Supplementary Material

The development and physicochemical characterization of *Linum usitatissimun* oil nanoemulsion loaded with macela extract (*Achyrocline satureioides)* was carried out to optimize its efficacy and stability (Pinheiro Machado et al., 2020). Different formulations were prepared and tested to identify the most efficient composition in terms of physicochemical stability and antimicrobial activity. After analyzing the evaluated parameters, the formulation called NE-ML 1:5 showed the best performance and was selected for the investigation in this manuscript, which aims to help elucidate the mechanism of action of this formulation. The information on the characterization of this formulation is summarized in Supplementary Table 1, which describes the results obtained for each parameter analyzed and highlights the most relevant aspects of the formulation in terms of its stability and antimicrobial efficacy against *S. aureus*.

Supplementary Table 1. Physicochemical characterization and antimicrobial efficacy of NE-ML 1:5 nanoemulsion formulation.

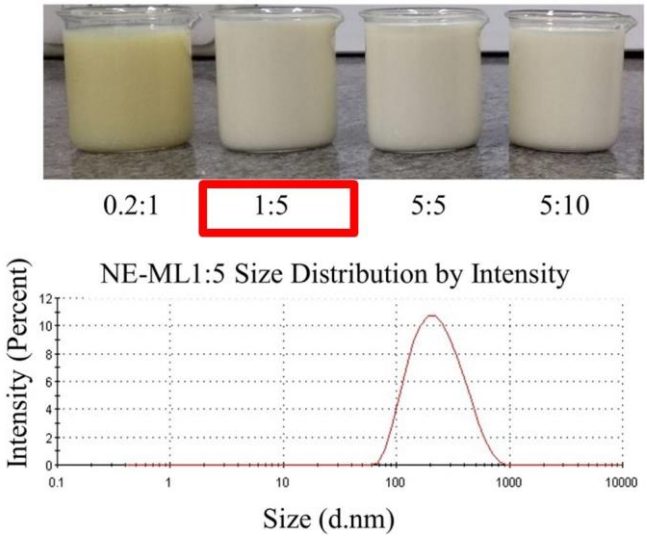
Parameter	Method	Description	Reference
Composition of macela-loaded nanoemulsion (NE-ML)	High-pressure homogenization (HPH)	Tween 80 (1%, w/v) Flaxseed Oil (5%, w/v) Extractive macela solution (20%, v/v)[a] Water (100 q.s. to) (mL)	Machado et al., 2020
Chemical characterization of macela extract	High-performance liquid chromatography (HPLC)	3-O-methylquercetin (187.3 ± 0.1 μg ml^{-1}) ACB (155.4 ± 11.6 μg ml^{-1}) Quercetin (76.3 ± 0.1 μg ml^{-1}) Luteolin (30.4 ± 0.0 μg ml^{-1})	Machado et al., 2020
Chemical characterization of flaxseed oil	Gas chromatography coupled to mass spectrometry (GC/MS)	Linoleic (LOA) (30.5% ± 0.1) Alpha-linolenic (30.7% ± 0.1) Oleic (25.7% ± 0.1) Palmitic acid (7.2%) Stearic acid (4.2%)	Machado et al., 2020
Encapsulation efficiency of macela compounds in nanoemulsions	Ultrafiltration and centrifugation technique	> 94%	Machado et al., 2020
Visual analysis of NE-ML	Visual description	Homogeneous formulation without precipitation or phase separation and yellowish milky appearance	Machado et al., 2020
Stability studies over time - storage at room temperature (Day 0)	Dynamic light scattering (DLS) and Laser Doppler Anemometry	Mean particle size (nm) 249.9 ± 2.6 Polydispersity index (PdI) 0.17 ± 0.0 Zeta potential ZP (mV) −39.9 ± 2.7 pH 5.1 ± 0.0	Machado et al., 2020
Stability studies over time - storage at room temperature (Day 160)	Dynamic light scattering (DLS) and Laser Doppler Anemometry	Mean particle size (nm) 240.2 ± 0.4 Polydispersity index (PdI) 0.18 ± 0.0 Zeta potential ZP (mV) −38.2 ± 1.5 pH 4.6 ± 0.0	Machado et al., 2020
Analysis of the accelerated physical stability of the nanoformulation	Dispersion analyzer	Instability index between 0.2–0.6	Machado et al., 2020

Antimicrobial activity (Planktonic bacteria)	Broth microdilution method	MIC_{50} 1.2% (v/v) MIC_{90} 5% (v/v)	Machado et al., 2020
Evaluation of biofilm prevetion	96-well microplate	NE-ML1:5 prevented 100% of biofilm formation at concentrations of 10% and 25% (v/v) for 2 MRSA strains from mastitic bovine milk. At 25% (v/v) it reduced biofilm mass by over 64% for ATCC 25923 and 2 MRSA strains.	Machado et al., 2020
Permeation and retention of free and nano-encapsulated chemical markers of the macela extract	Franz diffusion cells	The final permeation of quercetin and 3-O-methylquercetin in NE-ML was 50.7 ± 3.2 and 111.2 ± 0.6 μg/cm^2, respectively, compared to the final permeation of free extract of 35.0 ± 0.6 and 48.9 ± 1.2 μg/cm^2, respectively	Pinheiro Machado et al., 2022
Cell viability of mammary epithelial cells (MAC-T)	MTT method (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidei)	After exposure to NE-ML (5 and 1.2% v/v), the percentage of apoptotic cells was reduced by ±30%	Pinheiro Machado et al., 2022
Necrotic or apoptotic cell death	Flow cytometry and exposure to H_2O_2 (2 mM)	In the H_2O_2 assay, the percentage of cells in necrosis was reduced by 40% after exposure to NE-ML at 1% (v/v) + 2 mM H_2O_2	Pinheiro Machado et al., 2022

[a] In the final composition of the macela nanoemulsions (NE-ML), the extract content corresponded to 2.5 mg mL^{-1}.

Visually, the formulation was homogeneous, with no precipitation or phase separation, and presented a milky yellow appearance, as shown in Supplementary Figure 1. This figure illustrates the different treatments applied during the development of the nanoformulation, with the NE-ML 1:5 treatment being the one selected for this manuscript due to its superior performance.

Supplementary Figure 1. The physical appearance of the nanoemulsion loaded with macela (20%, v/v equivalent to 2.5 mg ml^{-1} of extract; NE-ML) and droplet size distribution of NE-ML1:5.



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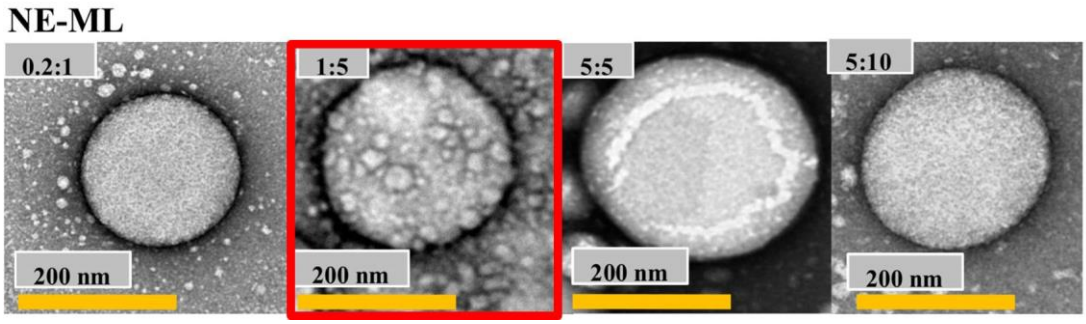
In addition to the visual inspection, the morphological characterization of the macela nanoemulsion was performed using transmission electron microscopy (TEM) (JEOL JEM 1011 TEM), which

allowed a detailed observation of the particle structure. Supplementary Figure 2 shows the TEM micrographs of the different formulations tested, including the NE-ML 1:5 treatment, which was selected for this study. The micrographs showed that the particles in the selected formulation were spherical, which is a key feature contributing to its physicochemical stability and antimicrobial efficacy. This structural analysis supports the findings summarized in Table 1 and reinforces the selection of the NE-ML 1:5 formulation for further investigation in this manuscript.

Supplementary Figure 2. Transmission electron micrographs of the nanoemulsion loaded with macela (NE-ML 1:5).



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References

Machado, G.T.P., Veleirinho, M.B., Honorato, L.A., and Kuhnen, S. (2020). Formulation and evaluation of anti-MRSA nanoemulsion loaded with Achyrocline satureioides: a new sustainable strategy for the bovine mastitis. *Nano Express* 1 (3), 030004. doi.org/10.1088/2632-959X/abbcac. License CC BY 4.0

Pinheiro Machado, G.T., Veleirinho, M.B., Ferreira, R.G., Zuglianello, C., Lemos-Senna, E., and Kuhnen, S. (2022). Protection of bovine mammary epithelial cells by a nanoemulsion of the medicinal herb *Achyrocline satureioides* (Lam.) DC and its capacity of permeation through the mammary epithelium. *Journal of Dairy Research* 89 (1), 80–85. doi.org/10.1017/S0022029922000139