Protection of bovine mammary epithelial cells by *Achyroline satu*reioides (Lam.) DC. nanoemulsion and its capacity of permeation through mammary epithelium

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SUPPLEMENTARY FILE

Material and methods

Macela samples and extract preparation

Commercial samples of macela were acquired from "*Entre Ervas"*, a company in southern Brazil that produces aromatic and medicinal herbs (30°02'14.89"S/51°12'39.08"W). The plants were taxonomically identified and a voucher specimen deposited at the FLOR Herbarium (Universidade Federal de Santa Catarina) under number FLOR66429. The activity to access to Genetic Heritage was registered in the National System of Management of Genetic Heritage and Traditional Knowledge Associated (SisGen) under number A7300B9.

Characterization of nanoemulsions

Table S1. Composition and physicochemical characteristics of nanoemulsions with flaxseed oil and macela extract developed by high-pressure homogenization (HPH).

Variables	**NE-BL$_{0.2:1}$**	**NE-BL$_{1:5}$**	**NE-BL$_{5:5}$**	**NE-BL$_{5:10}$**
Tween 80 (%, w/v)	0.2	1	5	5
Flaxseed oil (%, w/v)	1	5	5	10
Extractive macela solution (%, v/v) *	-	-	-	-
Water (q.s. to) (mL)	100	100	100	100
Size (nm)	251.4±6.0	227.0±3.6	212.8±2.5	187.7±3.5
PdI*	0.19±0.0	0.19±0.0	0.21±0.0	0.23±0.0
Zeta potential (mV)	-37.6±1.8	-30.6±0.9	-27.1±1.2	-26.1±1.7
pH	4.7±0.2	4.9±0.2	5.1±0.1	5.2±0.1
Variables	**NE-ML$_{0.2:1}$**	**NE- ML$_{1:5}$**	**NE-ML$_{5:5}$**	**NE- ML$_{5:10}$**
Tween 80 (%, w/v)	0.2	1	5	5
Flaxseed oil (%, w/v)	1	5	5	10
Extractive macela solution (%, v/v)*	20	20	20	20
Water (q.s. to) (mL)	100	100	100	100
Size (nm)	229.8±4.8	249.9±2.6	222.3±2.0	203.9±2.7
PdI*	0.19±0.0	0.17±0.0	0.18±0.0	0.23±0.0
Zeta potential (mV)	-40.3±1.0	-39.9±2.7	-38.6±2.5	-34.5±0.9
pH	4.8±0.0	5.1±0.0	4.6±0.0	4.7±0.0

*In the final composition of the macela nanoemulsions (NE-ML), the extract content corresponded to 2.5 mg mL^{-1}. Proportions following NE-B and NE-ML refer to the concentration of Tween 80 and flaxseed oil in the formulations. * PdI: polydispersity index, suggests the homogeneity of the system. Values ≤ 0.2 indicate greater sample homogeneity.

Determination of macela phenolic compounds by high-performance liquid chromatography with diode-array detection (HPLC/DAD)

Analysis of the phenolic composition of the macela extract was performed by HPLC/DAD, using a Thermo Scientific UltiMate 3000 RS Dual System (Thermo Fisher Scientific, San Jose, CA) with a Thermo Scientific C18 reverse-phase column (4.6 x 150 mm; 5 µm; 120Å; Acclaim™, Thermo Scientific©) at 25°C, operating at 240, 270, 320 and 375 nm. The mobile phase consisted of Milli-Q® water acidified to pH 2.3 (A) and methanol (B) eluted at a flow of 1.0 mL/min using the following gradient program: 0-5 min, 90% A; 5-25 min, 30% A; 25-37 min, 90%A. The identification of quercetin, 3-O-methylquercetin, and luteolin was performed by comparison with retention times of the commercial standards obtained from Sigma-Aldrich (São Paulo, Brazil, Q4951) according to a previously developed method (Pinheiro Machado et al. 2020) that has shown to be specific, precise and linear for quercetin assay (range of 0.97 –1000 µg mL^{-1}, r^2> 0.999, y = 0.168x). The detection limit (DL) was 0.19 µg mL^{-1}, whereas the quantification limit (QL) was 0.63 µg mL^{-1}. To the standard deviation of the response (σ), the *y*-intercepts obtained from three calibration curves were considered, and to S, the slope of the mean calibration curve was considered. For the glandular epithelium permeation and retention experiments, the specificity of the method was determined by analyzing the neat extract of the glandular epithelium at the same conditions in triplicate. The intra-day precision was assessed by six determinations at the test concentration (established at 4.0 µg mL^{-1}, corresponding to 27.8 µg/cm^2 of the membrane for retention experiments), and it was repeated on another day to assess the inter-day precision. These values were reported as relative standard deviation (RSD) between determinations. The accuracy, in terms of percent of recovery of an amount of

analyte added in the sample, was assessed by nine determinations at three concentration levels (3.5; 7.0 and 14.0 µg mL^{-1}).

Viability of MAC-T cells

Bovine epithelial cells of the MAC-T (mammary alveolar cells -T) lineage from the Cell Bank of Rio de Janeiro (BCRJ) were maintained in culture. Briefly, MAC-T cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) and supplementedat 37°C and 5% CO_2 in a humidified incubator. When the cells reached 70% confluence, they were treated with 0.25% trypsin in 1 mM EDTA to prepare the cellular suspension (10^5 cells/mL). The formed formazan on the MTT method was dissolved with dimethyl sulfoxide (DMSO) to give a purple color with characteristic absorption at 540 nm. Intensity of purple color is directly proportional to the number of viable cells, thus indicating cell viability.

In vitro mammary glandular epithelial permeation and retention analysis

Mammary explants of dry cows intended for disposal were obtained from a local meat processor (Águas Mornas, Brazil) immediately after slaughter. Later they were cleaned with ultrapure water, packed in phosphate-buffered saline (PBS, pH 7.4), and stored at 10°C until processing. The epithelial glandular tissue was detached from the explants and inserted between the donor and receptor compartments of Franz cells with the apical side facing up. The receptor chamber was filled with 50% hydroethanolic solution (11.0 mL) in order to maintain sink conditions (Fasolo *et al*. 2009; Zorzi *et al*. 2016). The receptor medium was maintained under magnetic stirring at 37°C during the experiments. To assess permeation, 1 mL of NE-ML1:5, or the aqueous extract of macela containing 77 µg mL^{-1} of quercetin, 188 µg mL^{-1} of 3-O-methylquercetin, and 30 µg mL^{-1} of luteolin, was added to the donor compartment. Aliquots of receptor

medium (1.0 mL) were collected at predetermined times (0.5, 1, 2, 4, 6, 8, 10 and 12 h) to assess the compounds of interest. The same volume of fresh receptor medium was immediately restored to the cells to maintain sink conditions. The samples were filtered through regenerated cellulose membranes of 0.45 µm and analyzed by HPLC/DAD as described in the online Supplementary File. After 12 h of the experiment, the excess formulation was removed from the donor compartment with a piece of cotton. Tissue slices were then removed from the Franz cells and fragmented for quantification of the compounds retained in the epithelium. The compounds of interest were extracted from the fragments with methanol (2.0 mL), using an ultrasound probe for 2 min followed by an ultrasound bath for 10 min. The method showed intra- and inter-day precision (RSD), respectively, of 2.7% and 2.3% and recovery of 103.0 ± 7.1%, 97.0 ± 1.3% and 105.0 ± 4.3% from the smallest to the highest level tested (respectively 3.5; 7.5 and 14.0 µg mL^{-1}).

RESULTS

Viability of MAC-T cells

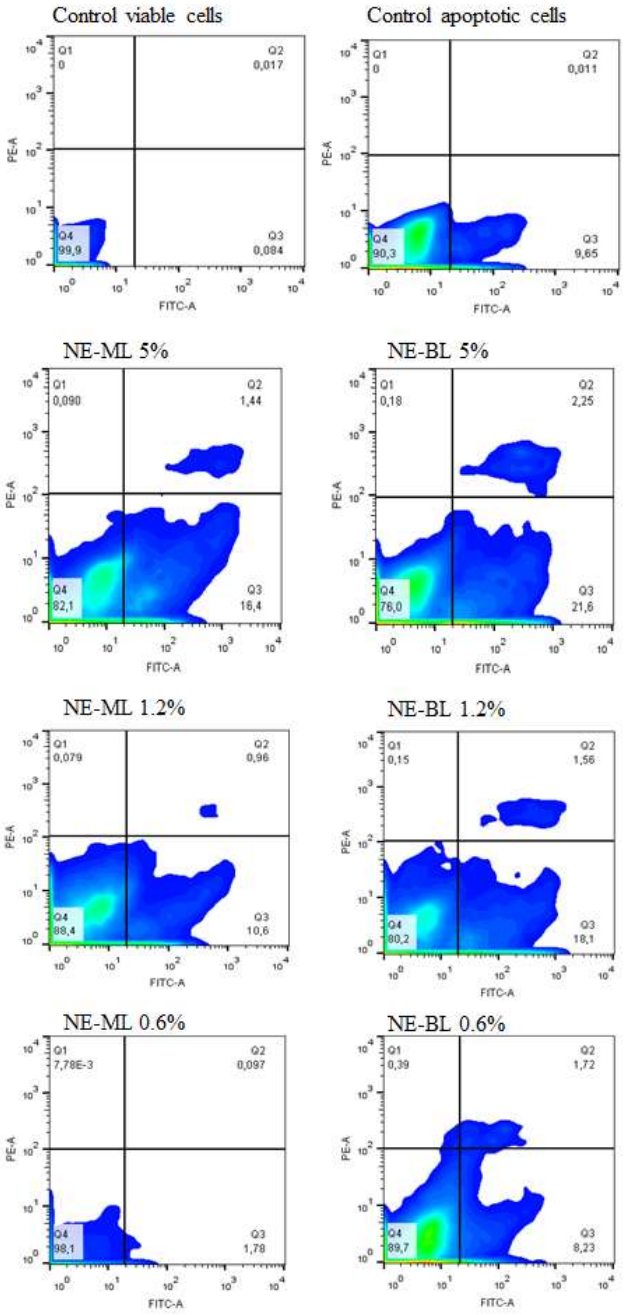
Table S2. Percentage of viable bovine epithelial cells from the MAC-T lineage (mean ± SEM) after exposure to different concentrations of macela-nanoemulsions (NE-ML) and blank-nanoemulsions (NE-BL), flaxseed oil and macela extract.

Concentration (mg/mL)	Control	Flaxseed oil	Macela extract
4	100 ± 0.0^{a}	33.6 ± 4.5^{bA}	0.0 ± 0.3^{cB}
2	100 ± 0.1^{a}	78.8 ± 4.2^{bA}	0.0 ± 0.3^{cA}
1	100 ± 0.0^{a}	87.0 ± 2.1^{bA}	0.0 ± 0.2^{cB}
0.7	100 ± 0.0^{a}	88.0 ± 2.8^{bA}	$9.3 \pm 0.^{3cB}$
0.3	100 ± 0.2^{a}	93.6 ± 4.2^{aA}	26.8 ± 1.7^{cB}
0.1	100 ± 0.3^{a}	94.0 ± 4.9^{aA}	44.0 ± 2.2^{bB}
0.09	100 ± 0.2^{a}	100 ± 0.5^{aA}	58.3 ± 4.0^{bB}
0.04	100 ± 0.1^{a}	100 ± 0.2^{aA}	78.3 ± 4.1^{bB}
0.02	100 ± 0.0^{a}	100 ± 0.6^{aA}	93.0 ± 3.6^{aA}
0.01	100 ± 0.1^{a}	100 ± 0.0^{aA}	99.3 ± 4.2^{aA}

The results shown are the average of three independent experiments. Different lowercase letters in the same row indicate significant statistical difference between treatments and control. Different uppercase letters in the same row indicate statistically significant difference between treatments ($P < 0.05$).

Apoptosis and necrosis induction

Figure S1. Graphical representation of the percentage of normal (lower left quadrant), initial apoptotic (lower right quadrant), late apoptotic (upper right quadrant), and necrotic (upper left quadrant) cells after exposure to different concentrations of the nanoemulsion NE-$ML_{1:5}$ and NE-$BL_{1:5}$.



In vitro mammary glandular epithelial permeation and retention analysis

Table S3. Lag time (Tlag), steady state flow (J), and permeability coefficient (Kp) obtained for quercetin and 3-O-Methylquecetin in a permeation experiment on bovine mammary gland explants.

	Tlag (h)	**J (μg/cm^2/h)**	**Kp (cm/h)**
Quercetin/Extract	n.a.	3.4 ± 1.2^{a}	$4.7 \times 10^{-2} \pm 5.4 \times 10^{-4\ a}$
Quercetin/NE-ML	3	4.6 ± 0.2^{b}	$6.0 \times 10^{-2} \pm 2.5 \times 10^{-3\ b}$
3-O-Methyl/Extract	n.a.	4.5 ± 0.1^{a}	$2.4 \times 10^{-2} \pm 3.0 \times 10^{-4\ a}$
3-O-Methyl/NE-ML	6	8.3 ± 0.2^{b}	$4.4 \times 10^{-2} \pm 1.4 \times 10^{-3\ b}$

n.a.: not applicable. Values expressed as an average ± SD. Different lowercase letters in the column represent significant pairwise differences ($P < 0.05$).

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