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Thymus mastichina (L.) L. and *Cistus ladanifer* L. for skin application: chemical characterization and *in vitro* bioactivity assessment

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ABSTRACT

Ethnopharmacological relevance: Thymus mastichina (L.) L. (TM) and *Cistus ladanifer* L. (CL) are two Portuguese autochthonous species with traditional skin application in folk medicine. TM is majorly known for its antiseptic and wound healing properties, as an external anti-inflammatory agent and for its application in folk cosmetics and hygiene products. Its use in *acne vulgaris* has also been reported. CL is traditionally used in remedies for wounds, ulcers and other skin ailments such as psoriasis and eczema. Its application has been found useful due to its anti-inflammatory, astringent, wound healing and antiseptic properties.

Aim of the study: With this work, we aimed to investigate relevant bioactivities related with the traditional application of TM and CL essential oils (EOs) and hydrolates (by-products of EO production) in skin ailments. Specifically their *in vitro* antioxidant, anti-inflammatory, cytotoxic, wound healing and antimicrobial properties were evaluated. The chemical composition of both EOs and respective hydrolates was also characterized.

Materials and methods: Chemical characterization of EOs and hydrolates was performed by GC-FID and GC-MS. Cellular biocompatibility was evaluated using the MTT assay in macrophages (RAW 264.7) and fibroblasts (L929) cell lines. Anti-inflammatory activity was investigated by studying nitric oxide (NO) production by macrophages with Griess reagent. Wound healing potential was evaluated with the scratch-wound assay. The antioxidant potential was studied by the DPPH scavenging method. Antimicrobial activity was evaluated by broth microdilution assay against relevant microbial strains and skin pathogens, namely *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Cutibacterium acnes*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Candida albicans* and *Aspergillus brasiliensis*.

Results: The major compounds present in TM and CL EOs were 1,8-cineole and α-pinene, respectively. 1,8-cineole and *E*-pinocarveol were the major compounds in the correspondent hydrolates. CL EO presented the highest anti-inflammatory potential [EC_{50} = 0.002% (v/v)], still with significant cytotoxicity [IC_{50} = 0.012% (v/v)]. TM preparations presented anti-inflammatory potential, also presenting higher biocompatibility. The same profile was present on fibroblasts regarding biocompatibility of the tested preparations. CL EO and hydrolate increased fibroblasts' migration by 155.7% and 148.4%, respectively. TM hydrolate presented a milder activity than CL hydrolate, but wound healing potential was still present, increasing cell migration by 125.1%. All preparations presented poor antioxidant capacity. CL EO presented higher antimicrobial activity, with MICs ranging from 0.06% (v/v) to 2% (v/v), against different microorganisms.

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Conclusions: Anti-inflammatory and skin repairing potential were present for CL preparations. TM hydrolate presented an interesting biocompatible profile on both cell lines, also presenting anti-inflammatory potential. Furthermore, EOs from both species presented antimicrobial activity against a panel of different microorganisms. These *in vitro* bioactivities support some of their traditional skin applications, specifically regarding their antiseptic, wound healing and anti-inflammatory uses.

Abbreviations

5-LOX	5-Lipoxygenase;
A. brasiliensis	*Aspergillus brasiliensis*;
ATCC	American-Type Culture Collection;
BHI	Brain Heart Infusion Broth;
C. acnes	*Cutibacterium acnes*;
C. albicans	*Candida albicans*;
CFU	Colony Forming Units;
CL	*Cistus ladanifer* L.;
CLSI	Clinical and Laboratory Standards Institute;
DMSO	Dimethyl sulfoxide;
DPPH	2,2-Diphenyl-1-picrylhydrazyl;
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen;
DMEM	Dulbecco′s Modified Eagle′s Medium;
EC_{50}	Half-maximal effective concentration;
E. coli	*Escherichia coli*;
EDTA	Ethylenediaminetetraacetic acid;
eNOS	Endothelial nitric oxide synthase;
EO(s)	Essential oil(s);
EUCAST	European Committee on Antimicrobial Susceptibility Testing;
FBS	Fetal bovine serum;
GC-FID	Gas chromatography with flame ionization detection;
GC-MS	Gas chromatography-mass spectrometry;
IC_{50}	Half-maximal inhibitory concentration;
iNOS	Inducible nitric oxide synthase;
ISO	International Organization for Standardization;
LPS	Lipopolysaccharide;
MHB	Mueller Hinton Broth;
MIC	Minimum inhibitory concentration;
MLC	Minimum lethal concentration;
MTT	3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide;
NO	Nitric oxide;
P. aeruginosa	*Pseudomonas aeruginosa*;
PBS	Phosphate-buffered saline;
PDA	Potato Dextrose Agar;
RPMI 1640	Roswell Park Memorial Institute 1640;
S. aureus	*Staphylococcus aureus*;
sBHI	Brain Heart Infusion Broth supplemented with 5% glucose;
S. epidermidis	*Staphylococcus epidermidis*;
SDA	Sabouraud Dextrose Agar;
SDS	Sodium dodecyl sulfate;
SNAP	*S*-Nitroso-*N*-acetylpenicillamine;
TLR4	Toll-like receptor 4;
TM	*Thymus mastichina* (L.) L.;
TSA	Tryptic Soy Agar.

1. Introduction

Plant preparations, including essential oils (EOs), hydrolates and plant extracts, have been used in traditional medicine due to their unique properties and as a source of bioactive compounds (Ferraz et al., 2022). In fact, preparations like herbal infusions are known for their medicinal properties since ancient times and have a very long tradition in medicine systems worldwide (Tschiggerl and Bucar, 2012). Among different plant preparations, EOs have also gained major attention, and their use as ingredients for cosmetics, perfumes, flavors or health promoters, is known since antiquity (Bohn, 2012). EOs are volatile, natural and complex compounds characterized by having a strong odour and are formed by aromatic plants as secondary metabolites (Bakkali et al., 2008). Less information is available regarding hydrolates, by-products of EO isolation. Simplistically, these diluted aromatic aqueous solutions result from the solubilization of the most hydrophilic volatile compounds in the condensation water generated during the steam distillation process used in the isolation of EOs. Still, hydrolates have recently gained the attention of different industries and the scientific community due to the increasing evidence for new uses and novel opportunities to aggregate value to waste or by-products of the EO industry (Di Vito et al., 2021).

One of the most important application of plant preparations, including EOs and hydrolates, is on products for skin application, either as promotors of overall skin health or to treat different skin ailments. In fact, approximately one-third of all traditional medicines that include different plant preparations, are for treating wounds or skin disorders (Zeng et al., 2017). This gains major relevance considering that skin diseases are the fourth most common cause of all human diseases and affect almost one-third of the world's population, with their burden being underestimated, despite their visibility (Flohr and Hay, 2021). The application of plant preparations toward skin conditions is dependent on their activity as antimicrobial, anti-inflammatory, anti-proliferative, wound healing and photo-protective agents (Reuter et al., 2010).

This increasing interest in plant preparations and the associated increase in their demand has led to an increase in the investment and production of specific plants by plant producers. Still, with the paradigm shift concerning sustainability, it is essential to recognize the role of autochthonous species and their benefits to the ecosystem, as these species are highly adapted to their habitats and present advantages to biodiversity. In addition, autochthonous plants typically have traditional knowledge associated with their use. Therefore, it is important to valorize this knowledge by scientifically supporting the biological activities related to their folk use.

Thymus mastichina (L.) L. (Lamiaceae) is a small sub-woody shrub that grows in land clearings, especially in the thickest zones. It is an autochthonous species of Portuguese territory and unique in the Iberian Peninsula (Iberian endemism) (Faleiro et al., 2003; Méndez-Tovar et al., 2015). This widespread Mediterranean species is considered a medicinal plant, although some other common uses have been reported, specifically for food purposes in local cuisines. Regarding its medicinal application, this species is known for its use in folk medicine due to its anti-inflammatory and antiseptic properties, being typically used for external inflammations and skin diseases (Cutillas et al., 2018; Pardo-de-Santayana et al., 2007). Ethnopharmacological studies have reported its use in skin burns and corrosions, in wounds of unspecified body regions, and as folk cosmetics, disinfectants and antiseptics (González et al., 2012; Rivera et al., 2019). The Spanish Inventory of Traditional Knowledge Related to Biodiversity reported its widespread

dermatological application as an antiseptic and wound healing agent and also its use for acne treatment (Pardo de Santayana et al., 2014). Apart from skin applications, other medicinal properties are attributed to this species particularly for the respiratory and gastrointestinal systems, with infusions of dried material and herbal teas with the fresh plant being used for colds, coughs, throat irritations and for indigestion and stomach ache, respectively (Barros et al., 2010; Pardo de Santayana et al., 2014).

Another autochthonous species from the Iberian Peninsula with a marked traditional application is *Cistus ladanifer* L. (Cistaceae). This species is a resinous and fragrant evergreen shrub, very abundant among other wild species in the forest and uncultivated areas, and is well known for its resin, labdanum (Deforce, 2006; Frazão et al., 2018; Kivi, 2022; Raimundo et al., 2018). Traditionally, labdanum was collected from the beards and fur of goats that grazed amongst *Cistus* shrubs or by walking through the *Cistus* shrubs with a traditional bow-shaped instrument with strips wrapped in wool (Deforce, 2006). Nowadays, labdanum is mainly extracted in Portugal and Spain and the oleoresin, EO and other extracts of *C. ladanifer* are used as fixatives in perfumery and in cosmetics (Frazão et al., 2018, 2022; Gaweł-Bęben et al., 2020). Its folk application as a medicinal plant is majorly related to its wound healing activity and its EO is used for wounds, skin ulcers, to stop hemorrhages, to avoid secondary infections and for other skin ailments such as psoriasis and eczema (Adadi et al., 2022; Hewitt, 2017; Pardo de Santayana et al., 2014). In addition to its application to treat superficial wounds, its antiseptic use is also reported in ethnopharmacological studies (Novais et al., 2004; Rivera et al., 2019). Apart from its relevance for skin application, other medicinal applications in other target organs have been reported for its EOs, and/or extracts as anti-ulcerogenic, anti-acid, anti-diarrheal, antispasmodic, diuretic, analgesic, hypoglycemic, antihypertensive and vasodilator (Adadi et al., 2022).

With this study, we aimed to characterize the potential of EOs and hydrolates from two Portuguese autochthonous species, namely *T. mastichina* (TM) and *C. ladanifer* (CL), as bioactive ingredients for skin application. Specifically, we intended to characterize their chemical profile along with their *in vitro* antioxidant, anti-inflammatory, cytotoxic, wound healing and antimicrobial properties against selected microorganisms, in order to support their beneficial roles and traditional knowledge for skin use in different ailments. By studying not only EOs but also the hydrolates, by-products of EO production, we further intended to contribute to the added value of these products, thus promoting a circular economy.

2. Materials and Methods

2.1. Chemicals

The microbiological culture media Tryptic Soy Agar (TSA), Mueller Hinton Broth (MHB), Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA) were supplied by VWR Chemicals (VWR, Radnor, Pennsylvania, USA). Brain Heart Infusion Broth (BHI) and Bacteriological Agar type E were supplied by Himedia (L.B.S. Marg, Mumbai, India). Glucose, Fetal bovine serum (FBS), sodium bicarbonate, lipopolysaccharide (LPS), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), sodium nitrite, sulphanilamide, phosphoric acid, *N*-(1-naphthyl)-ethylenediamine dihydrochloride, dexamethasone, ciprofloxacin, gentamicin, tetracycline, benzylpenicillin, ampicillin, erythromycin, fluconazole and amphotericin B were purchased from Sigma-Aldrich (St. Louis, MO, USA). The anaerobic generating system Anaerocult® A was supplied by Merck (Darmstadt, Germany). Roswell Park Memorial Institute (RPMI) 1640, streptomycin, trypsin/ethylenediaminetetraacetic acid (EDTA) solution and Dulbecco's Modified Eagle's Medium (DMEM) were supplied by Biowest (VWR, Radnor, Pennsylvania, USA). Dimethyl sulfoxide (DMSO) and methanol were purchased from Honeywell (Charlotte, North Carolina, USA). Crystal violet stain was acquired from Amresco (Solon, OH, USA). 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was supplied by Alfa Aesar (Massachusetts, USA). Propan-2-ol (or isopropanol), acetic acid and ascorbic acid were supplied by Fisher Chemicals (Fisher, Chicago, IL, USA). Sodium dodecyl sulfate (SDS) was purchased from PanReac AppliChem (Barcelona, Spain) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) was acquired from Biogen, Tocris (Bristol, UK). Allantoin was supplied by Guimana (Valencia, Espanha).

2.2. Plants and plant preparations

Plant names for TM (common name: mastic thyme or 'tomilho bela-luz') and CL (common name: rockrose or 'esteva') were checked with the new version of https://www.theplantlist.org, specifically, http://www.worldfloraonline.org.

The studied plant preparations were commercially acquired from Portuguese companies. TM EO (batch: TM010719) and hydrolate (batch: HTM072020), produced from the distillation of leaves, flowers and thin branches were obtained from Planalto Dourado® company (Portugal). Plants were produced under organic farming, as certified by PT-BIO-04.

CL EO and respective hydrolate (batches: 6), produced from steam distillation of leaves, flowers and thin branches, were acquired from Proentia® company (Portugal). Plants were produced under organic farming, as certified by PT-BIO-03.

2.3. Chemical analyses of essential oils and hydrolates

The composition of EOs and hydrolates were determined by a combination of gas chromatography with flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS) following the methodology and the conditions previously described (Oliveira et al., 2021).

Briefly, each hydrolate was added to *n*-dodecane as an internal standard and submitted to liquid/liquid partition with *n*-pentane: diethyl oxide [93:7 (v/v)]. The organic fraction was concentrated at low pressure and room temperature and set to final volume with *n*-pentane. EO samples were diluted (1:8) in *n*-pentane. GC-FID analysis was performed in a Hewlett-Packard 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph with a single injector and two flame ionization detectors for simultaneous sampling on two different columns (SPB-1: polydimethylsiloxane 30 m × 0.20 mm i.d., film thickness 0.20 μm; and SupelcoWax-10: polyethyleneglycol 30 m × 0.20 mm i.d., film thickness 0.20 μm) (both from Supelco, Bellefonte, PA, USA). GC-MS analyses were performed with an Agilent 6890 gas chromatograph interfaced with a mass selective detector MSD 5973 (Agilent Technologies). An HP1 column (polydimethylsiloxane 30 m × 0.25 mm i.d., film thickness 0.25 μm) was used. For GC-FID and GC-MS parameters refer to (Oliveira et al., 2021). EOs samples were injected (0.2 μL) in split mode (1:40). Hydrolate samples were injected (0.2 μL) in splitless mode.

Identification of the hydrolates and EOs components were achieved by considering, concurrently: 1) the acquired retention indices on both SPB-1 and SupelcoWax-10 columns determined by linear interpolation relative to the retention times of C8–C24 of *n*-alkanes and compared with reference data from authentic products (available in the laboratory database of the Faculty of Pharmacy, University of Coimbra) and literature data (Adams, 2007; Linstrom and Mallard, 2019); 2) the acquired mass spectra compared with reference data from the Wiley/NIST library (McLafferty, 2009).

The relative amounts (%) of each component of the EOs and the hydrolates' volatile fraction were calculated from GC-FID raw data without any correction. Absolute concentrations of each component of the hydrolates were calculated using the internal standard method and expressed as *n*-dodecane.

2.4. Antioxidant potential – DPPH radical scavenging assay

The antioxidant potential was determined by evaluating the free radical scavenging potential for DPPH using the original Molyneux method, with the scale down adaptation for microplates, proposed by Prieto (Molyneux, 2004; Prieto, 2012).

Serial dilutions of plant preparations were prepared in a 96 multiwell plate and mixed with an equal volume of a DPPH solution (0.05 mg mL^{-1}), prepared in methanol, achieving a final DPPH concentration of 0.025 mg mL^{-1}. Plates were incubated for 30 min at room temperature, protected from the light. After incubation, absorbance measurements were performed at 517 nm using a microplate reader (xMarkTM Microplate Absorbance Spectrophotometer, Bio-Rad, California, USA). The DPPH radical scavenging potential of the tested plant preparations was calculated as described elsewhere, following equation (1) (Rocha et al., 2021). Ascorbic acid was used as a positive control for antioxidant potential, and the negative control was composed of a DPPH solution at 0.025 mg mL^{-1}. CL and TM EOs were tested at concentrations ranging from 0.05% (v/v) to 5% (v/v) and from 0.1% (v/v) to 10% (v/v), respectively. Both hydrolates were tested from 0.5% (v/v) to 50% (v/v).

$$\text{Reduction}\ (\%) = 100 - \left(\frac{\text{Abs sample} - \text{Abs blank}}{\text{Abs control}} \times 100\right) \quad (1)$$

The concentration of each plant preparation necessary to reduce 50% of the free radical activity (IC_{50}) was extrapolated from a calibration curve [concentration vs. DPPH reduction (%)]. The antioxidant activity was expressed by the antioxidant activity index (AAI), as described by Scherer and Godoy, obtained by dividing the final concentration of DPPH (0.025 mg mL^{-1}) by the estimated IC_{50} of each sample. The AAI allow the categorization of the plant preparations under the following categories: AAI <0.5 – poor antioxidants; 0.5 < AAI <1 – moderate antioxidants; 1 < AAI <2 – strong antioxidants; AAI >2 – very strong antioxidants (Scherer and Godoy, 2009).

2.5. Cell culture

Mouse leukemic monocyte macrophages (RAW 264.7 cell line) from American Type Culture Collection (ATCC-TIB-71™) were used as an *in vitro* model for determining the anti-inflammatory potential of the plant preparations. Cells were cultured in DMEM, supplemented with 10% (v/v) of non-inactivated FBS, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, glucose to the final concentration of 4.5 g L^{-1} and 1.5 g L^{-1} of sodium bicarbonate. Cells were split using a cell scrapper and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, according to ATCC recommendations.

L929 NCTC, a mouse fibroblasts' cell line derived from normal subcutaneous areolar and adipose tissue (Sigma-Aldrich, St. Louis, MO, USA), was used for cytotoxicity and wound healing assays. Cells were cultured in DMEM, supplemented with 10% (v/v) of FBS, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 1.5 g L^{-1} of sodium bicarbonate, at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Semi-confluent cells were split using a solution of trypsin/EDTA according to the supplier's recommendations.

2.6. In vitro cytotoxicity

Cellular viability was determined for mouse fibroblasts using the MTT assay, according to International Organization for Standardization (ISO) guideline ISO/EN 10993–5 (International Organization for Standardization, 2009). Cells were seeded at a density of 1×10^4 cells *per* well onto a 96-well plate and left to adhere for 24 h. After adherence, the cells were exposed for 24 h to freshly prepared dilutions of the plant preparations, prepared in DMEM culture medium. After exposure, the culture medium was removed, and an additional washing step was included with sterile pre-warmed phosphate-buffered saline (PBS) to remove traces of the plant preparations that could interfere with MTT conversion. A MTT solution (1 mg mL^{-1}) was freshly prepared in incomplete culture medium and added to the cells, and the plates were incubated at 37 °C for 2 h. Then, the MTT solution was removed, and the formed formazan crystals were dissolved with 100 μL of isopropanol. Absorbance was measured at 570 nm, with a reference wavelength of 650 nm, using an xMarkTM Microplate Absorbance Spectrophotometer (Bio-Rad, California, USA).

EOs were tested at a concentration range from 0.002% (v/v) to 0.25% (v/v) and hydrolates from 0.78% (v/v) to 50% (v/v). SDS at 2% and culture medium alone were used as positive and negative controls for cytotoxicity, respectively. DMSO was also tested, as solvent control, at the highest applied concentration of 0.25% (v/v). Ultra-pure water, at 50% (v/v), was used to mimic the aqueous dilution obtained with the highest tested concentration of the hydrolates.

Absorbance measurements were used to estimate the concentration that decreased 50% of cellular viability (IC_{50}) when compared with the negative control.

2.7. Anti-inflammatory activity

2.7.1. Cellular biocompatibility

The cellular biocompatibility of plant preparations with macrophages was first determined to evaluate the biocompatible concentration range to pursue with further studies. Cellular biocompatibility was determined as disclosed for fibroblasts in the section "2.6. *In vitro* cytotoxicity", using 2.5×10^4 cells *per* well for this cell line, as described elsewhere (Oliveira et al., 2021).

2.7.2. Nitric oxide (NO) production

NO production was measured through a colorimetric assay using Griess reagent, as previously described (Green et al., 1982; Silva et al., 2020). Macrophages were plated (1×10^5 cells *per* well) and left to stabilize for 24 h. After this period, the cells were exposed to LPS at 1 μg mL^{-1} and serial dilutions of biocompatible concentrations of the plant preparations for an additional 24 h period. Controls for NO production were included, composed of cells exposed only to the culture medium (basal NO production) and culture medium and LPS (highest expected NO production). Dexamethasone, an anti-inflammatory drug, was included as a positive control for anti-inflammatory activity. After incubation, culture supernatants were collected and equal volumes of Griess reagent [1% (w/v) sulphanilamide in 5% (w/v) phosphoric acid and 0.1% (w/v) *N*-(1-naphthyl)-ethylenediamine dihydrochloride] were mixed and incubated at room temperature for 30 min. Absorbance measurements were performed at 550 nm, and nitrite concentration was calculated through a sodium nitrite standard curve. NO production was normalized to the highest NO production, obtained in the Control+LPS condition. The estimated concentration of each plant preparation necessary to reduce 50% of NO production (EC_{50}) was calculated.

2.7.3. Nitric oxide scavenging activity

The NO scavenging potential of the four plant preparations was evaluated using the SNAP method, as previously described (Roxo et al., 2020). Serial dilutions of plant preparations, prepared in LPS-free DMEM culture medium (final volume of 300 μL), were exposed to the NO donor SNAP (300 μM) and incubated for 3 h at 37 °C in the dark. Plant preparations were tested at the same concentration range as for NO determination. A negative control, composed of culture medium alone, without the addition of the NO donor, was also included. Nitrite levels were quantified as referred in section '2.7.2. Nitric oxide (NO) production'.

2.8. Wound healing potential

For wound healing potential assessment, cell migration was determined by the scratch-wound assay, as previously described, with minor

adjustments (Oliveira et al., 2020; Rocha et al., 2021). Before the assay, two parallel lines were carved on the underside of each well with a scalpel, acting as a guidance axis. Fibroblasts were seeded at a density of 2.5×10^5 cells *per* well in complete culture medium using a 12-well plate. Cells were left to adhere for 24 h, until a semi-confluent monolayer was reached. An artificial wound was made by perpendicularly scratching the cells with a 20 μL pipette tip. Cells were carefully washed twice with pre-warmed sterile PBS to remove cell debris and exposed to plant preparations prepared in a low serum culture medium (2% FBS) to reduce the proliferation rate during the experiment. Four representative photographs *per* well were taken after adding the stimulus (t=0) using an inverted microscope (Olympus Model IX51) with a 5 × 10 amplification. After 12 h, the same area was photographed, using the craved axis as a guide. The cell-free (wound) area was quantified using FIJI software, and the percentage of wound healing was determined using the following equation (2), as described by Rocha et al. (2021). Wound healing percentages were normalized to control (cells exposed to culture medium only). Allantoin at 1 μg mL^{-1}, freshly prepared in pre-warmed ultrapure water, was used as a positive control for wound healing. The effect of the solvent (DMSO) at the highest concentration was also determined.

$$\text{Wound closure} = \left[100\% - \left(\frac{\text{open wound area at t} = 0\text{h}}{\text{open wound area at t} = 12\text{h}}\right) \times 100\right] \quad (2)$$

2.9. Antimicrobial activity

2.9.1. Microbial strains

The antimicrobial activity of the plant preparations was evaluated against a panel of representative microorganisms using strains from American Type Culture Collection (ATCC). Additionally, two relevant skin pathogens namely *Staphylococcus epidermidis* and *Cutibacterium acnes,* obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), were also included. Details on enrolled strains and their relevance to the study are presented in Table 1.

Purity and viability assessment was performed by sub-culturing aerobic bacteria on TSA, anaerobic bacteria on BHI supplemented with 15 g L^{-1} of agar and 5% glucose, yeast on SDA, and fungi on PDA. Bacteria and yeasts were incubated at 37 °C and filamentous fungi at room temperature. For the growth of anaerobic bacteria, an anaerobic environment was generated using Anaerocult® A anaerobic system.

2.9.2. Minimum inhibitory concentration

The antimicrobial activity was performed according to Clinical and Laboratory Standards Institute M07-A10, M11-A8, M27-A3 and M38-A2 microdilution methods for testing aerobic bacteria, anaerobic bacteria, yeast and filamentous fungi, respectively (CLSI, 2008a, 2008b; CLSI, 2012a, 2012b).

Briefly, a 0.5 MacFarland suspension was prepared from bacterial and yeast cultures using sterile NaCl at 0.85% (w/v), and proper dilutions were made with different culture media. Specifically, MHB was used for aerobic bacteria, Brain Heart Infusion broth supplemented with 5% glucose (sBHI) was used for anaerobic bacteria, and RPMI was used for yeast. Plant preparations were serially diluted in 96-well microdilution plates before adding the microbial suspensions. Sterile DMSO was used as a surfactant to allow the dilution of EOs in the different culture media. EOs were tested at a concentration range from 0.03% (v/v) to 2% (v/v), and hydrolates were tested from 0.78% (v/v) to 50% (v/v). The adjusted microbial suspensions were exposed to serial dilutions of plant preparations resulting in a 2-fold dilution of both plant preparations and microbial suspensions.

For filamentous fungi, seven-day-old colonies were covered with 1 mL of sterile PBS and a drop of DMSO, and mixed to prepare a fungal suspension that was transferred to a sterile tube and left to set for 3–5 min. The upper part was isolated, vortexed briefly and used to prepare a fungal suspension with an adjusted OD_{600} of 0.09–0.11 that was further diluted using RPMI media. Finally, the diluted fungal suspension was mixed with dilutions of the plant preparations, as described for bacteria and yeast.

Appropriate growth and sterility controls were included, composed of microbial suspensions inoculated in specific culture media (expected maximum growth) and non-inoculated media, respectively. DMSO was also tested at the maximum used concentration to discard a possible interference from the surfactant. Different antibiotic and antifungal agents, with different spectrums of action, were included as positive controls of antimicrobial activity. Concentration ranges were selected based on susceptibility breakpoints described by European Committee on Antimicrobial Susceptibility Testing (EUCAST) for the different microorganisms. The following antibiotics were included: ampicillin (max. 16 μg mL^{-1}), erythromycin (max. 16 μg mL^{-1}), gentamicin (max. 4 μg mL^{-1}), tetracycline (max. 4 μg mL^{-1}), ciprofloxacin (max. 2 μg mL^{-1}) and benzylpenicilin (max. 1 μg mL^{-1}). Fluconazole and amphotericin B were tested at maximum concentrations of 8 μg mL^{-1} and 64 μg mL^{-1}, respectively.

The minimum inhibitory concentration (MIC) was defined as the first concentration that caused a complete absence of microbial growth, determined by visual inspection of the multiwell plates after 24 h, 48 h and 72 h for aerobic bacteria, fungi and anaerobic bacteria, respectively. Due to the inter-operator variability of MIC determination associated with visual inspection methods, absorbance was also measured at 600 nm. The lowest concentration in test capable to decrease 50% of microbial growth (MIC_{50}) by spectrophotometric evaluation, was also determined for *C. albicans*, as recommended by EUCAST. The microorganisms understudy were classified as susceptible (S), resistant (R) or susceptible with increased exposure (I) to each antimicrobial agent, based on updated MIC breakpoints (The European Committee on Antimicrobial Susceptibility Testing, 2022, 2020).

2.9.3. Minimum lethal concentration

Minimum lethal concentration (MLC) was determined by plating on TSA, SDA, PDA or sBHI agar, 5 μl of the content of each well that presented an absence of microbial growth. Plates were incubated for an additional 24 h, 48 h or 72 h period for aerobic, fungi and anaerobic bacteria, respectively. After the correspondent incubation period, MLC was defined as the lowest concentration with the absence of microbial colonies.

2.10. Anti-biofilm activity

The effect of plant preparations on biofilm growth forms of the relevant skin pathogen *C. acnes* was further determined, using two different protocols as described (de Canha et al., 2020; Di Lodovico et al., 2020; Oliveira et al., 2022).

A bacterial pre-inoculum was grown for three days in sBHI under anaerobic conditions, and used to prepare bacterial suspensions. To evaluate the effect on the biofilm's adhesion to polystyrene surfaces,

Table 1
Details and classification of the included microbial strains.

Species	Strain collection number	Classification
Staphylococcus aureus	ATCC 6538	Gram-positive aerobic bacteria
Staphylococcus epidermidis	DSM 28764	Gram-positive aerobic bacteria
Cutibacterium acnes	DSM 1897	Gram-positive anaerobic bacteria
Pseudomonas aeruginosa	ATCC 9027	Gram-negative aerobic bacteria
Escherichia coli	ATCC 8739	Gram-negative aerobic bacteria
Candida albicans	ATCC 10231	Yeast
Aspergillus brasiliensis	ATCC 16404	Filamentous fungi

C. acnes bacterial suspensions were plated in 96-well flat-bottom microdilution plates, simultaneously with different concentrations of each plant preparation, achieving a 2-fold dilution of the samples and approximately 10^6 CFU mL^{-1}. The plates were incubated at 37 °C for 72 h, allowing biofilm adhesion. Following incubation, the medium was removed and plates were washed twice with sterile PBS.

Alternatively, to evaluate the effect of plant preparations on mature (preformed) biofilms, the bacterial suspension was diluted in sBHI to

Table 2
Qualitative and quantitative composition of EOs from *T. mastichina* (TM) and *C. ladanifer* (CL).

RI[a]	Ref. RI[a]	RI[b]	Ref. RI[b]	Compounds*	Relative amount (%) in TM EO	Relative amount (%) in CL EO
921	921	1020	1030	Tricyclene	–	2.1
922	922	1072	1028	α-Thujene	–	0.6
929	929	1030	1030	α-Pinene	3.4	50.0
943	943	1075	1073	Camphene	0.4	10.1
950	953	1127	–	Thuja-2,4-(10)-diene	–	1.2
964	964	1124	1124	Sabinene	3.3	0.4
970	970	1118	1118	β-Pinene	5.0	0.8
980	980	1161	1162	Myrcene	1.8	0.3
997	997	1171	1171	α-Phellandreme	0.1	0.1
1001	998	1138	1138	δ-2-Carene	0.1	0.3
1007	1010	1179	1081	α-Terpinene	0.5	0.7
1011	1011	1275	1275	*p*-Cymene	16.8	2.1
1018	1017	1310	1309	2,2,6-Trimethyl-cyclohexanone	–	1.8
1020	1020	1215	1215	1,8-Cineole	43.5	0.6
1020	1020	1204	1205	Limonene	0.8	1.7
1035	1035	1253	1253	*E*-β-Ocimene	0.3	–
1046	1046	1249	1249	γ-Terpinene	0.8	1.1
1050	1050	1459	1462	*E*-Sabinene hydrate	0.4	–
1055	1058	1439	1439	*Z*-Linalool oxyde (THF)	0.1	–
1076	1076	1288	1288	Terpinolene	0.1	0.3
1082	1082	1543	1542	Linalool	3.8	0.3
1094	1096	1357	1358	*cis*-Rose oxide	–	0.3
1108	1106	n.d.	–	*cis-p*-Menth-2-en-1-ol	T	–
1102	1102	1487	1487	Campholenal	–	1.2
1119	1119	1647	1647	Pinocarveol	–	2.5
1135	1135	1563	1562	Pinocarvone	–	1.4
1118	1118	1515	1515	Camphor	0.1	0.8
1149	1144	1671	1667	δ-Terpineol	2.0	–
1150	1151	1700	*1698*	*p*-Mentha-1,5-diene-8-ol	–	0.9
1158	1157	1845	1845	Cymene-8-ol	T	–
1158	1158	1597	1597	Terpinene-4-ol	0.5	1.1
1169	1169	1692	1692	α-Terpineol	3.9	0.5
1177	1177	1698	1698	Verbenone	–	0.2
1205	1207	1830	1830	*E*-Carveol	–	0.1
1179	1180	n.d.	–	*Z*-Dihydrocarvone	0.2	–
1181	1182	1624	1624	*E*-Dihydrocarvone	0.1	–
1226	1223	1601	1601	Carvacrol methyl oxide	0.3	–
1244	1240	1555	1555	Linalyl acetate	0.1	–
1264	1264	1574	1574	Bornyl acetate	T	3.4
1274	1275	2214	2215	Carvacrol	0.8	–
1364	1368	1466	–	Cyclosativene	–	0.4
1369	1368	1487	1487	α-Copaene	–	0.4
1376	1376	1517	1517	β-Bourbunene	0.3	–
1384	1382	1585	1585	β-Elemene	0.1	–
1401	1401	1526	1527	α-Gurjunene	0.1	–
1410	1409	1590	1590	*E*-Caryophyllene	0.4	0.1
1430	1428	1604	1604	Aromadendrene	0.1	0.7
1440	1440	1666	1662	α-Humulene	T	–
1444	1447	1638	1637	*allo*-Aromadendrene	T	0.6
1479	1486	1725	1732	Eremophilene	1.4	–
1466	1466	1699	1699	Germacrene D	2.3	–
1486	1483	1689	1696	Ledene	–	0.5
1487	1487	1733	1735	Bicyclogermacrene	0.1	–
1501	1497	1708	1722	β-Bisabolene	1.4	–
1508	1507	1751	1751	δ-Cadinene	0.2	–
1553	1551	2113	2113	Spathulenol	0.4	–
1571	1569	2068	2073	Viridiflorol	0.7	2.3
1584	1582	2026	2024	Ledol	–	1.1
1628	1628	2218	2220	α-Cadinol	0.5	–
				Monoterpene hydrocarbons	33.4	71.8
				Oxygen-containing monoterpenes	55.8	13.3
				Sesquiterpene hydrocarbons	6.4	2.7
				Oxygen-containing sesquiterpenes	1.6	3.4
				Other compounds	–	1.8
				Total identified	97.2	93.0

Exp. RI[a]: Experimental retention indices on the SPB-1 column relative to C8–C24 *n*-alkanes. Ref. RI[a]: Reference retention indices in nonpolar column (Linstrom and Mallard, 2019). Exp. RI[b]: Experimental retention on the SupelcoWax-10 column relative to C8–C23 *n*-alkanes. Ref. RI[b]: Reference retention indices in polar column (Linstrom and Mallard, 2019). T: trace amounts. * Compounds listed in order to their elution on the SPB-1 column.

achieve 10^6 CFU mL^{-1} and plated onto 96-well flat-bottom microdilution plates. The plates were incubated for 72 h to allow biofilm formation. After incubation, culture media was removed, and the plates were gently washed twice with sterile PBS to remove planktonic cells. Next, dilutions of plant preparations, prepared in sBHI, were added to the wells and incubated for an additional 72 h period.

The following steps, used to determine the biofilm biomass, were similar for the two protocols. Briefly, biofilms were fixed for 15 min using 100 μL of methanol. After, the methanol was discarded and plates were allowed to air dry. The adherent phase was stained with 0.5% crystal violet for 20 min. After staining, the plates were gently rinsed under running water to remove the non-bound stain. The bound crystal violet was dissolved with 150 μL of acetic acid at 33% (v/v), and the optical density was measured at 590 nm using an xMarkTM Microplate Absorbance Spectrophotometer (Bio-Rad, California, USA).

Plant preparations were tested at concentrations of one-fourth, half, once and twice the obtained planktonic MIC value for *C. acnes*. Negative controls (in the absence of plant preparations) corresponding to the highest biofilm biomass, and sterility controls (non-inoculated medium) were included in both protocols. The possible effect of the solvents was also evaluated.

2.11. Statistics

In vitro experiments were carried out in at least triplicates and in a minimum of three independent experiments. Mean values ± SD are expressed as percentages of the respective controls in the absence of the plant preparations. Statistical analyses were performed using one-way ANOVA with Tukey's multiple comparisons test or student's *t*-test, and a p-value <0.05 was accepted as denoting statistical significance. Half-maximal inhibitory or effective concentrations (IC_{50}/EC_{50}) were calculated for different bioactivities by logistic regression using GraphPad Prism version 8 for Windows. For DPPH radical scavenging assay, the IC_{50} was estimated by a calibration curve generated by Microsoft Excel (Microsoft Office Professional Plus 2016) for Windows, and the statistical analyses were performed using GraphPad Prism version 8 for Windows.

3. Results

3.1. Composition of plant preparations

The compositions of TM and CL EOs are presented in Table 2. In brief, CL EO is chiefly composed of monoterpene hydrocarbons (71.8%), being α-pinene (50%) and camphene (10.1%) the major constituents.

Differently, in the TM EO, oxygen-containing monoterpenes are dominant (55.8%), being 1,8-cineole (43.5%) the major one. The second major compound from TM EO is the monoterpene *p*-cymene (16.8%). Sesquiterpene hydrocarbons and oxygen-containing sesquiterpenes are present in both EOs, as presented in Table 2.

As expected, the organic fractions of both hydrolates are exclusively composed of oxygen-containing compounds, some of them not detected or detected in trace amounts in the correspondent EOs. Qualitative and quantitative compositions of the volatile fraction of the hydrolates from TM and CL are presented in Table 3. In TM hydrolate, 1,8-cineole (43.9%), α-terpineol (21.1%) and borneol (11.5%) are dominant, and their absolute concentrations in the hydrolate (expressed as *n*-dodecane) are 662 ppm, 379 ppm and 173 ppm, respectively. Regarding the CL hydrolate, *E*-pinocarveol (25.3%), borneol (14.0%) and terpinene-4-ol (9.7%) are the most representative compounds in the organic fraction, and their absolute concentrations in the hydrolate are 123 ppm, 68 ppm and 47 ppm, respectively.

3.2. Antioxidant activity of plant preparations

The antioxidant capacity of the plant preparations was determined through the DPPH radical scavenging method and they were classified as poor, moderate, strong or very strong antioxidants based on the obtained antioxidant activity indexes (AAI). The IC_{50}, AAI, and grading as antioxidant for each plant preparation are presented in Table 4. CL EO

Table 3
Qualitative and quantitative composition of the volatile fraction of the hydrolates from *T. mastichina* (TM) and *C. ladanifer* (CL).

Compound	TM hydrolate		CL hydrolate	
	Relative amount (%) in the volatile fraction	Absolute concentration (ppm) expressed as *n*-dodecane	Relative amount (%) in the volatile fraction	Absolute concentration (ppm) expressed as *n*-dodecane
1,8-Cineole	43.9	662	5.7	28
Acetophenone	–	–	5.4	26
Octan-3-one	–	–	1.1	5
2,6-Trimethylcyclohexanol	–	–	8.0	39
cis-Linalool oxide	0.5	7	–	–
trans-Linalool oxide	0.4	6	–	–
Linalool	3.6	54	–	–
Phenylethyl alcohol	0.5	7	–	–
3,5,5-Trimethyl-3-cyclohexen-1-one	0.2	4	–	–
E-Pinocarveol	–	–	25.3	123
Borneol	11.5	173	14.0	68
Terpinene-4-ol	4.5	68	9.7	47
p-Cymene-8-ol	–	–	2.6	13
α-Terpineol	21.1	379	2.2	11
Myrtenol	–	–	6.0	29
Verbenone	–	–	4.9	24
Dihydrocarvone	0.2	4	–	–
endo-2-Hidroxycineole	0.7	10	–	–
E-Carveol	0.2	3	2.5	12
Z-Carveol	–	–	1.0	5
exo-2-Hidroxycineole	0.5	8	–	–
2-Cyclohexen-1-one	–	–	0.5	2
Geraniol	–	–	0.6	3
Bornyl acetate	–	–	0.8	4
Carvacrol	1.6	24	0.5	3
4-Hidroxi-3-methylacetophenone	–	–	2.7	13
Viridiflorol	–	–	0.7	3

Table 4
Antioxidant capacity and correspondent antioxidant activity indexes (AAI).

Preparation/ compound	IC_{50} (Mean ± SD)	AAI (Mean ± SD)	Antioxidant capacity
TM EO % (v/v)	0.975 ± 0.02*	0.0026 ± 0.00005*	Poor
CL EO % (v/v)	0.691 ± 0.09*	0.0037 ± 0.00045*	Poor
TM hydrolate % (v/v)	33.48 ± 2.75*	0.0001 ± 0.00001*	Poor
CL hydrolate % (v/v)	43.00 ± 1.55*	0.0001 ± 0.000002*	Poor
Ascorbic acid (μg mL^{-1})	2.156 ± 0.36	11.793 ± 1.797	Very strong

* Indicates a significant result when compared with ascorbic acid (p-value < 0.05).

presented a higher DPPH scavenging potential when compared with TM EO [IC_{50} of 0.69 ± 0.09% (v/v) *versus* 0.98 ± 0.02% (v/v)], respectively. For the hydrolates, the results were the opposite, with TM hydrolate presenting a lower IC_{50} value of 33.48 ± 2.75% (v/v) when compared with CL hydrolate [43.00 ± 1.55% (v/v)]. As expected, both EOs presented much lower IC_{50} values, when compared with their corresponding hydrolates.

Regarding the antioxidant classification, based on the obtained AAI, the four plant preparations were classified as poor antioxidants (AAI <0.5) when compared with the positive control, ascorbic acid, a very strong antioxidant (AAI >2), being the difference in their AAI and IC_{50} values statistically significant.

3.3. *In vitro cytotoxicity of plant preparations*

EOs and hydrolates were tested for their cytotoxicity on two different cell lines, namely L929 fibroblasts, the recommended cell line for the determination of *in vitro* toxicity by ISO/EN 10993–5; and on macrophages, to estimate the biocompatible concentration range to pursue with further experiments.

Considering the results obtained for fibroblasts, the four plant preparations presented a dose-dependent response on cellular viability, as presented in Fig. 1. TM EO was slightly more biocompatible than CL EO, thus presenting a higher IC_{50} value (Table 5). The same pattern was also present for the corresponding hydrolates. Interestingly, a small but significant increase (≈10%) in cellular viability was obtained for CL EO compared with cells maintained in DMEM culture medium only, at the lowest tested concentration of 0.002% (v/v).

Considering the murine macrophage cell line, similar results were obtained, as presented in Fig. 2. From the EOs, TM EO was more biocompatible than CL EO, presenting toxicity (<70% of cellular viability) only at the two higher concentrations under test, specifically 0.125 and 0.25% (v/v). For the hydrolates, the same profile was obtained, with TM hydrolate presenting higher biocompatibility than CL hydrolate.

3.4. *Anti-inflammatory activity of plant preparations at biocompatible concentrations*

After obtaining the biocompatible concentration range, macrophages were used as an *in vitro* model for the anti-inflammatory potential of the plant preparations by quantifying the NO production after a simultaneous stimulus with LPS. A decrease in NO production was present for all plant preparations, with a dose-dependent response, as presented in Fig. 3. Regarding the EOs, CL EO presented a higher anti-inflammatory potential by causing higher reductions in NO production. Moreover, this EO was able to reduce NO to approximately 50%, even at the lowest tested concentration of 0.002% (v/v), thus producing comparable results with the ones obtained for dexamethasone at 10 μM. For TM EO, the anti-inflammatory potential was not as high as for CL EO, presenting a higher EC_{50} value [0.006% (v/v) *versus* 0.002% (v/v)], respectively, as presented in Table 5. Still, for TM EO, this value was ≈15 times lower than the correspondent cytotoxicity IC_{50} value obtained for this cell line.

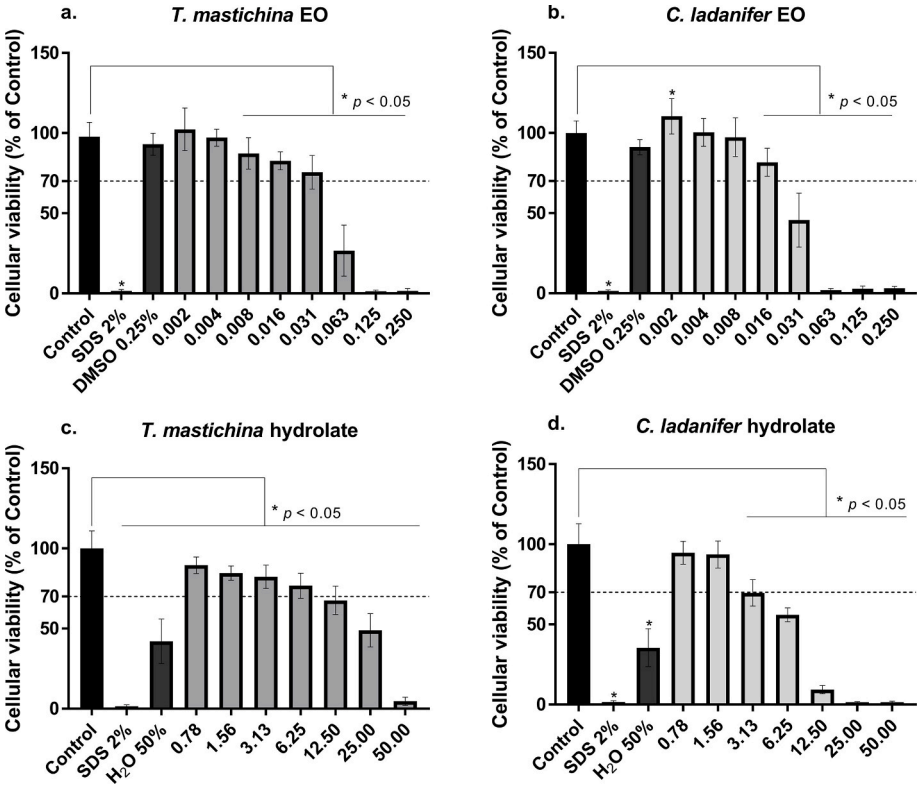

Fig. 1. Effect of EOs and hydrolates from TM (a, c) and CL (b, d) on cellular viability of L929 fibroblasts after 24 h of contact with serial dilutions of the plant preparations. EOs were tested from 0.002% to 0.25% (v/v) and hydrolates from 0.78% to 50% (v/v). A plant preparation was considered cytotoxic at a determined concentration when cellular viability was <70%, compared with negative control, as evaluated by the MTT reduction assay. SDS at 2% was included as a positive control for cytotoxicity. DMSO, used as a solvent in the assays with the EOs, was also tested at its maximum concentration, to discard a possible contribution to cellular cytoxicity. Ultra-pure water was used to mimic the dilution factor achieved when testing the maximum hydrolate concentration of 50% (v/v). * represents a statistically significant result when compared with control, as determined by one-way ANOVA with Dunnett's multiple comparisons test.

Table 5
Comparison of estimated half-maximal inhibitory concentrations for cellular viability (IC_{50}) obtained for the two tested cell lines and estimated half-maximal effective concentrations for NO production in macrophages (EC_{50}).

Preparation	Fibroblasts (L929)		Macrophages (RAW 264.7)				
	Cellular viability		Cellular viability		NO production		IC_{50}/EC_{50} ratio[a]
	IC_{50}	95% CI	IC_{50}	95% CI	EC_{50}	95% CI	
TM EO	0.043	0.039–0.046	0.091	0.08–0.10	0.006	0.005–0.007	15.16
CL EO	0.027	0.024–0.029	0.012	0.011–0.013	0.002	0.002–0.003	6.00
TM hydrolate	17.02	14.77–19.58	33.38	30.29–37.11	3.35	3.134–3.599	9.96
CL hydrolate	5.71	5.33–6.10	8.57	8.02–9.14	0.79	0.75–0.82	10.84

[a] Ratio between half-maximal inhibitory concentration for cellular cytotoxicity and half-maximal effective concentration for NO production in macrophages. A higher ratio represents a larger difference between these two values, indicative of a wider range of biocompatible and effective concentrations.

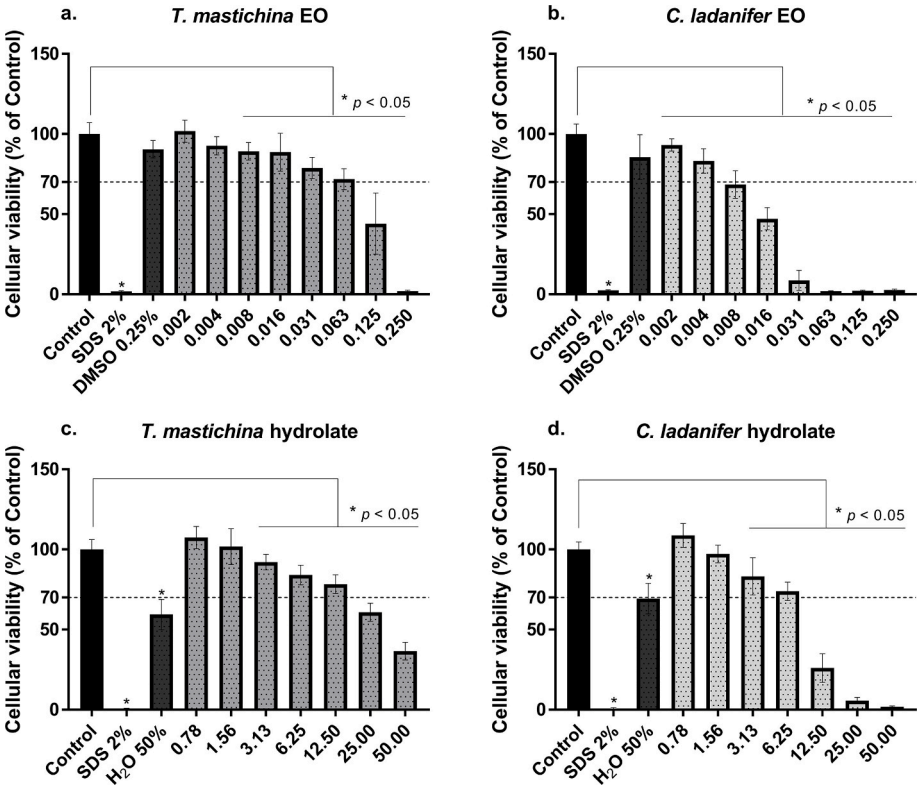

Fig. 2. Effect of EOs and hydrolates from TM (a, c) and CL (b, d) on cellular viability of RAW 264.7 macrophages after 24 h of contact with serial dilutions of the plant preparations. EOs were tested from 0.002% to 0.25% (v/v) and hydrolates from 0.78% to 50% (v/v). A plant preparation was considered cytotoxic at a determined concentration when cellular viability was <70%, compared with negative control, as evaluated by the MTT reduction assay. SDS at 2% was included as a positive control for cytotoxicity. DMSO, used as a solvent in the assays with the EOs, was also tested at its maximum concentration, to discard a possible contribution to cellular cytoxicity. Ultra-pure water was used to mimic the dilution factor achieved when testing the maximum hydrolate concentration of 50% (v/v). * represents a statistically significant result when compared with control, as determined by one-way ANOVA with Dunnett's multiple comparisons test.

Regarding hydrolates, a similar profile was obtained. CL hydrolate presented a higher anti-inflammatory potential when compared with TM hydrolate, with a much lower EC_{50} value [0.79% (v/v) *versus* 3.35% (v/v)]. Additionally, and similarly to its EO, CL hydrolate was able to reduce NO production to similar levels to the ones obtained for dexamethasone, at the lowest tested concentration of 0.78% (v/v).

3.5. NO scavenging potential

In order to better understand the mechanism behind the anti-inflammatory potential of the plant preparations at biocompatible concentrations, we investigate the presence of a possible direct NO scavenging potential by exposing each preparation to a NO donor (SNAP). As presented in Fig. 4, all plant preparations at the tested concentration range, failed to significantly scavenge NO from SNAP.

3.6. Effect of plant preparations on cellular migration

For the evaluation of the wound healing potential of EOs and hydrolates, an *in vitro* model of cell migration was used. A similar biocompatible concentration was selected for each type of plant preparation. Considering the increase in cellular viability of skin fibroblasts obtained with CL EO at 0.002% (v/v), this concentration was selected to pursue with wound healing studies. Results obtained for EOs, tested at 0.002% (v/v) and hydrolates, tested at 2% (v/v) are disclosed in Fig. 5. Representative images of cellular migration are presented in Fig. 6.

CL EO was able to significantly promote cellular migration compared with control (155.7%), presenting comparable results with the positive control allantoin (149.2%). Contrarily, TM EO did not significantly promote cellular migration at the tested concentration.

Regarding hydrolates, both TM and CL promoted a significant increase in cellular migration by 125.1% and 148.4%, respectively, when compared with negative control. Similar to its EO, CL hydrolate also produced comparable results with allantoin. Although TM hydrolate also promoted cellular migration, the response was weaker compared with the positive control.

3.7. Antimicrobial activity of plant preparations against a panel of representative microorganisms and selected skin pathogens

Antimicrobial activity was evaluated against a spectrum of selected

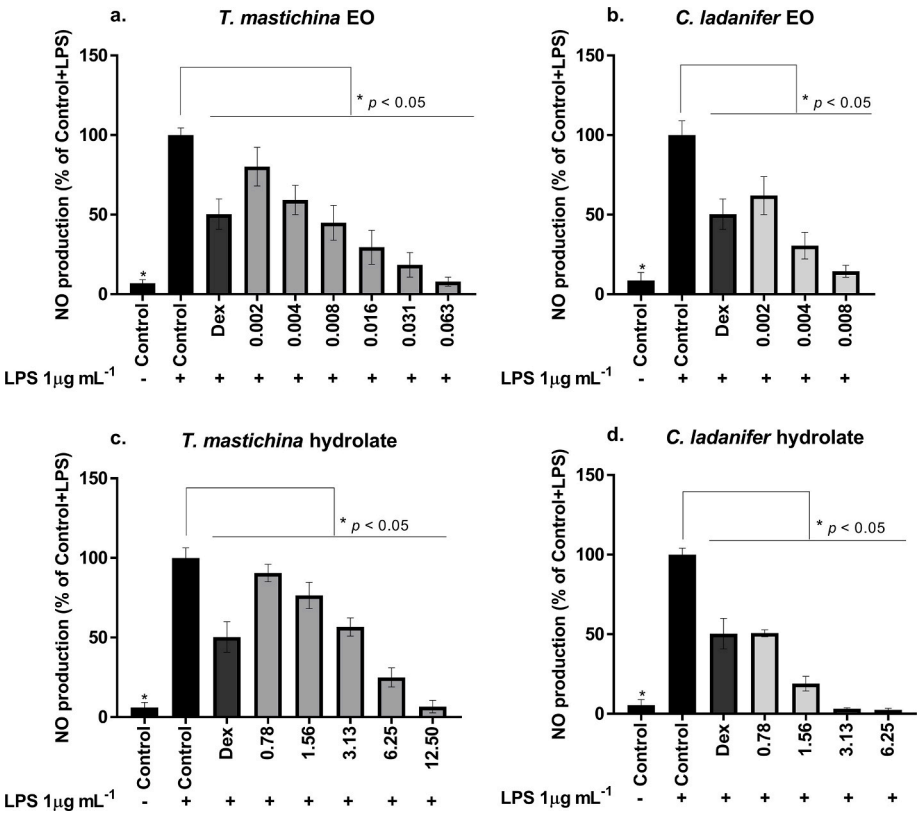

Fig. 3. Decrease in NO production obtained at biocompatible concentrations of EOs and hydrolates from TM (a, c) and CL (b, d) upon a pro-inflammatory stimulus with LPS (1 μg mL^{-1}). NO stable metabolites in cell culture supernatants were measured with Griess reagent. Dexamethasone (Dex) at 10 μM was included as a positive control for anti-inflammatory activity. A control without any stimulus (culture medium only) was also included to evaluate the basal state of the cells. *$p < 0.05$ was considered a statistically significant NO reduction when compared with cells treated only with LPS (maximum expected NO production), as determined by one-way ANOVA with Dunnett's multiple comparisons test.

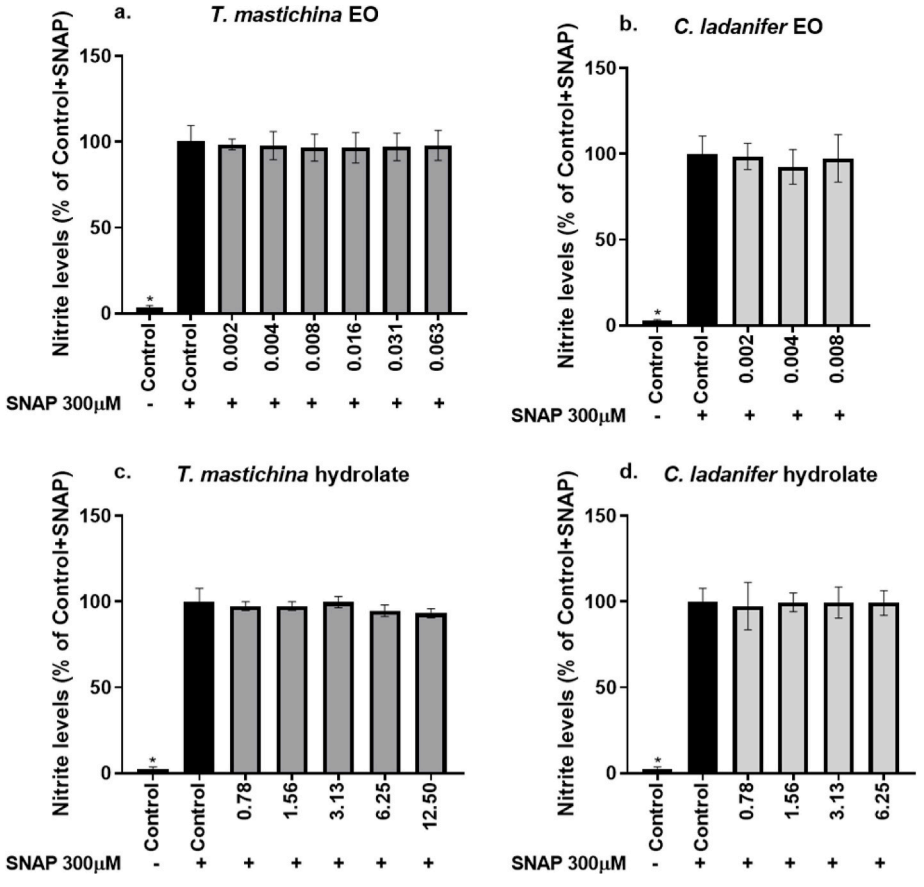

Fig. 4. Capacity of EOs and hydrolates from TM (a, c) and CL (b, d) to scavenge NO from donor SNAP, measured with Griess reagent. The tested concentrations were the same as the ones applied in the cellular NO production assay. A control without SNAP (culture medium only) was included to address basal nitrite presence in the medium. One-way ANOVA with Dunnett's multiple comparisons test was performed to find statistical significant reductions in nitrite levels when compared with culture media in the presence of SNAP, with no significant results.

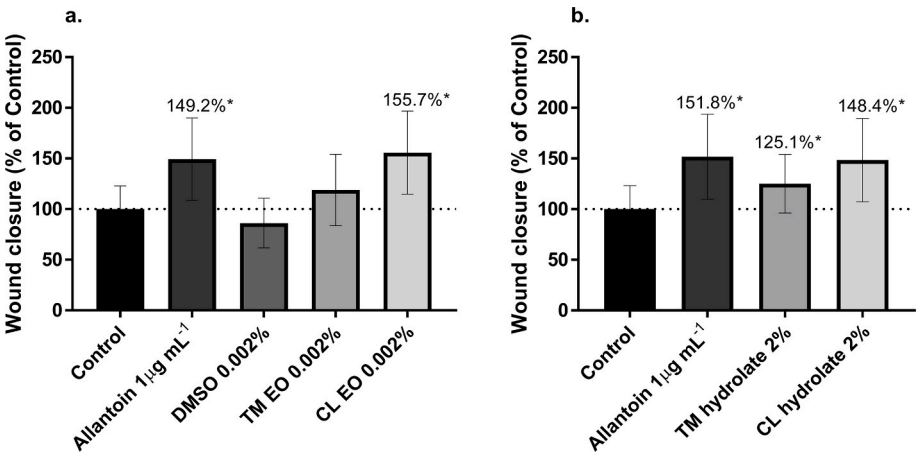

Fig. 5. Effect of EOs (a) and hydrolates (b) from TM and CL on cellular migration of an artificial wound created by scratching a cellular monolayer of L929 fibroblasts. After the mechanical injury, cells were exposed to biocompatible concentrations of the plant preparations (prepared in a low serum culture medium) and to controls for 12 h. The results express the percentage of wound closure after the experiment course, normalized to the control (low serum culture medium only). Allantoin at 1 μg mL^{-1} was used as a positive control for wound healing. $^{*}p < 0.05$ was considered a statistically significant result when compared with control, as determined by student *t*-test.

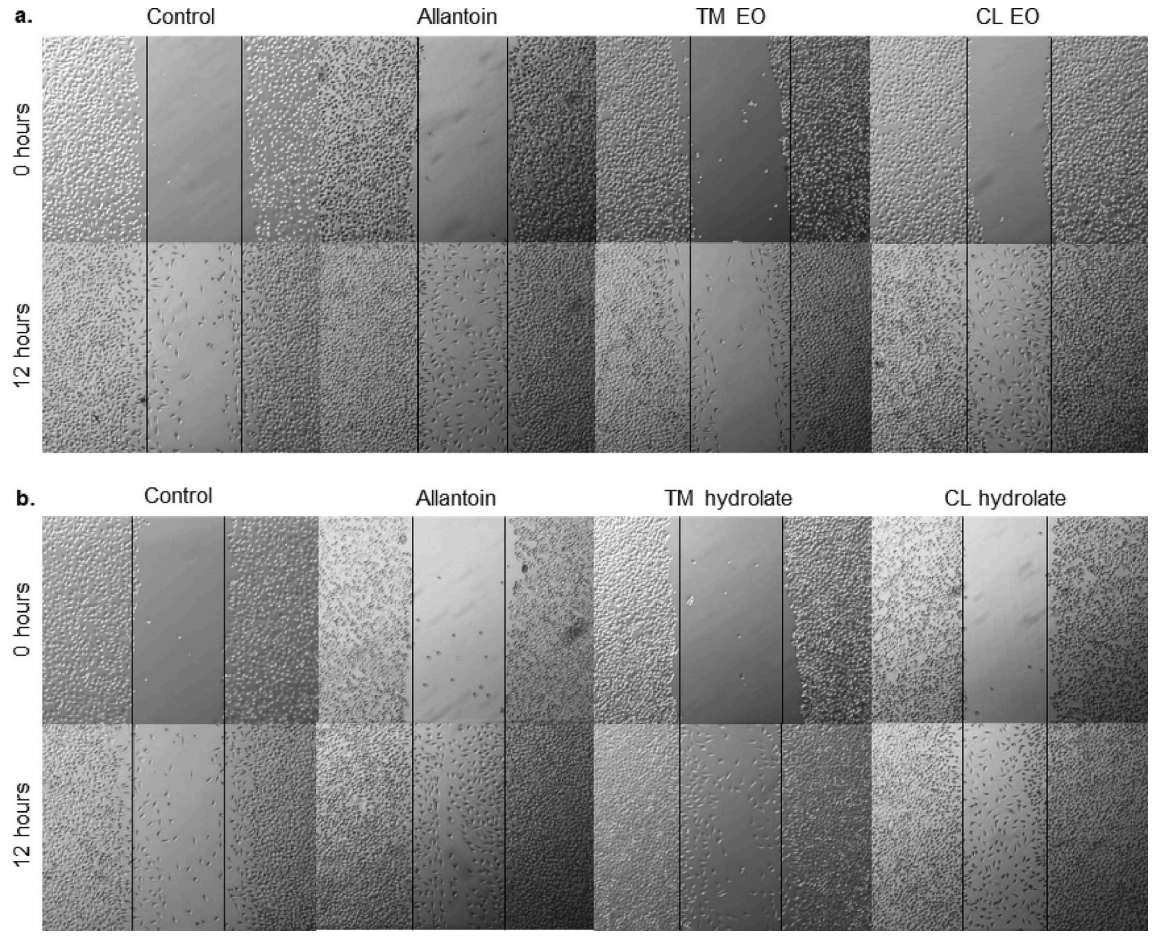

Fig. 6. Representative images of increased migration of fibroblasts when exposed to EOs (a) and hydrolates (b) from TM and CL. Allantoin (1 μg mL^{-1}) was included as a positive control for wound healing. Images were acquired at the beginning of the experiment (0 h) and after 12 h of exposure to different plant preparations and controls. Photographs were taken using an inverted microscope (Olympus Model IX51) with a 5 × 10 amplification.

microorganisms, including gram-positive and gram-negative bacteria, yeast and filamentous fungi.

Visual MIC and MLC values obtained for bacteria with EOs and antibiotic controls are disclosed in Table 6. Spectrophotometric MIC_{50}, visual MIC and MLC values for yeast and filamentous fungi are represented in Table 7. Graphical representations of the effect of plant preparations and antimicrobial agents on microbial growth are presented in the Supplementary Figs. S1–S7.

Overall, the tested strains presented a susceptible or susceptible with increased exposure profiles to the classic antibiotics, with exception of multi-resistant *S. epidermidis*, which was resistant to five of the six included antibiotics. Regarding EOs, results were independent from the antibiotic susceptibility profiles and related mostly with the bacteria cell wall.

For CL EO, of the tested bacteria, gram-positives presented lower MIC values when compared to gram-negatives. The lower MIC value of 0.06% (v/v) was obtained for multi-resistant *S. epidermidis*. For all gram-positive bacteria, MLC values were higher than the obtained MICs,

Table 6
Visual minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) values for EOs from *T. mastichina* (TM) and *C. ladanider* (CL) and for different antibiotic controls against tested bacteria. Susceptibility profile of the tested bacteria to each antibiotic is represented in brackets next to the obtained MIC value.

Strain	TM EO % (v/v)		CL EO % (v/v)		Ciprofloxacin (μg mL^{-1})		Gentamicin (μg mL^{-1})		Tetracycline (μg mL^{-1})		Benzylpenicillin (μg mL^{-1})		Ampicillin (μg mL^{-1})		Erythromycin (μg mL^{-1})	
	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC
S. aureus	>2	>2	1	2	0.125 (I)	0.25	0.5 (S)	1	0.25 (S)	>4	0.016 (S)	0.03	0.06 (S)*	0.06	0.25 (S)	16
S. epidermidis	>2	>2	0.06	1	2 (R)	2	>4 (R)	>4	1 (S)	>4	>1 (R)	>1	8 (R)*	8	>16 (R)	>16
E. coli	2	2	>2	>2	0.008 (S)	0.016	1 (S)	1	–	–	–	–	2 (S)	2	–	–
P. aeruginosa	2	2	2	2	0.06 (I)	0.06	1 (IE)	1	–	–	–	–	–	–	–	–
C. acnes‡	1	2	0.25	0.5	0.5	1	2	>4	0.5	>4	0.004 (S)	0.008	0.03	0.06	0.016	1

S – Susceptible; I – Susceptible with increased exposure; R – Resistant; - Susceptibility testing is not recommended. IE – no breakpoints described as there is insufficient evidence that the microorganism is a good target for the agent. * Classification based on susceptibility profile to benzylpenicillin. ‡ From the tested antibiotics, *C. acnes* susceptibility profile was only available for benzylpenicillin in EUCAST guidelines.

Table 7
Visual minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) values for EOs from *T. mastichina* (TM) and *C. ladanider* (CL) and different antifungal controls against *C. albicans* (yeast) and *A. brasiliensis* (filamentous fungi). Susceptibility classification to each antifungal is represented in brackets next to the obtained MIC value.

Strain	TM EO % (v/v)			CL EO % (v/v)			Fluconazole (μg mL^{-1})			Amphotericin B (μg mL^{-1})	
	MIC_{50}	MIC	MLC	MIC_{50}	MIC	MLC	MIC_{50}	MIC	MLC	MIC	MLC
C. albicans	1	2	>2	0.5	1	>2	1 (S)	#	#	16 (R)	32
A. brasiliensis	0.25	2	>2	0.25	0.5	>2	–	–	–	32 (IE)	64

S – Susceptible; R – Resistant; - Susceptibility testing is not recommended. IE – no breakpoints described as there is insufficient evidence that the microorganism is a good target for the agent. # Endpoint not considered for this species.

presenting a bacteriostatic effect. No antimicrobial activity was present for this EO against gram-negative bacteria *E. coli,* at the tested concentration range. Antimicrobial activity was also present for fungi and yeast being CL EO fungistatic, as no MLC value was determined at the tested concentration range.

TM EO presented milder antimicrobial activity, when compared with

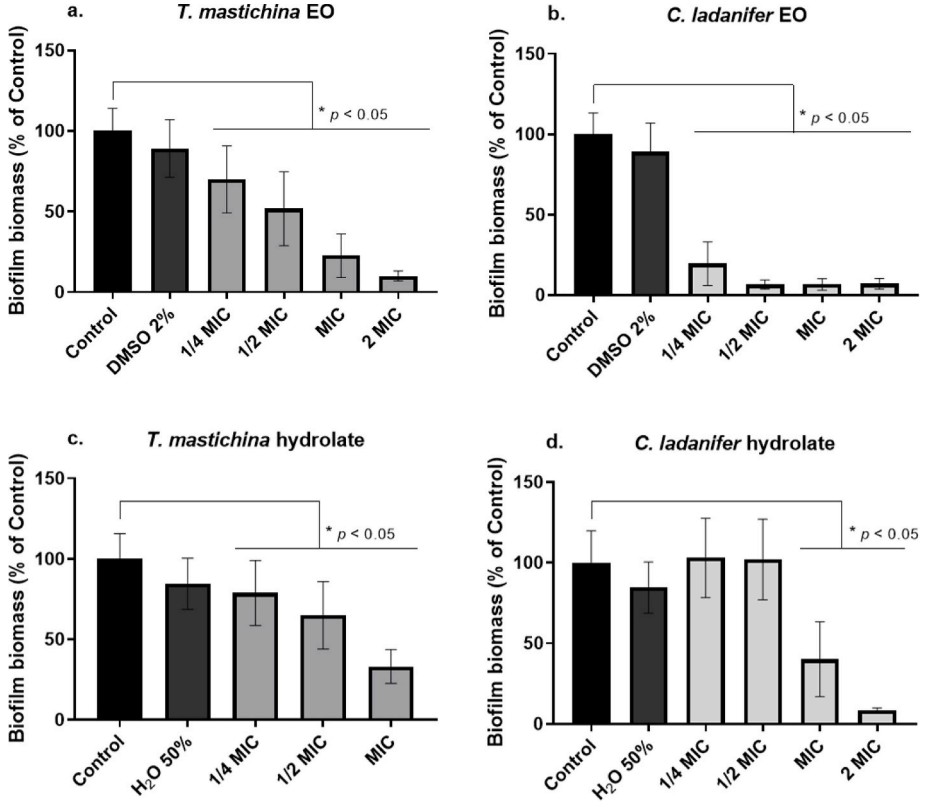

Fig. 7. Effect of EOs and hydrolates from TM (a, c) and CL (b, d) on *C. acnes* biofilm adhesion, determined by staining biofilm biomass with crystal violet. DMSO was used at the maximum tested concentration of 2% (v/v) as solvent control, and ultrapure water was used at 50% (v/v) to mimic the dilution obtained when testing the hydrolates at the maximum concentration. *p value < 0.05 was considered statistically significant.

CL EO. Considering gram-positive bacteria, only *C. acnes* presented a visual MIC value of 1% (v/v), being the EO bacteriostatic at this concentration. For the remaining gram-positive bacteria, no visual MIC was obtained. Still, the EO produced an effect in microbial growth particularly at higher concentrations, as represented in Supplementary Figs. S1 and S2. Regarding gram-negatives, TM produced visual MICs for both bacteria, with equal MLC values, thus being bactericidal at this concentration. Amphotericin B-resistant *C. albicans* and *A. brasiliensis* presented equal MIC values of 2% (v/v), which corresponded to the highest concentration in test.

Concerning the hydrolates, overall, they did not present relevant antimicrobial activity, with visual MIC values only present against *C. acnes* bacteria. Specifically, TM presented a MIC of 50% (v/v), and CL presented a visual MIC of 25% (v/v). Still, no lethal activity was present as no MLC was observed. Although no visual MICs were observed, reductions in microbial growth were present with both hydrolates for all tested microorganisms, as represented in Supplementary Figs. S1–S7.

3.8. Effect of plant preparations on C. acnes biofilms

Due to the overall higher susceptibility of *C. acnes*, a relevant skin pathogen associated with *acne vulgaris* disease, we pursue by studying the effect of each plant preparation in the prevention of biofilm formation and in the promotion of mature biofilm disruption of biofilms produced by *C. acnes*.

3.8.1. Biofilm formation

Considering the ability of the plant preparations to inhibit biofilm adhesion to polystyrene surfaces, both EOs were able to reduce this step of biofilm formation, as presented in Fig. 7. TM EO produced a reduction on biofilm biomass in a dose-dependent manner ranging from 30% to 90%, for one-fourth and for twice planktonic MIC values, respectively. Contrarily, CL EO presented a remarkable effect in inhibiting biofilm formation at all tested concentrations.

Regarding hydrolates from both plants, the effect on biofilm adhesion was milder when compared with the correspondent EO, as represented in Fig. 7. Still, for TM hydrolate, the reductions of biofilm formation ranged from 20% at one-fourth the planktonic MIC, to 65% at MIC value. Twice MIC value was not tested for TM hydrolate since the MIC corresponded to the highest concentration in study. For CL hydrolate, reductions on biofilm biomass were only present at MIC and twice MIC values, ranging from 60% to approximately 95%, respectively.

3.8.2. Mature biofilm disruption

Considering the effect of plant preparations on the disruption of mature biofilms, a slight reduction on their efficacy was present when compared with the effect against biofilm formation, as shown by Fig. 8. Still, high biofilm disruptions were present for MIC and twice the MIC values for TM EO, with reductions ranging between 75% and 85%, respectively. For CL EO, biofilm disruption followed a dose-dependent pattern, ranging from 20% at one-fourth the MIC value, to 80% obtained at once and twice the MIC.

Regarding the effect of hydrolates on biofilm disruption, TM hydrolate presented a higher activity when compared to CL hydrolate, achieving reductions that ranged from 25% at one-fourth the MIC, to 55% at MIC value. Reductions with CL hydrolate ranged from no reduction at one-fourth the MIC to a 55% reduction obtained at twice the MIC value.

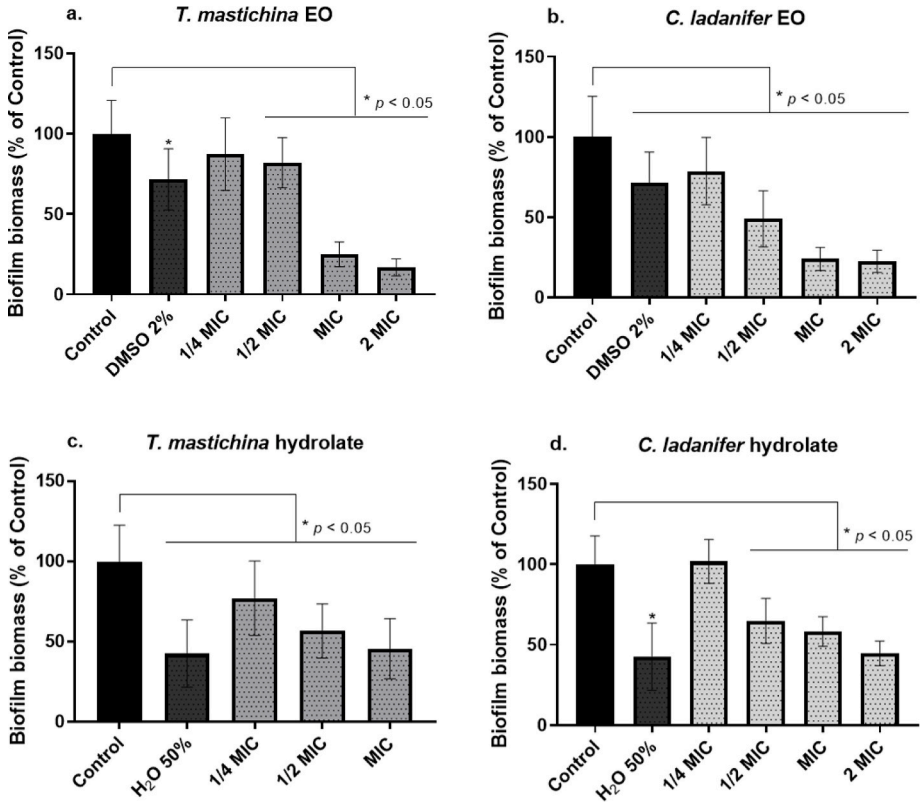

Fig. 8. Effect of EOs and hydrolates from TM (a, c) and CL (b, d) on 72 h mature biofilms from *C. acnes*, determined by staining biofilm biomass with crystal violet. DMSO was used at the maximum tested concentration of 2% (v/v) as solvent control, and ultrapure water was used at 50% (v/v) to mimic the dilution obtained when testing the hydrolates at the maximum concentration understudy. *p value < 0.05 was considered statistically significant.

4. Discussion

This study intended to valorize two Portuguese autochthonous species, TM and CL, with skin applications in folk medicine, thus scientifically supporting their traditional use. In addition, by studying not only their EOs but also their hydrolates, it was also intended to contribute to the valorization of these by-products, as they are typically discarded by EO producers. These preparations are characterized by having a much softer scent and lower biological activity than the corresponding EOs (Di Vito et al., 2021). They are also regarded as safe, as results obtained from toxicological tests did show. The worth of hydrolates gains relevance in the case of raw materials with a low EO content, thus leading to low EO yields, as in the case of CL (Frazão et al., 2018; Prusinowska et al., 2016).

As the biological activity of plant preparations is intrinsically related to their composition, we started by characterizing the compositional profile of both EOs and respective hydrolates. These results also allowed us to better understand the consistency of these preparations regarding their composition, when compared with others from the same species grown in Portugal. As disclosed in the results section, the main component present in TM EO was 1,8-cineole (43.5%). This result is in agreement with the composition of several EOs from TM of Portuguese origins reported in the literature, and from other Portuguese commercial samples for which 1,8-cineole was reported as the major compound (Aazza et al., 2016; Miguel et al., 2015; Pina-Vaz et al., 2004; Queiroga et al., 2018; Rodrigues et al., 2020). It is also in agreement with the requirements for chromatographic profile reported in ISO 4728:2003 for TM, where 1,8-cineole relative amount is required to be between 30 and 68% (International Organization for Standardization, 2003).

For CL EO, two monoterpenic hydrocarbons, α-pinene (50%) and camphene (10.1%) were the major components. This profile is also in agreement with the composition of CL EOs obtained from plants collected in Portugal. Tavares et al. reported the same major components in CL EOs obtained from steam and hydrodistillation, with variations depending on the harvest month and extraction methods (Tavares et al., 2020). Vieira et al. and Luis et al. also reported α-pinene (36%–39.25%) and camphene (8.22%–12%), as the major components in their EOs (Luís et al., 2020; Vieira et al., 2017). The EO here reported presented a higher percentage of α-pinene compared to the ones previously described. Still, this relative amount is in accordance with the maximum percentage described by Tavares et al. (59.5%) regarding Portuguese commercial EO samples from this species (Tavares et al., 2020). These differences in the relative amounts, expected and justifiable, can result from factors that range from plant characteristics, edaphoclimatic factors, cultivation management, plant material processing and EO isolation conditions, among others (Toncer et al., 2017).

Considering hydrolates, less information was found regarding these by-products, as much of the available information is focused on the correspondent EOs. Still, for CL hydrolate, information is available in the literature regarding this species, also harvested in Portugal. Tavares et al. reported that the major component in CL hydrolate obtained from steam distillation, was 2,6,6-trimethyl cyclohexanone (9.1%–12.4%) or *trans*-pinocarveol (5%–12.6%), depending on the harvest month (Tavares et al., 2020, 2022). *Trans*-pinocarveol was also reported as major component in CL hydrolate obtained from hydrodistillation (Tavares et al., 2020). Borneol was reported as one of the major components of the hydrolates, with relative amounts varying from 3.1% to 8.5%. Comparing our results with the ones described in the literature, we found higher relative amounts of the reported major components. As the plant material from both studies was collected from the same geographical location, other factors such as cultivation management or material processing can account for this difference. For TM hydrolate, no information was available in the literature regarding the chemical composition of hydrolates from Portuguese origins. Still, results from a hydrolate of Japanese origin also reported 1,8-cineole as the major component, still with higher relative amounts (75% *versus* 43.9%) (Inouye et al., 2008).

We further pursued a range of *in vitro* studies using a multidisciplinary approach in order to scientifically support the plants' traditional application in skin conditions. As some of the traditional uses of both plants are associated with an inflammatory state, we evaluated the preparations' anti-inflammatory potential by measuring NO production by LPS-stimulated macrophages, at biocompatible concentrations. The pro-inflammatory mediator NO is released by LPS-stimulated macrophages, after LPS binding to Toll-like receptor 4 (TLR4). This leads to the activation of intracellular cascades that culminated in the expression of inducible nitric oxide synthase (iNOS) which produces NO (Sun et al., 2019). This pro-inflammatory mediator plays an important role in regulating cutaneous inflammation, and high iNOS and endothelial nitric oxide synthase (eNOS) levels have been linked to skin inflammatory diseases (Man et al., 2022). Thus, NO production by LPS-stimulated RAW 264.7 cells has been widely used as an *in vitro* model of anti-inflammatory potential. As disclosed in the results section, all studied plant preparations were able to impair NO production, producing dose-dependent responses. Nevertheless, both the hydrolate and the EO from CL were effective even at very low concentrations, thus highlighting a more potent effect of CL for this application. In fact, the anti-inflammatory activity of CL extracts has been previously reported in the literature. Tavares et al. reported the anti-inflammatory potential of CL hydrolate *in vitro* using an albumin denaturation assay (Tavares et al., 2020). Also, Frazão et al. reported the effect of different extracts from CL resin, labdanum, on the reduction of NO production using the same method (Frazão et al., 2022). As neither of our tested preparations presented intrinsic NO scavenging activity, we hypothesized that the anti-inflammatory potential could be related to the catalysis of iNOS. For TM preparations, the anti-inflammatory potential was not as pronounced. However, both preparations presented higher biocompatibility with this cell line, when compared with CL. Therefore, despite not being as effective, when considering the difference between toxic and effective concentrations, TM EO was able to reduce 50% of NO production at concentrations ≈15 times lower than the ones required to reduce cell viability in the same extent. Only a few reports on the anti-inflammatory activity of this plant can be found in the literature and are related to its activity on the 5-lipoxygenase (5-LOX) enzyme, thus associating its anti-inflammatory potential with the LOX pathway (Cutillas et al., 2018). Still, our results led us to hypothesize that other pathways, as the iNOS pathway, may be involved.

We also evaluated the cytotoxicity of the preparations on L929 fibroblasts, not only to pursue with the determination of wound healing potential but also to comply with the ISO guideline ISO/EN 10993–5 for cytotoxicity determination (International Organization for Standardization, 2009). As expected, hydrolates presented higher biocompatibility when compared with correspondent EOs. As these preparations have much lower concentration of terpenes, molecules with associated toxicity, this could explain their higher biocompatibility even when tested at much high concentrations (Zárybnický et al., 2018). In addition, the hydrophilic nature of such preparations may also contribute to this result. Similarly to what was observed with the macrophage cell line, TM preparations were more biocompatible with fibroblasts when compared with preparations from CL. As these two preparations present 1,8-cineole as a major compound, this could account for their lower toxicity, as this oxygenated monoterpene has been previously associated with less cytotoxicity in fibroblasts when compared with other molecules of the terpene family (Mendanha et al., 2013). Cytotoxicity of preparations from the studied plant species have been described in the literature against different cell lines, mostly focusing on their cytotoxic activity against cancerous cells (Barrajón-Catalán et al., 2010; Gordo et al., 2012; Najar et al., 2020; Taghouti et al., 2020). Still, Kessler et al. studied the cytotoxic potential of the EO and hydrolate from TM on a monkey non-tumorous kidney cell line (Vero) and also reported a high biocompatible profile of the hydrolate compared with the correspondent EO (Kessler et al., 2022). Considering skin applications, specifically for cosmetic formulations, Gaweł-Beben et al. studied the cytotoxic

potential of methanolic extracts from CL against a noncancerous human keratinocytes cell line (HaCaT). Differently from our results, the extracts did not showed cytotoxic potential on the studied cells. Due to the different nature of the preparations, and therefore of their composition on bioactive compounds, no direct comparisons can be drawn. Still, the authors ultimately concluded about the interest of this species for skin application, considering the portrayed *in vitro* bioactivities.

As one of the traditional applications of both species is related to its ability to promote the cicatrization of superficial wounds, we tested all plant preparations regarding their effect on cellular migration, an important step when studying skin repairing/wound healing potential. Our results appear to support the traditional application of CL for this specific outcome as, for both plant preparations, there was a significant increase in cellular migration. To the best of our knowledge, no studies addressed the skin repairing potential of CL EO and hydrolate in the literature, thus not permitting a direct comparison. Still, other preparations are also used for wound healing purposes and there is a report of increased proliferation and viability of skin keratinocytes after exposure to a fraction released from plasters coated with CL extract, important *in vitro* markers for wound healing (Haponska et al., 2021). The wound healing potential of compounds present in our preparations, as α-pinene has also been reported (Pérez-Recalde et al., 2018; Salas-Oropeza et al., 2021). Despite the wound healing potential of *E*-pinocarveol, the main component of CL hydrolate, has not been reported, the wound healing potential of this extract may be related to other components, such as borneol, for which the wound healing capacity is well established (Barreto et al., 2016; Mai et al., 2003). Regarding TM, a milder but significant positive result on wound healing was found for hydrolate but not for the EO. Despite 1,8-cineole have a recognized wound healing capacity, as elegantly reviewed by Mercedes Pérez-Recalde et al., as the EO was used in a much lower concentration than the hydrolate, this higher dilution of the bioactive component could be one accountable factor for this difference (Pérez-Recalde et al., 2018).

We also investigated the antimicrobial and antioxidant activities of the two species, both important features for skin application (Pérez-Recalde et al., 2018). Regarding the antioxidant potential of the tested plant preparations, CL EO presented a higher antioxidant potential than TM EO, with hydrolates presenting an opposite profile. Although several studies have been performed on the antioxidant capacity of TM, as recently reviewed by Rodrigues M. et al., most of the studies evaluated the activity of extracts of aqueous or alcoholic nature, which typically performed better in the DPPH radical scavenging method (Arantes et al., 2017; Rodrigues et al., 2020). As our work focused on EOs and hydrolates, a lower antioxidant potential for TM is here described when compared with the literature. For CL, the absence of a relevant antioxidant capacity of the hydrolate has already been reported (Oliveira et al., 2018). Contrarily, its EO has been described to possess some potential to act as an antioxidant in the DPPH and β-carotene methods (Luís et al., 2020). Nonetheless, all of the tested preparations acted as poor antioxidants when compared with the included positive control, ascorbic acid.

Finally, focusing on the antimicrobial activity of the plant preparations, we evaluated their activity against relevant pathogens associated with skin conditions and against a panel of representative microorganisms, relevant when aiming for a skin application (International Organization for Standardization, 2014). As presented in the results section, CL EO performed better in terms of antimicrobial activity by presenting lower visual MIC values for a higher number of species. It also presented antimicrobial activity, at a low concentration, against a collection strain of *S. epidermidis*, isolated from human skin, which was multi-resistant to classical antibiotics. As classic antibiotics present mechanisms of action on specific targets, contrarily to EOs that act trough non-specific mechanisms, we did not expected a direct relation between the susceptibility profiles of the antibiotics and the plant preparations. This prediction was corroborated with the obtained results for EOs, for which the effect was proven dependent of the bacterial cell wall. Hydrolates did not presented relevant antimicrobial activity except against *C. acnes,* for which they were able to prevent bacterial growth, still at high concentrations. This low antimicrobial activity of CL hydrolate has been previously reported, specifically against *S. aureus*, *E.coli* and *C. albicans* (Tavares et al., 2020).

The activity of both plant preparations against *C. acnes*, a relevant pathogen in one of the most common skin diseases, combined with the report of the traditional application for acne treatment, motivated us to further investigate their anti-acne potential (Pardo de Santayana et al., 2014). Thus we pursued by studying the effect of the plant preparations on *C. acnes* biofilms, structures associated with disease severity (Honraet et al., 2013). To the best of our knowledge, no studies have been conducted on the anti-acne potential of these plant species regarding their effect on planktonic or biofilm growth forms of *C. acnes*. Other thyme species have been studied for the anti-acne potential of their EOs, specifically, *Thymus* x *citriodorus* and *Thymus quinquecostatus,* presenting lower MIC values of 0.06% (v/v) and 0.04% (v/v), respectively, thus presenting higher activity than TM (Oh et al., 2009; Oliveira et al., 2022). Main differences in chemical composition among these thyme species may be accountable for this difference, as 1,8-cineole (the major compound in TM EO) is reported to be deprived of *C. acnes* activity (Raman et al., 1995). Still, surprising results were obtained with CL EO, which presented the capacity to inhibit the formation and to disrupt *C. acnes* biofilms. The anti-acne potential of CL preparations or extracts was not yet described, thus not allowing a direct comparison. Still, some of its main components, particularly α-pinene in the EO, and terpinene-4-ol in hydrolate, are reported to possess activity against *C. acnes* and *S. epidermidis* species (Raman et al., 1995).

5. Conclusions

Plant preparations are widely applied in different fields due to their wide range of bioactivities. Still, is essential to valorize the available endemic or autochthonous species when studying their bioactive potential, preserving and validating the traditional knowledge associated with their application. In addition, the valorization of local production of such species presents advantages to local producers and to the entire ecosystem, since they are well adapted to their origin.

Our results support the usefulness of TM and CL, two Portuguese autochthonous species, as bioactive ingredients for skin application. CL preparations presented *in vitro* anti-inflammatory, skin repairing and antimicrobial potential, important indicators considering their traditional application for the healing of superficial wounds. In addition, an interesting potential to impair *C. acnes* virulence uncovered a possible new application for this species, specifically when combining this activity with its anti-inflammatory and skin repairing potential, important features for anti-acne applications. Considering TM, this species did not present a demarked bioactive potential, especially when compared with CL. Nevertheless, antimicrobial activity and wound healing capacity were present for its EO and hydrolate, respectively. Also, TM preparations, particularly its hydrolate, showed an interesting biocompatible profile, with associated anti-inflammatory activity, thus supporting their value to be used as active ingredients to improve skin health in inflammatory skin conditions such as *acne vulgaris*. Additionally, their capacity to impair *C. acnes* biofilms also supports the traditional knowledge related to this application.

Additional research, using close-to-disease models, must be pursued to further confirm the applications of different preparations from CL and TM, validating the benefits of these species for skin application.

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CRediT authorship contribution statement

Ana S. Oliveira: Investigation, Methodology, Formal analysis, Writing – original draft. **Joana Rolo:** Methodology, Validation, Writing – review & editing. **Carlos Gaspar:** Methodology, Validation, Writing – review & editing. **Leonor Ramos:** Methodology, Writing – review & editing. **Carlos Cavaleiro:** Investigation, Formal analysis, Writing – review & editing. **Lígia Salgueiro:** Investigation, Formal analysis, Writing – review & editing. **Rita Palmeira-de-Oliveira:** Methodology, Writing – review & editing. **João Paulo Teixeira:** Writing – review & editing, Formal analysis, Supervision. **José Martinez-de-Oliveira:** Conceptualization, Writing – review & editing, Supervision. **Ana Palmeira-de-Oliveira:** Conceptualization, Writing – review & editing, Funding acquisition, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2022.115830.

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