hydrophilic and hydrophobic compounds. In addition to that, nanoemulsions can be formulated in gels, creams, foams, aerosols, and sprays by using low-cost standard operative processes and also be taken orally, topically, intravenously, intranasally, and intraocularly.

Thus, the objective of this work is to develop a nanoemulsion for topical application, combining the essential oil of *Lippia origanoides* (EO-LO) with the fixed oil of *Syagrus coronata* (FO-SC), to prove its action against *S. aureus*. Both natural products, from caatinga biome, have a high concentration of carvacrol (EO-LO) and lauric acid (FO-SC), which have proved antimicrobial, anti-inflammatory, and antioxidant action.

2 MATERIAL & METHODS

Essential oil of *Lippia origanoides* Kunth, chemotype B (LAPRON/UEFS, Feira de Santana-Ba, Brazil), Polysorbate 80 (tween 80) (Croda do Brasil Ltda. Campina-SP, Brazil), deionized water and strain of *S. aureus* multi-resistant CCMB263 (Resistance to Novobiocin) obtained from the Collection of Microorganism Cultures of Bahia (CCMB).

The nanoemulsion (NE) was formulated using the low energy consumption method using the spontaneous emulsification technique. For this purpose, two phases (aqueous and organic) were prepared separately, with the aqueous phase consisting of purified water (75%). The organic phase (OF) was generated with a blend of OE-LO (6%), fixed oil SC (4%) and tween 80 (15%), under magnetic stirring at 500 rpm/20 minutes. Subsequently, the OF was poured into the FA, by dripping, at a flow rate of 2 mL/min under stirring (500 rpm/20 minutes). Three batches were formulated (Lots: E01, E02 and E03) and analyzed for physical-chemical characteristics (pH, refractive index, absolute density, conductivity, morphology, droplet size, polydispersity, zeta potential and active content). Measurements were performed in triplicate. The nanoemulsion stability evaluation was carried out for 30 days, under room temperature (25 ± 2 ◦C) and refrigeration (4 ± 2 ◦C).

Morphology was analyzed by negative stain transmission electron microscopy (JEM 2800, Jeol, Akishima, Japan) at 80 kV, according to literature with modifications[7]. Antimicrobial assessment was determined by the Clinical and Laboratory Standards Institute broth microdilution method[8-10]. The tests were performed in Müeller–Hinton (MH) culture medium. Permeation in Franz cells (Logan, Model: DHC-6T, New Jersey, USA) was performed with a cellulose dialysis membrane (33mm) and the data obtained were cumulative % over the pre-established time interval for collections. (24h). The statistical treatment of the data was based on analysis of variance (ANOVA) and its means ± standard deviation (SD) compared using the Tukey test at 5% probability of error ($p<0.05$).

3 RESULTS & DISCUSSION

The O/W nanoemulsion formulation containing EO-LO and FO-SC was homogeneous, translucent with a bluish and slightly yellowish background, a color characteristic of the lipid components (essential oil of *L. origanoides* and fixed oil of *S. coronata*). Data referring to pH, density, conductivity and refractive index,

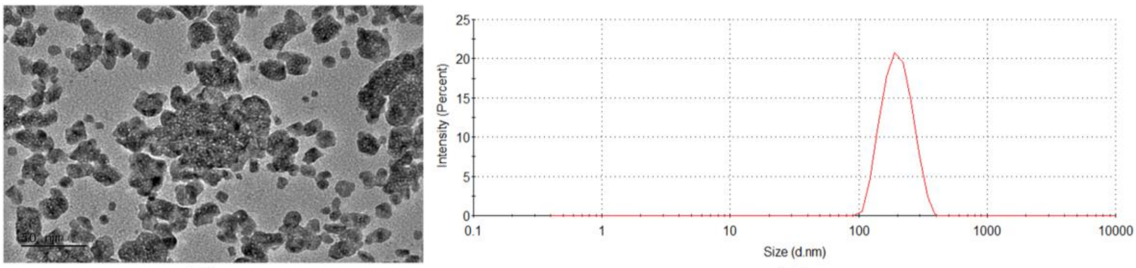
Table 1 – O/W Nanoemulsion Characterization (Batch E2): pH, density (D), conductivity (cond), refractive index (RI), Active content (AC), droplet size (DS), Polidispersity index (PDI) and zeta potential (PZ). n=3 with p<0.05

pH	D (g/mL)	Cond (µS/cm)	RI	AC (%)	DS (nm)	PDI	PZ (mV)
5.13 ± 0.03	1.02 ± 0.00	139.40 ± 0.47	1.363 ± 0.002	96.23 ± 0.33%	184.13 ± 0.26	0.15 ± 0.02	-8,32± 0.89

Nanoemulsion pH values reflects the pH values of the components and presents acidic characteristics, which favors the chemical stability. The density (d), conductivity (cond) and refractive index (RI) values are in agreement with O/W nanoemulsion type. Nanoemulsion presented monomodal profile and mean diameter < 200nm. Zeta potential values were slightly negative, probably due to surfactant (tween 80) placed on the droplets surface. However, according to literature, PZ values between -30mV and +30mV can make NE more susceptible to flocculation, because the attractive van der Waals forces are stronger than the repulsive forces[12]. That point of instability does not impact the NE efficiency but can reduce the useful life of the product[11-15].

NE stability remained better under refrigeration, with no statistically significant change (183.3 ± 1.4 nm) ($p>0.05$) during the 30 days. NE stored at room temperature has showed physical instability, with mean diameter varying from 181.3 ± 1.5 nm to 409.7 ± 3.3 nm in the 7th day ($p<0.05$). The refractive index and density, in both storage conditions, were similar during the evaluated period. The pH values did not change during the 30 days.

The morphology of NE droplets showed irregular shape with clear contours and nuclei (**Figure 1A**). Some aggregation was observed probably due to the sample preparation technique for analysis. The nanodroplets size is compatible to those measured with DLS with monomodal profile of size distribution (**Figure 1B**).



(A) (B)

Figura 1: Morphology Batch E2 - **(A)** Negative stain transmission electron microscopy image of the nanoemulsion and **(B)** Nanoemulsion size distribution by DLS.

In the antibacterial activity against the gram-positive bacterium *S. aureus* (CCMB263 - Novobiocin Resistance), it was observed that the oils (essential oil from *L. origanoides* and fixed oil from *S. coronata*) presented MIC and MBC higher than the maximum concentration tested in this study (>2000 µg/mL) (Table 2). Oils in their free form and oils entrapped into NE showed lower concentrations for MIC and MBC (Table 2). Such results may be related to the synergism between the lipid components. When LO-EO and FO-SC were entrapped into NE, the antibacterial activity was improved. The results found in the literature (MIC=600 µg/ml and MBC = 1500 µg/mL) [17,18] for free LO-EO were lower than those found in the present study. Another study reported that EO of *L. origanoides* (thymol chemotype) showed greater bactericidal activity (CBM = 0.5 mg/mL[18]. The encapsulation of bioactives from essential oil in nanoemulsion favored the dose reduction with desired therapeutic effect.

Table 2 - Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of the o/w nanoemulsion (Batch E2) against *Staphylococcus aureus* CCMB263

SAMPLES MICRORGANISM - SA CCMB263	**LO-EO**	**FO-SC**	**EO:SC**	**NE**	**C+**
MIC (µg/mL)	R >2000	R >2000	937,5/625	360/241	93,8
MBC (µg/mL)	R >2000	R >2000	1875/1250	469/313	750

Legend: Legend: EO – essential oil *L. origanoides*; SC – fixed oil of *S. coronata*; EO:SC – essential oil:fixed oil 1:1; NE – nanoemulsion; C+ - positive control (Chloramphenicol); SA – *Staphylococcus aureus* CCMB263; CCMB - Collection of Microorganism Cultures of Bahia

The permeation of NE at 15 minutes, 30 minutes, 1h, 2h, 4h, 8h, 12h and 24h, increased considerably, close to 80%, when compared to free oils (C1 – LO-EO + OF-SC) and C2 (LO-EO) whose values were below 40% after 24h. Therefore, the improved performance can be attributed to the nanostructured system[19].

4 CONCLUSION

Nanoemulsion was succesfully produced using a low energy method. NE was stable for 30 days, especially under refrigeration. Furthermore, it was found to be a nanostructured formulation with potential topical application in the treatment of mastitis due to improved antimicrobial action of *Lippia. origanoides* essential oil.

REFERENCES

1 CANESCHI, Alice et al. Plant essential oils as a tool in the control of bovine mastitis: an update**. Molecules**, v. 28, n. 8, p. 3425, 2023.

2 Embrapa Gado de Leite, Rentero, N. Projeção de tendências para o leite aqui e lá fora. **Anuário leite 2023**. Embrapa gado de leite. 2023.

3 Sharma N, Jeong DK. Stem cell research: a novel boulevard towards improved bovine mastitis management. **Int J Biol Sci** 2013, 9, 818-829.

4 NUÑEZ, M., Karen Vanessa et al. Antimicrobial Activity of Selected Essential Oils against *Staphylococcus aureus* from Bovine Mastitis. **Dairy**, v. 5, n. 1, p. 54-65, 2024.

5 OIE. World Organization for Animal Health. Risk assessment for antimicrobial resistance arising from the use of antimicrobials in animals. In: OIE. Terrestrial animal health code. Paris: **OIE**, 2008. (Capítulo 6.8. p. 16). Disponível em <httD://www.oie.intlena/normes/Mcode/ensommaire.htm.> Acesso em 13 de novembro de 2023.

6 MUSHTAQ, Sadaf et al. Natural products as reservoirs of novel therapeutic agents**. EXCLI journal**, v. 17, p. 420, 2018.

7 Budama-Kilinc, Y.; Gok, B.; Kecel-Gunduz, S.; Altuntas, E. Development of Nanoformulation for Hyperpigmentation Disorders: Experimental Evaluations, in Vitro Efficacy and in Silico Molecular Docking Studies. **Arab. J. Chem**., 2022, 15 (12), 104362.

8 CLSI. Clinical and Laboratory Standards Institute. Method for broth dilution antifungal susceptibility testing of yeasts: approved standard-third edition M27-A3. **CLSI**, Wayne, PA, USA, 2008.

9 CLSI. Clinical and Laboratory Standards Institute. Methods for dilution in antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard M07- A9, 9 ed., n. 2. Pensilvânia: **CLSI**, 2012a. 68p.

10 CLSI. Reference Method for Broth Dilution Antifungal Susceptibility testing of Filamentous Fungi. 3rd Ed. **CLSI** standard M38 [Internet]. 2017. Available from: www.clsi.org

11 ANVISA. Guia n. 28, de 11 de novembro de 2019: Guia de Estudos de Estabilidade. https://pesquisa.anvisa.gov.br/upload/surveys/15455/files/Guia%20de%20Estudos%20de%20Estabiliade(1).PDF. **Anvisa:** Brasilia-DF 2019.

12 ALMEIDA, A. C. de; et al. Atividade Antisséptica Do Óleo Essencial de *Lippia Origanoides* Cham. (Alecrim-Pimenta) Na Presença de Leite Bovino. **Pesqui. Veterinária Bras**., 2016, 36, 905–911.

13 JAFARI, Seid Mahdi; MCCLEMENTS, D. Julian (Ed.). Nanoemulsions: formulation, applications, and characterization. **Academic Press**, 2018.

14 Barreto, S.M.A.G., et al. Evaluation of in vitro and in vivo safety of the by-product of Agave sisalana as a new cosmetic raw material: Development and clinical evaluation of a nanoemulsion to improve skin moisturizing, **Ind. Crops Prod**. 108 (2017) 470–479, https://doi.org/10.1016/j.indcrop.2017.06.064

15 Mahdi, E.S., A.M. Noor, M.H. Sakeena, G.Z. Abdullah, M.F. Abdulkarim, M. A. Sattar, Formulation and in vitro release evaluation of newly synthesized palm kernel oil esters-based nanoemulsion delivery system for 30% ethanolic dried extract derived from local *Phyllanthus urinaria* for skin antiaging, **Int. J. Nanomed**.6 (2011) 2499–2512.

16 Viana, V. C. R.; Machado, F. P.; Esteves, R.; Duarte, J. A. D.; Enríquez, J. J. S.; Campaz, M. L. M.; Oliveira, E. E.; Santos, M. G.; Ricci-Junior, E.; Ruppelt, B. M.; et al. Green Nanobioinsecticide of a Brazilian Endemic Plant for the *Aedes Aegypti* Control. **Sustain. Chem. Pharm**., 2023, 32, 100992.

17 GÓMEZ-SEQUEDA, Nicolás et al. Antimicrobial and antibiofilm activities of essential oils against *Escherichia coli* O157: H7 and methicillin-resistant *Staphylococcus aureus* (MRSA). **Antibiotics**, v. 9, n. 11, p. 730, 2020.

18 CACERES, Marlon et al. Metabolomic Analysis of the Effect of *Lippia origanoides* Essential Oil on the Inhibition of Quorum Sensing in *Chromobacterium violaceum*. **Antibiotics**, v. 12, n. 5, p. 814, 2023.

19 HARWANSH, Ranjit K.; DESHMUKH, Rohitas; RAHMAN, Md Akhlaquer. Nanoemulsion: Promising nanocarrier system for delivery of herbal bioactives. **Journal of Drug Delivery Science and Technology**, v. 51, p. 224-233, 2019.

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