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# Chemical characterization and bioactive potential of *Thymus* × *citriodorus* (Pers.) Schreb. preparations for anti-acne applications: Antimicrobial, anti-biofilm, anti-inflammatory and safety profiles

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## ABSTRACT

*Ethnopharmacological relevance: Thymus* × *citriodorus* (Pers.) Schreb. is an interspecific hybrid between *Thymus pulegioides* and *Thymus vulgaris,* known for its pharmacological activities as diaphoretic, deodorant, antiseptic and disinfectant, the last mostly related with its antimicrobial activity. The folk use of other extracts, as hydrolates, have also been disseminated, as regulators of oily skin with anti-acne effect.

*Aim of the study:* We aimed to evaluate the anti-acne potential of two *Thymus x citriodorus* (TC) preparations, the essential oil (EO) and the hydrolate, to be used as active ingredients for skin applications. Specifically, we intend to validate their anti-acne potential by describing their activity on acne related bacteria, bacterial virulence, antioxidant and anti-inflammatory potential, and biocompatibility on inflammatory cells. Additionally, we aimed to report their ecotoxicity under the Globally Harmonized System of Classification and Labelling of Chemicals (GHS), thus focusing not only on the consumer, but also on environmental safety assessment.

*Materials and methods:* Minimum inhibitory concentration (MIC) against *C. acnes*, *S. aureus* and *S. epidermidis* was evaluated. Minimum lethal concentration (MLC) was also determined. The effect on *C. acnes* biofilm formation and disruption was evaluated with crystal violet staining. Anti-inflammatory activity was investigated on LPS-stimulated mouse macrophages (RAW 264.7), by studying nitric oxide (NO) production (Griess reagent) and cellular biocompatibility through MTT assay. *In-vitro* NO and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging potential were also evaluated. The ecotoxicity was evaluated using *Daphnia magna* acute toxicity assays.

*Results:* EO presented direct antimicrobial activity, with visual MICs ranging from 0.06% for *S. epidermidis* and *C. acnes* to 0.125% for *S. aureus.* MLCs were higher than the obtained MICs. Hydrolate revealed visual MIC only for *C. acnes*. TC essential oil was effective in preventing biofilm formation and disrupting preformed biofilms

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even at sub-inhibitory concentrations. Hydrolate showed a more modest anti-biofilm effect. Regarding anti-inflammatory activity, TC hydrolate has a higher cellular biocompatibility. Still, both plant preparations were able to inhibit at least 50% of NO production at non-cytotoxic concentrations. Both EO and hydrolate have poor anti-oxidant activities. Regarding the ecotoxicity, TC essential oil was classified under acute 3 category, while the hydrolate has proved to be nontoxic, in accordance to the GHS.

*Conclusions:* These results support the anti-acne value of different TC preparations for different applications. TC hydrolate by presenting higher biocompatibility, anti-inflammatory potential and the ability to modulate *C. acnes* virulence, can be advantageous in a product for everyday application. On the other hand, EO by presenting a marked antimicrobial, anti-biofilm and anti-inflammatory activities, still with some cytotoxicity, may be better suited for application in acute flare-ups, for short treatment periods.

## 1. Introduction

Plant extracts have been considered interesting natural medicinal alternatives and have been traditionally used as therapeutic agents (Palmeira-De-Oliveira et al., 2012). In fact, in the last years, consumers had increasingly search for "green" cosmetic products and/or medical devices (Kamel et al., 2020), without the addition of synthetic chemical molecules, as natural alternatives for the promotion of skin health. In this increasing trend towards natural products, essential oils (EO) from numerous plants have made a remarkable entrance into both the therapeutic and cosmetic fields (Barbaud and Lafforgue, 2021). This increasing demand of plant based products is, among other factors, believed to be due to the inherent perception that such products are safer and more efficient compared with modern medicine ("WHO | WHO traditional medicine strategy: 2014–2023," 2015). Although, despite the medical benefits of different plant based products, as essential oils, there have been many reports on various toxic effects following prolonged consumption or application of these products (Md Zin et al., 2018). Additionally, due to the high demand by the consumer and a spread increase in their use and production, essential oils and their by-products are often discharged into the environment. Thus, it becomes essential to characterize their safety profile not only to the consumer, but also regarding their environmental toxicity.

*Thymus* genus includes several aromatic species with major interest due its medicinal and non-medicinal uses. *Thymus* L. is a large genus of the Lamiaceae family, comprising 300–400 species widely distributed around the globe, particularly in the Mediterranean region (Pereira et al., 2013b). Several preparations of *Thymus* spp. have been used for the treatment of gastrointestinal, respiratory and skin disorders (Roxo et al., 2020). Also, volatile oils from species of this genus are often used in pharmaceuticals, as well as in the production of perfumes and cosmetics (Raudone et al., 2017). The use of some species as herbal medicinal ingredients is harmonized by the European Union through a EU monograph issued by the European Medicines Agency and object of attentive reports of the European Scientific Cooperative on Phytotherapy and World Health Organization (EMA, 2007; ESCOP, 2003; WHO, 1999).

One particular thyme species that has gained interest in the last years is *Thymus x citriodorus* (Pers.) Schreb. (TC) or lemon-(Lundgren and Stenhagen, 1982; Ntalli et al., 2020; Rita et al., 2018; Taghouti et al., 2020). This attractive medicinal and aromatic perennial subshrub is reported to be a hybrid of two *Thymus* species, *T. pulegioides* and *T. vulgaris* (Jurevičiūtė et al., 2019; Omidbaigi et al., 2005). The pleasant citric aroma characteristic of this plant, makes it one of the most used *Thymus* in the food industry (Pereira et al., 2013b).

The EO obtained from of the leaves of *Thymus x citriodorus*, is described to be a strong antiseptic, with deodorant and disinfectant properties (Omidbaigi et al., 2009; Pereira et al., 2013a), the last majorly related to its antimicrobial activity (Orłowska et al., 2015; Rita et al., 2018; Sacchetti et al., 2005). The essential oil is also described to be used in aromatherapy to treat asthma and other respiratory complaints, especially in children, being considered less irritant than other *Thymus* species (Horváth et al., 2006; Pereira et al., 2013a). In addition to the essential oil, different extracts of this plant, such as hydrolates, have been produced and their properties as regulators of oily skin and hair and anti-acne effect have been disseminated in folk medicine and among plant producers and distributors (Graber, 2012; "Hidrolato de Tomilho-Limão Biológico – Ministica," n.d., "Thyme - Lemon (Thymus citriodorus)," 2021, "Tomilho limão – Ervitas Catitas," n.d.; Li et al., 2019).

*Acne vulgaris*, also known as acne, is a common skin disease of the pilosabeceous unit that impacts the quality of life of millions worldwide, affecting majorly adolescents and, in lower extent, young adults (Claudel et al., 2019; Lim et al., 2018; Pineau et al., 2019). This skin disease is characterized by an inflammatory state of the pilosebaceous unit and has specific hallmarks that contribute to its development, namely: excessive sebum production, hyperkeratinization, increased release of inflammatory mediators and overgrowth of the bacteria *Cutibacterium acnes (C. acnes)* (Lee et al., 2017; Lim et al., 2018; Zhu et al., 2020). *C. acnes* is a gram-positive rod of particular importance in acne progression, as it contributes to modulate keratinocyte proliferation, secrete virulent enzymes, and activating skin innate immunity (de Canha et al., 2020). Also, the biofilm growth form of *C. acnes* plays an important role in antibiotic resistance and pathogenesis, with biofilm-forming strains being associated with more severe cases of *acne vulgaris* (Coenye et al., 2012). Still, the presence of other bacterial species in acne lesions has also been reported (Jusuf et al., 2020). From those, *Staphylococcus aureus* and *Staphylococcus epidermidis* have been identified, along with and *C. acnes,* as the main acne-related bacteria (Claudel et al., 2019; Yamaguchi et al., 2009).

Due to its multifactorial pathogenicity, several drugs are used to control *acne vulgaris* progression, ranging from sebum production regulators, keratolytics, non-steroid anti-inflammatory drugs and antibiotics (de Canha et al., 2020). Isotretinoin, a classical drug used to treat *acne vulgaris*, has ability to counteract the various hallmarks of the disease. However, safety concerns are associated with this drug due to its teratogenic potential (Chen et al., 2020). Other therapeutic agents include azelaic acid, which is often associated with skin depigmentation, and retinoic and salicylic acids, that can also cause skin irritation and desquamation (Woolery-Lloyd et al., 2013). Lastly, treatment of *acne vulgaris* with antibiotics, has increased drug resistance of *C. acnes* to such drugs (Pineau et al., 2019). Therefore, the need for active and safe alternatives to control the progression of the disease is mandatory.

With this work, we aim to highlight the potential of *Thymus x citriodorus* (Pers.) Schreb. essential oil and also the by-product of its production (hydrolate), to be used as active ingredients for skin applications. Specifically, we intend to validate their anti-acne potential by studying their ability to control growth and virulence of acne related bacteria, and to modulate inflammation and oxidation. Additionally, we intend to explore their ecotoxicological hazard, thus contributing to fill the gap on the existing information about the environmental toxicity of these plant preparations.

## 2. Methods and materials

### 2.1. Plant preparations

TC essential oil, isolated by steam distillation, and the correspondent

hydrolate (batch numbers: 07TC20) were obtained from Ervitas Catitas™ company (Portugal). Plants were produced under organic farming, as certified by PT-BIO-04. Plant names were checked with https://www.theplantlist.org.

### 2.2. Essential oil and hydrolate chemical analyses

GC was performed in a Hewlett-Packard 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph, with a single injector and two flame ionization detectors. A divider (Agilent Technologies, part no. 5021–7148) was used for simultaneous sampling into two different fused silica capillary columns: SPB-1 (polydimethylsiloxane 30m × 0.20 mm i.d., film thickness 0.20 μm) and SupelcoWax-10 (polyethyleneglycol 30m × 0.20 mm i.d., film thickness 0.20 μm) (both from Supelco, Bellefonte, PA, USA). An HP GC ChemStation Rev. A.05.04 data system was used for operation and data handling. Oven temperature program: 70–220 °C (3 °C.min$^{-1}$), 220 °C (15 min); injector temperature: 250 °C; carrier gas: helium, adjusted to a linear velocity of 30 cm s$^{-1}$; detectors temperature: 250 °C. GC-MS analysis was performed with an Agilent 6890 gas chromatograph interfaced with a mass selective detector MSD 5973 (Agilent Technologies), both operated by HP Enhanced ChemStation software, version A.03.00. A HP1 fused silica column (polydimethylsiloxane 30m × 0.25 mm i.d., film thickness 0.25 μm) was used. GC parameters were as above described; MSD parameters: interface temperature: 250 °C; MS source temperature: 230 °C; MS quadrupole temperature: 150 °C; ionization energy: 70 eV; ionization current: 60 μA; scan range: 35–350 units; scans.s$^{-1}$: 4.51.

For the essential oil analysis, samples were diluted (1:8) in *n*-pentane and then injected (0.2 μL) in split mode (1:40).

For the hydrolate analysis, sample (100 mL) was added of *n*-dodecane as internal standard and submitted to liquid/liquid partition with (3 x 30 mL) *n*-pentane/diethyl oxide [93/7 (v/v)]. The organic fraction was concentrated at low pressure and room temperature and further adjusted to 10 mL with *n*-pentane. Solutions were injected (0.2 μL) in splitless.

Identifications of the essential oil and hydrolate 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–C23 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) the acquired mass spectra compared with reference data from the Wiley/NIST library (McLafferty, 2009).

For the TC essential oil, the relative amount of each component was estimated from GC peaks areas without corrections to FID responses. For the TC hydrolate both, the relative amount of each constituent in the volatile fraction (%) (calculated from GC-FIC raw data without any correction) and the absolute concentration of each constituent (expressed by reference to *n*-dodecane) were calculated.

### 2.3. Bacterial strains

Activity of both preparations was evaluated against acne related bacteria, using collection strains from American-Type Culture Collection specifically *S. aureus* (ATCC 6538), and from Deutsche Sammlung von Mikroorganismen und Zellkulturen, namely *S. epidermidis* (DSM 28764) and *C. acnes* (DSM, 1897). In order to assess culture purity and viability, microorganisms were subcultured twice on tryptic soy agar -TSA (VWR, Radnor, Pennsylvania, USA) for aerobic bacteria; and on Brain Heart Infusion broth - BHI (Himedia, L.B.S. Marg, Mumbai, India), supplemented with 15 g.L$^{-1}$ of agar (Himedia, L.B.S. Marg, Mumbai, India) and 5% glucose (Sigma-Aldrich, St. Louis, MO, USA) for anaerobic bacteria. For the growth of *C. acnes*, an anaerobic environment was generated using Anaerocult® A (Merck, Darmstadt, Germany).

### 2.4. Antimicrobial activity

The antimicrobial activity against aerobic and anaerobic bacteria was performed according to Clinical and Laboratory Standards Institute M07-A10 and M11-A6 microdilution methods, respectively, with small modifications (Clinical and Laboratory Standards Institute., 2004; Clinical and Laboratory Standards Institute, 2015). Briefly, a 0.5 MacFarland suspension was prepared from bacterial cultures using sterile phosphate buffer solution and proper dilutions were made with Mueller Hinton broth [MHB (VWR Chemicals)] for *S. aureus* and *S. epidermidis*, and with Brain Heart Infusion broth supplemented with 5% glucose (sBHI) for *C. acnes*. Bacterial suspensions were exposed to serial dilutions of essential oil and hydrolate, resulting in a 2-fold dilution of both plant preparations and bacterial suspensions. TC essential oil was diluted in sterile DMSO (Honeywell, Charlotte, North Carolina, USA) while TC hydrolate was used directly.

Minimum inhibitory concentration (MIC) was defined as first extract concentration with an absence of microbial growth, determined by visual inspection, after 24h and 72h for aerobic and anaerobic bacteria, respectively. Additionally, due to the inter-operator variability of MIC determination and the possible interference with turbidity generated by some preparations (Tomás et al., 2021), absorbance was measured at 600 nm and compared with the controls, to confirm the visual MIC results. $MIC_{50}$ value (concentration estimated to inhibit 50% of microbial growth) was also calculated.

Minimum lethal concentrations (MLC) were determined by inoculating 5 μl of the content of each well where no bacterial growth was visually assessed, on TSA or sBHI agar, and incubating for 24h and 72h for aerobic and anaerobic bacteria, respectively. MLC was defined as the first concentration where no bacterial colonies were present, after the correspondent incubation period.

In addition to growth control (bacteria in the respective culture medium), a sterility control (un-inoculated medium) was also included, in addition to a solvent control (DMSO) in the maximum tested concentration (2%) to discard its influence on bacterial growth.

### 2.5. Effect on biofilm adhesion

The effect of TC essential oil and hydrolate against *C. acnes* biofilm adhesion to polystyrene surfaces was determined as previously described, with minor adjustments (de Canha et al., 2020; Di Lodovico et al., 2020). Briefly, *C. acnes* was used to prepare a bacterial pre-inoculum, for 3 days in sBHI, under anaerobic conditions. This bacterial suspension was diluted and mixed with concentrated TC preparations to yield a dilution of the plant preparations and approximately $10^6$ CFU.mL$^{-1}$. The bacterial suspension in the presence of plant preparations, was inoculated in a 96-well flat-bottom microdilution plate. Plant preparations were tested at twice, once, half and one-fourth of the obtained planktonic MIC. TC essential oil firstly was diluted in sterile DMSO (Honeywell, Charlotte, North Carolina, USA) while TC hydrolate was used directly, as described for 'Antimicrobial activity'.

The plates were incubated at 37 °C for 72h under anaerobic environment, allowing biofilm formation. Following incubation, medium was removed, and plates were washed twice with 100 μL of sterile PBS, to remove planktonic phase. Adhered cells were fixed for 15 min using 100 μL of 99% methanol (Honeywell, Charlotte, North Carolina, USA) and, afterwards, methanol was discarded and plates were allowed to air dry. Quantification of biofilm biomass was performed by staining adherent phase with 0.5% crystal violet (amresco, Solon, OH, USA) for 20 min and, afterwards, plates were gently rinsed under running water to remove the excess stain. The bound crystal violet was dissolved using 150 μL of a 33% acetic acid solution and the optical density was measured at 590 nm using xMarkTM Microplate Absorbance Spectrophotometer (Bio-Rad, California, USA).

A positive control referring to 100% adhesion (untreated *C. acnes*) and a negative control (un-inoculated medium) were also included. The

possible effect of the solvent was also assessed. $EC_{50}$ value, corresponding to the estimated concentration necessary to cause a 50% decrease in biofilm adhesion, was calculated. Representative images of the effect of TC preparations on biofilm formation were taken using an inverted microscope (Olympus Model IX51) with a 10x10 amplification.

### 2.6. Effect on biofilm disruption

The ability of TC essential oil and hydrolate to disrupt *C. acnes* preformed biofilms was also determined as described elsewhere (Gaspar et al., 2021; Palmeira-De-Oliveira et al., 2012). *C. acnes* pre-inoculum was prepared as described in the adhesion protocol. Approximately $10^6$ CFU.mL$^{-1}$ were plated in 96-well flat-bottom microdilution plates and incubated for 72 h to allow biofilm formation. Afterwards, media was removed, and the plates were gently washed twice with sterile PBS to remove planktonic cells. Following the washing step, 200 μL of TC essential oil or hydrolate, prepared in sBHI at the final concentrations, were added to the wells and incubated for an additional 72 h period. After incubation, disrupted biofilms were treated as described for the adhesion protocol. $EC_{50}$ value, corresponding to the estimated concentration able to disrupt 50% of the preformed biofilm, was also calculated. Representative images of the effect of TC preparations on the disruption of preformed biofilms were taken using an inverted microscope (Olympus Model IX51) with a 10x10 amplification.

### 2.7. Cell culture

Raw 264.7, a mouse leukaemic monocyte macrophage cell line from American Type Culture Collection (ATCC-TIB-71), was kindly supplied by Dr. Teresa Gonçalves (Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal). Cells were cultured in Dulbecco's Modified Eagle's Medium - DMEM (Gibco, Alfagene, Lisboa Portugal), supplemented with 10% (v/v) of non-inactivated Fetal Bovine Serum - FBS (Gibco, Alfagene, Lisboa Portugal), 100 U.mL$^{-1}$ penicillin, 100 μg.mL$^{-1}$ streptomycin (Gibco, Alfagene, Lisboa Portugal), Glucose (Sigma-Aldrich, St. Louis, MO, USA) up to the final concentration of 4.5 g.L$^{-1}$, and 1.5 g.L$^{-1}$ of sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA), at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$. The cells were used from passage 8 to passage 17. Before reaching confluence, the cells were sub cultured using a cell scrapper according to ATCC recommendations.

### 2.8. Effect of TC preparations on cellular viability

Cellular viability was assessed by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Alfa Aesar, Massachusetts, USA) assay, as previously described (Oliveira et al., 2020) and according to ISO/EN 10993-5 (International Organization for Standardization, 2009), with minor adaptations, as follows. TC essential oil was diluted in sterile DMSO (Honeywell, Charlotte, North Carolina, USA) prior the assay, while TC hydrolate was used directly, as described in the previous sections. 2.5x10$^4$ cells *per* well were seeded in a 96-well plate and, after 24h, were exposed to 2-fold dilutions of TC preparations, prepared in DMEM culture medium, for an additional 24h period. Thereafter, medium was removed and cells were washed with 150 μl of sterile pre-warmed PBS, to remove traces of TC preparations that could interfere with MTT. Freshly prepared MTT solution (prepared in incomplete culture medium) was added to each well (1 mg.mL$^{-1}$) and the plates were incubated, in the dark, at 37 °C for 4h. MTT-containing medium was removed and 100 μl of propan-2-ol (Fisher Chemical, Chicago, IL, USA) was added to dissolve the formazan crystals. Dissolved formazan was quantified using an xMarkTM Microplate Absorbance Spectrophotometer (Bio-Rad, California, USA) at 570 nm, with a reference wavelength of 630 nm. $EC_{50}$ value, corresponding to the estimated concentration that impair 50% of cellular viability, when compared with culture medium only (Control) was calculated. Sodium dodecyl sulfate [(SDS), PanReac AppliChem, Barcelona, Spain] at 2% was also included as a positive control for cytotoxicity.

### 2.9. Nitric oxide (NO) production

For NO determination cells were plated at a density of 1x10$^5$ *per* well. After 24h, the cells were simultaneously exposed to 1 μg.mL$^{-1}$ of lipopolysaccharide-LPS (Sigma-Aldrich, St. Louis, MO, USA) and 2-fold dilutions of TC preparations, prepared as described in the previous sections, for 24 h. Afterwards, NO production was determined by measuring nitrite accumulation in the culture supernatants, through a colorimetric assay using the Griess reagent, as described elsewhere (Silva et al., 2020). Briefly, equal volumes of cell culture supernatants and 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. The absorbance was measured at 550 nm and nitrite concentration was calculated through a sodium nitrite standard curve. $EC_{50}$ value, corresponding to the estimated concentration able to reduce 50% of NO production, when compared with culture medium in the presence of LPS (Control + LPS), was also calculated. An anti-inflammatory drug, Dexamethasone (Sigma-Aldrich, St. Louis, MO, USA) at 10 μM, was included as positive control.

### 2.10. Determination of NO scavenging activity

NO scavenging activity was evaluated as described (Roxo et al., 2020) by adding a NO donor - S-nitroso-N-acetylpenicillamine [(SNAP), Biogen, Tocris, Bristol, UK] at 300 μM, to 300 μl of each dilution of TC hydrolate and essential oil, prepared in DMEM culture medium, as described in the previous sections. The mixture was incubated for 3h in the dark. Nitrite levels in the medium were then quantified by Griess method, as described for NO production determination.

### 2.11. DPPH radical scavenging assay

The free radical-scavenging potential for 2,2-Diphenyl-1-picrylhydrazyl [(DPPH)- Sigma-Aldrich, St. Louis, MO, USA)] of both TC preparations was determined using the method originally proposed by Molyneux (n.d.), with the adaptation to a microplate assay, as proposed by Prieto (n.d.). Briefly, 100 μl of methanol DPPH solution (stock solution: 0.05 mg.mL$^{-1}$) was incubated with 100 μl of serial diluted TC hydrolate and essential oil, yielding a 2-fold dilution. The negative control (without sample - Control) was composed of DPPH stock solution mixed with methanol (final concentration: 0.025 mg.mL$^{-1}$) and Ascorbic acid (Fisher Chemical, Chicago, IL, USA) was used as positive control. Plates were incubated for 30 min in the dark at room temperature and afterwards, the absorbance was read in a microplate reader at 517 nm. DPPH radical-scavenging activity of the tested samples was calculated using the following equation (1), as described elsewhere (Rocha et al., 2021):

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

Where Abs sample is the absorbance of DPPH in the presence of the sample, Abs blank is basal absorbance of the sample in methanol and Abs control is the absorbance of the negative control.

The $IC_{50}$ (estimated concentration of an antioxidant necessary to cause a 50% inhibition of free radical activity) was determined using a calibration curve by plotting sample concentrations versus the corresponding percentage of DPPH reduction. The antioxidant activity was expressed using the antioxidant activity index (AAI), as described by Scherer et al. (Scherer and Godoy, 2009), that was calculated using the following equation (Equation (2)):

$$AAI = \frac{Final\ concentration\ of\ DPPH}{IC_{50}\ of\ the\ sample} \quad (2)$$

Samples were considered to show poor antioxidant activity when AAI <0.5, moderate antioxidant activity when AAI was between 0.5 and 1.0, strong antioxidant activity when AAI was between 1.0 and 2.0, and very strong when AAI >2.0 (Scherer and Godoy, 2009).

### 2.12. Acute toxicity assays with Daphnia magna

TC essential oil and hydrolate acute toxicities were evaluated using the freshwater crustacean *Daphnia magna* (Strauss, clone K6) following the OECD guideline 202 (OECD, 2004). The organisms used were obtained from a culture maintained at our laboratory in ASTM hard water, fed daily with a *Raphidocelis capitata* suspension ($3.0 \times 10^5$ cells.mL$^{-1}$). The organisms were kept in controlled temperature chambers (20° ± 1) under a 16/8h light/dark cycle. Prior to the toxicity tests, parthenogenic neonates (<24h old) were selected from the 2nd to 5th brood and exposed for 48h to an increasing set of concentrations of the substances under study.

Due to poor solubility in the medium, TC essential oil was first diluted in a solution containing 1% DMSO in ASTM hard water. In order to comply with the Globally Harmonized System of Classification and Labelling of Chemicals (GHS), mg.L$^{-1}$ units of measurement were used in this assay. Successive dilutions were then performed until reaching the working concentrations (10, 15, 22.5, 33.75, 55.63 mg.L$^{-1}$) and ensuring that the solvent concentration was never higher than 0.1% (OECD, 2019). TC hydrolate was diluted in ASTM hard water and subsequently diluted until reaching the working concentrations. The concentrations used for hydrolate were 125, 250, 500, 1000, 2000 mg.L$^{-1}$. For the essential oil, a solvent control (DMSO 0.1% in ASTM hard water) was also performed, and for TC hydrolate only the ASTM control was performed as no solvent was used. The tests were performed in multi-well plates, each well containing 10 mL of test substance and five organisms (2 mL of test substance *per* organism as recommended in the OECD guideline). Each concentration had five replicates. The plates were then transferred to the test chamber maintaining the same conditions as for culture, and the organisms were not fed for the entire duration of the test, which was repeated five times. Immobilisation of the daphnids was observed (an organism was considered immobilised when no movement was observed for 15 s after gentle agitation of the test vessels) and recorded after 48h of exposure and the $EC_{50}$ value (concentration estimated to cause the measured effect in 50% of the organisms) was calculated.

### 2.13. Statistical analyses

*In vitro* assays were carried out in, at least triplicates, in three independent experiments, and means ± SD values were expressed as percentages of the respective negative controls (without treatments). One-way ANOVA with Tukey's multiple comparisons test or Student's *t*-test were performed to compare the effect of each concentration with the controls. A p value < 0.05 was accepted as denoting statistical significance. Half-maximal inhibitory or effective concentrations ($MIC_{50}$ or $IC_{50}$/$EC_{50}$), were calculated for different bioactivities by logistic regression using GraphPad Prism version 8 for Windows. $IC_{50}$ for DPPH radical scavenging was calculated using a calibration curve generated by Microsoft Excel for Windows. The EO and hydrolate $EC_{50}$ values causing acute toxicity upon *D. magna* were calculated by non-linear regression between the [Oil/Hydrolate] vs. normalized immobilisation response with the variable slope model using GraphPad Prism V8 software.

## 3. Results

### 3.1. Chemical composition

The detailed compositions of both preparations, TC essential oil and hydrolate, are presented in Tables 1 and 2, respectively. In brief, TC essential oil is composed of an high amount of oxygen containing

**Table 1**
Composition of the essential oil of *Thymus x citriodorus*.

| Exp. RI[a] | Ref. RI[a] | Exp. RI[b] | Ref. RI[b] | Compound* | Percent in sample (%) |
|---|---|---|---|---|---|
| **921** | 921 | 1030 | 1030 | α-Thuyene | 0.3 |
| **929** | 930 | 1030 | 1030 | α-Pinene | 1.6 |
| **943** | 943 | 1075 | 1073 | Camphene | 0.6 |
| **964** | 964 | 1226 | 1126 | Sabinene | 1.1 |
| **970** | 970 | 1118 | 1118 | β-Pinene | 2.0 |
| **962** | 962 | 1255 | 1253 | 3-Octanone | 1.3 |
| **980** | 980 | 1161 | 1162 | Myrcene | 0.7 |
| **982** | 984 | 1384 | 1384 | 3-Octanol | 0.7 |
| **997** | 997 | 1171 | 1171 | α-Phellandrene | 0.1 |
| **1007** | 1010 | 1179 | 1081 | α-Terpinene | 0.4 |
| **1011** | 1011 | 1275 | 1275 | *p*-Cymene | 3.7 |
| **1020** | 1019 | 1215 | 1215 | 1,8-Cineole | 16.3 |
| **1025** | 1025 | 1235 | 1235 | *Z*-β-Ocimene | 0.1 |
| **1046** | 1046 | 1249 | 1249 | γ-Terpinene | 2.1 |
| **1050** | 1051 | 1459 | – | *E*-Sabinene hydrate | 0.1 |
| **1076** | 1076 | 1288 | 1288 | Terpinolene | 0.2 |
| **1082** | 1082 | 1541 | 1542 | Linalool | 1.9 |
| **1118** | 1118 | 1515 | 1515 | Camphor | 0.1 |
| **1144** | 1144 | 1695 | 1695 | Borneol | 2.7 |
| **1158** | 1158 | 1597 | 1597 | Terpinen-4-ol | 0.6 |
| **1169** | 1169 | 1692 | 1692 | α-Terpineol | 2.2 |
| **1195** | 1194 | 1830 | – | *trans*-Carveol | 0.3 |
| **1209** | 1209 | 1797 | 1797 | Nerol | 1.2 |
| **1214** | 1214 | 1591 | 1583 | Thymylmethyl oxide | 0.3 |
| **1216** | 1214 | 1679 | 1679 | Neral | 3.4 |
| **1222** | 1223 | 1601 | 1614 | Carvacrylmethyl oxide | 0.3 |
| **1233** | 1233 | 1842 | 1842 | Geraniol | 27.5 |
| **1242** | 1240 | 1730 | 1743 | Geranial | 4.2 |
| **1264** | 1264 | 1574 | 1574 | Bornyl acetate | 0.1 |
| **1267** | 1267 | 2188 | 2188 | Thymol | 9.2 |
| **1275** | 1275 | 2215 | 2215 | Carvacrol | 1.3 |
| **1375** | 1375 | 1516 | 1517 | β-Bourbonene | 0.8 |
| **1375** | 1375 | 1751 | 1752 | Neryl acetate | 0.8 |
| **1381** | 1381 | 1585 | 1584 | β-Elemene | 0.1 |
| **1410** | 1409 | 1590 | 1590 | *E*-Caryophyllene | 3.3 |
| **1442** | 1442 | 1662 | 1662 | α-Humulene | 0.1 |
| **1466** | 1467 | 1699 | 1703 | Germacrene D | 0.1 |
| **1427** | 1428 | 1600 | 1602 | Aromadendrene | 0.2 |
| **1464** | 1464 | 1683 | 1683 | γ-Muurolene | 0.1 |
| **1479** | 1479 | 1726 | 1769 | Bicyclogermacrene | 0.9 |
| **1490** | 1492 | 1601 | 1607 | Eremophyllene | 0.9 |
| **1508** | 1508 | 1734 | 1736 | β-Bisabolene | 2.6 |
| **1506** | 1506 | – | – | α-Amorphene | 0.2 |
| **1508** | 1508 | 1751 | 1751 | δ-Cadinene | 0.2 |
| **1553** | 1552 | 2113 | 2112 | Spathulenol | 0.3 |
| **1557** | 1557 | 1975 | 1968 | Caryophyllene oxide | 0.5 |
| **1580** | 1582 | 2015 | 2204 | Ledol | 0.5 |
| **1588** | 1590 | – | 1925 | Geranyl isovalerate | 0.1 |
| | | | | **Monoterpene hydrocarbons** | 12.9 |
| | | | | **Oxygen containing monoterpenes** | 72.6 |
| | | | | **Sesquiterpene hydrocarbons** | 9.5 |
| | | | | **Oxygen containing sesquiterpenes** | 1.3 |
| | | | | **Other compounds** | 2.0 |
| | | | | **Total identified** | 98.3 |

Exp. RI[a]: Experimental retention indices on the SPB-1 column relative to C8–C23 n-alkanes. Ref. RI[a]: Reference retention indices in nonpolar column. Exp. RI[b]: Experimental retention on the SupelcoWax-10 column relative to C8 to C23 n-alkanes. Ref. RI[b]: Reference retention indices in polar column. * Compounds listed in order to their elution on the SPB-1 column.

**Table 2**
Composition of the volatile fraction of the TC hydrolate.

| Compound | Relative amount in the volatile fraction | Concentration in the hydrolate (expressed as *n*-dodecane) | | |
|---|---|---|---|---|
| | | Absolute concentration | | Relative amount |
| | (%) | (mg.100g$^{-1}$) | (ppm) | (%) |
| 1-Octen-3-ol | 0.4% | 0.217 | 2 | 0.0002% |
| 6-Methyl-5-heptene-2-one | 1.2% | 0.660 | 7 | 0.0007% |
| 3-Octanol | 0.6% | 0.313 | 3 | 0.0003% |
| 1,8-Cineole | 26.3% | 14.469 | 145 | 0.0145% |
| *Z*-Linalool oxide | 0.5% | 0.277 | 3 | 0.0003% |
| *E*-Linalool oxide | 0.4% | 0.234 | 2 | 0.0002% |
| Linalool | 24.3% | 13.354 | 134 | 0.0134% |
| Phenylethyl alcohol | 0.3% | 0.156 | 2 | 0.0002% |
| Borneol | 4.0% | 2.206 | 22 | 0.0022% |
| Terpinene-4-ol | 1.0% | 0.573 | 6 | 0.0006% |
| *p*-Cymene-8-ol | 0.5% | 0.252 | 3 | 0.0003% |
| α-Terpineol | 1.2% | 6.188 | 62 | 0.0062% |
| Nerol | 1.3% | 0.817 | 8 | 0.0008% |
| Geraniol | 13.9% | 7.675 | 77 | 0.0077% |
| Thymol | 9.1% | 5.025 | 50 | 0.0050% |
| Carvacrol | 3.2% | 1.743 | 17 | 0.0017% |
| Geranyl acetate | 0.6% | 0.326 | 3 | 0.0003% |

monoterpenes (72.6%), being geraniol (27.5%), 1,8-cineole (16.3%) and thymol (9.2%) the major ones. Monoterpene hydrocarbons (12.9%) and sesquiterpene hydrocarbons (9.5%) are also part of the composition being *p*-cymene (3.7%) the most abundant monoterpene and *E*-caryophyllene the major sesquiterpene hydrocarbon (3.3%).

In the organic fraction isolated from the TC hydrolate, oxygen-containing monoterpenes are also dominant. Major constituents are1,8-cineole (26.3%), followed by linalool (24.3%) and geraniol (13.9%). In the whole hydrolate the absolute concentration of these compounds are respectively, 145 ppm, 134 ppm and 77 ppm, expressed as *n*-tetradecane.

### 3.2. TC preparations present a direct antimicrobial activity against acne-related bacteria

The effect of TC preparations was evaluated against three bacteria described to be involved in the pathophysiology of *acne vulgaris*. Due to the higher content in bioactive molecules in the essential oil preparation and its hydrophobic nature, essential oil was tested at lower concentrations than its correspondent hydrolate.

Visual MIC and MLC values obtained for TC essential oil and hydrolate against *C. acnes*, *S. epidermidis* and *S. aureus* are present in Table 3. Overall, TC essential oil was more effective in impairing bacterial growth than TC hydrolate, since for the last, visual MIC was only obtained for *C. acnes* bacteria, and at the highest tested concentration (50% v/v). Moreover, no MLC was determined for this plant preparation in none of the tested bacteria. For TC essential oil, *C. acnes* and *S. epidermidis* were the most susceptible bacteria, presenting the lower visual MIC values (0.06%). MLC values were higher than the obtained MIC values for both bacteria, being 2 and 4-fold higher for *C. acnes* and *S. epidermidis*, respectively. *S. aureus* presented the higher MIC value (0.125% v/v), and the obtained MLC was twice the MIC, for this species. For all tested species, microbial growth was also impaired at sub-inhibitory concentrations, as presented in Figure S1 (supplementary material).

**Table 3**
Minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of TC preparations against acne related bacteria. MIC and MLC values are expressed as % (v/v).

| | TC Essential Oil | | TC Hydrolate | |
|---|---|---|---|---|
| | MIC | MLC | MIC | MLC |
| *C. acnes* | 0.06% | 0.125% | 50% | ND |
| *S. epidermidis* | 0.06% | 0.25% | ND | ND |
| *S. aureus* | 0.125% | 0.25% | ND | ND |

### 3.3. TC is effective in preventing C. acnes biofilm formation and in disrupting C. acnes preformed biofilms

Due to the relevance of *C. acnes* biofilm formation in bacterial pathogenesis, namely in *acne vulgaris* progression, we studied the effect of both plant preparations against biofilm adhesion and regarding their ability to disrupt preformed biofilms, using a concentration range from twice (for TC essential oil) to one-fourth of the obtained planktonic MICs. For TC hydrolate, twice-MIC concentration was not evaluated, since MIC was obtained at the highest tested concentration. The effect of both preparations in biofilm formation and representative inverted microscope images are represented in Figs. 1 and 2, respectively. TC essential oil was effective in reducing biofilm formation, even at sub inhibitory planktonic concentrations. Reductions on biofilm formation ranged from 35%, obtained at one-fourth the MIC, to 90% obtained at MIC and twice the MIC. Regarding TC hydrolate, a significant reduction on biofilm adhesion (approximately 70%) was present only at MIC value.

When testing the effect against preformed biofilms, TC essential oil was also effective in impairing the biofilm, with significant disruptions ranging from 35%, obtained at half-MIC, to 80% obtained at twice the planktonic MIC. TC hydrolate was also able to impair preformed biofilms with significant disruptions ranging from 40 to 55% at ½MIC and MIC value, respectively. Percentages of preformed biofilm disruption and representative inverted microscope images are represented in Figs. 3 and 4, respectively.

### 3.4. TC preparations affect cellular viability in a dose-dependent manner

The biocompatibility of both TC preparations was evaluated on a murine macrophage cell line, used as a model to evaluate general toxicity on macrophages. TC hydrolate was tested at the same concentrations as for antimicrobial activity, and TC essential oil was tested at the highest concentration in which the solvent (DMSO) did not cause a decrease in cell viability higher than 30% (data not shown). Both extracts impaired cellular viability in a dose dependent manner, as represented in Fig. 5. Still, TC hydrolate was more biocompatible when compared with TC essential oil, considering the range of tested concentrations.

### 3.5. Anti-inflammatory potential is related to a reduction in NO production, independent from NO scavenging activity

The anti-inflammatory activity of both TC essential oil and hydrolate was assessed by their ability to inhibit NO production in stimulated macrophages. LPS (at 1 μg.mL$^{-1}$) was used to produce an inflammatory stimulus and mimic a state of inflammation. NO stable metabolites (nitrites) after cell exposure to TC preparations were measured by the colorimetric Griess reaction and used as a parameter to determine their anti-inflammatory potential.

As presented in Fig. 6, both TC preparations were able to inhibit NO production, in LPS-stimulated macrophages, in a dose-dependent manner. Of note, both TC preparations were able to reduce NO production at non-cytotoxic concentrations, showing significant reductions at 0.004–0.002% for TC essential oil and at 1.56–6.25% for TC hydrolate.

TC essential oil was more potent in reducing NO production, when compared with TC hydrolate, showing high reductions at very low concentrations. Importantly, TC essential oil at the lowest tested

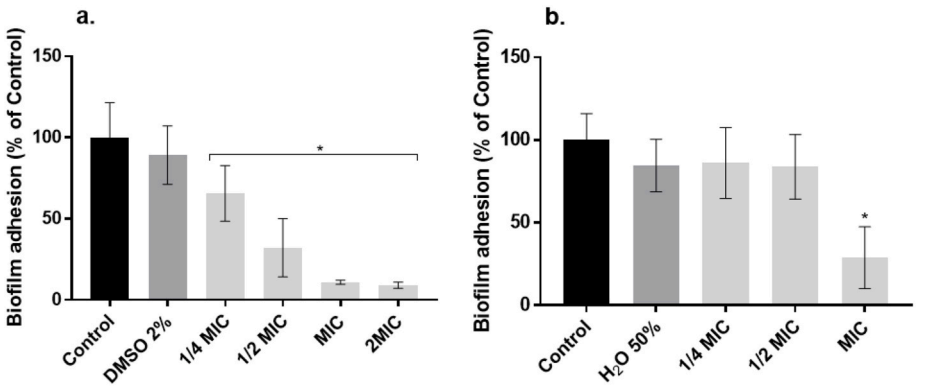


**Fig. 1.** Effect of TC essential oil (a) and hydrolate (b) on *C. acnes* biofilm adhesion, measured by biomass staining with crystal violet. Results are presented as percentage of Control (in the absence of plant preparations). Solvents used in their maximum concentration were also included to test the effect of the solvent. In TC hydrolate, since no solvent was used, ultrapure water was used at the maximum tested concentration to mimic the dilution of the culture medium caused by the hydrolate, at the highest tested concentration (50%). * represent statistical significance when compared with the Control, as determined by p value $< 0.05$ using One-away ANOVA.

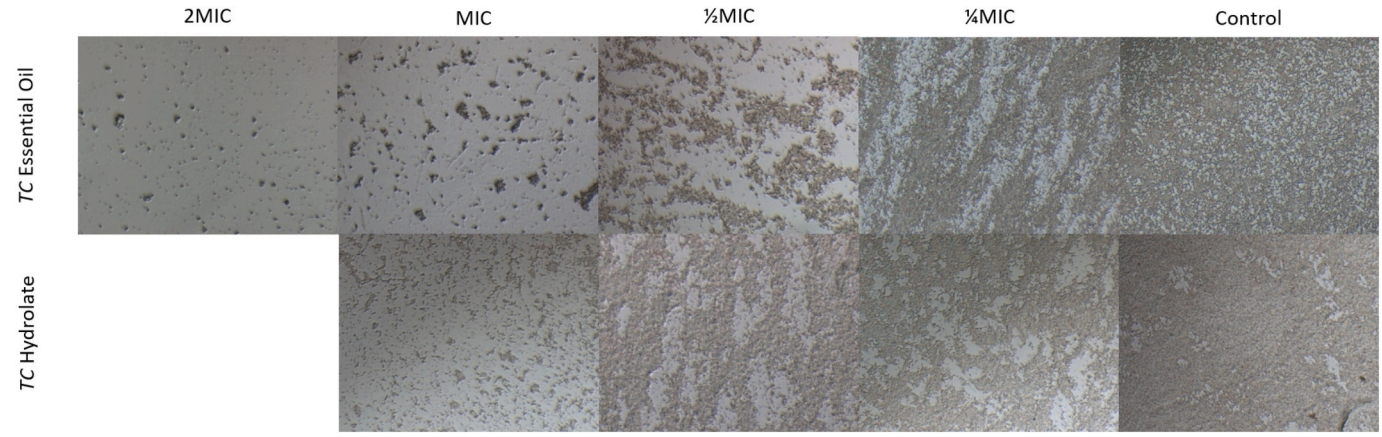


**Fig. 2.** Representative images of the prevention of *C. acnes* biofilm adhesion for TC essential oil and hydrolate at the tested concentrations. Since MIC value for TC hydrolate was obtained at the tested concentration (50%) no 2MIC condition was included in biofilm adhesion. Representative images were acquired after methanol fixation step, before crystal violet staining, using an inverted microscope with 10X10 amplification. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

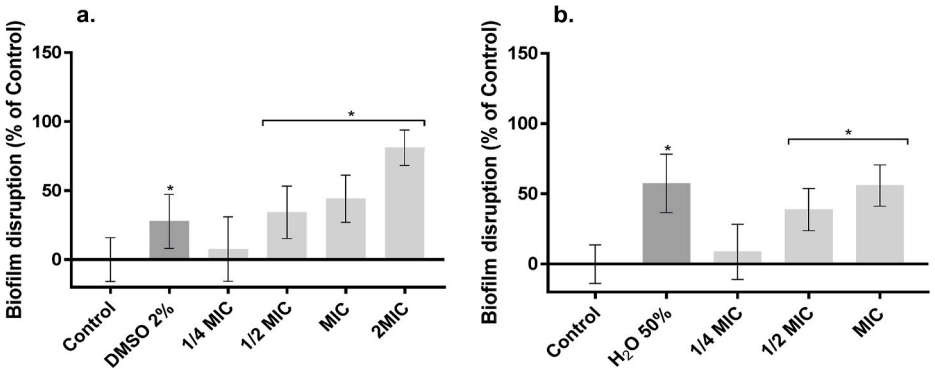


**Fig. 3.** Effect of TC essential oil (a) and hydrolate (b) on the disruption of *C. acnes* 72h biofilms, measured by biomass staining with crystal violet. Results are presented as percentage of Control (in the absence of plant preparations), which was taken as 0% disruption. Solvents used in their maximum concentration were also included to test the effect of the solvent. In TC hydrolate, since no solvent was used, ultrapure water was used at the maximum tested concentration to mimic the dilution of the culture medium caused by the hydrolate, at the highest tested concentration (50%). * represent statistical significance when compared with the Control, as determined by p value $< 0.05$ using One-away ANOVA.

concentration (0.002%) presented comparable reductions with the included positive control (Dexamethasone at 10 μM).

To clarify whether the reduction of NO was an actual reduction on NO cellular production or, alternatively, a direct scavenging potential of plant preparations, a reaction between a NO donor (SNAP) and different concentrations of the plant preparations was performed. As presented in Fig. 7, both plant preparations failed to scavenge NO from SNAP. Only a low (10%) significant reduction was obtained for TC hydrolate, at the highest tested concentration.

### 3.6. DPPH radical scavenging potential

The antioxidant properties of both TC preparations were evaluated using a second scavenging method, specifically the DPPH radical scavenging assay. $IC_{50}$ values and their antioxidant potential, as determined by their antioxidant activity index (AAI), are presented in Table 4.

Both TC preparations showed poor antioxidant activities, with TC hydrolate presenting a lower antioxidant potential ($IC_{50} = 20.08 \pm 1.25\%$; $AAI = 0.00013 \pm 0.00001$) when compared with correspondent

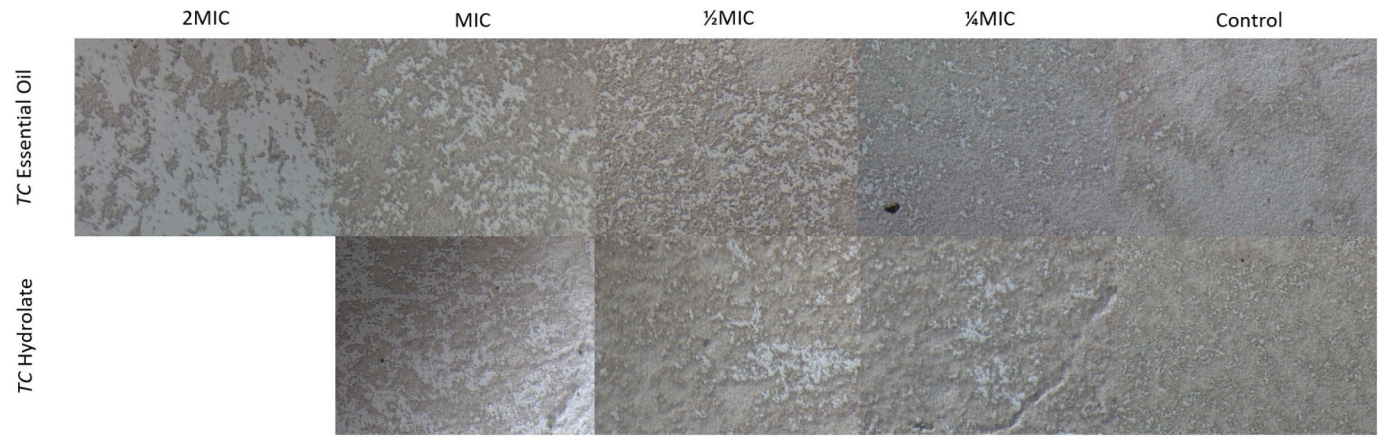


**Fig. 4.** Representative images of the disruption of *C. acnes* preformed biofilms caused by TC essential oil and hydrolate at the tested concentrations. Since MIC value for TC hydrolate was obtained at the tested concentration (50%) no 2MIC condition was included in biofilm adhesion. Representative images were acquired after methanol fixation step, before crystal violet staining, using an inverted microscope with 10X10 amplification. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

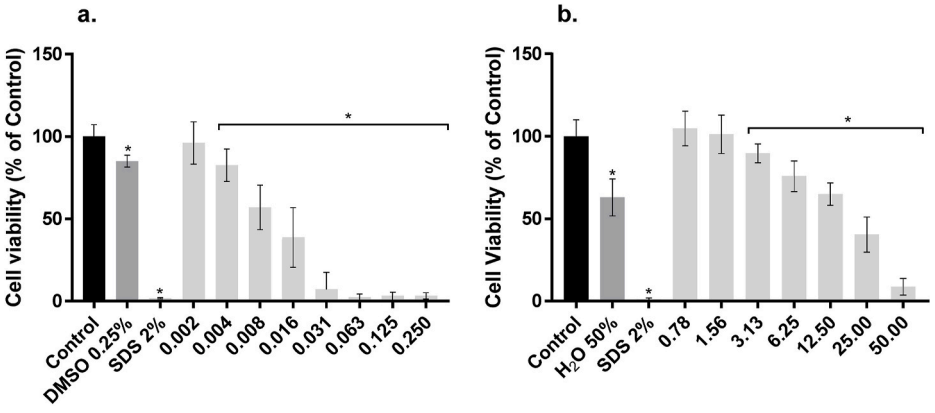


**Fig. 5.** Cellular viability of macrophage cell line, after 24h exposure to increasing concentrations of essential oil (a) and hydrolate (b) of TC, as determined by the MTT reduction assay. Solvents used in their maximum concentration were also included to test the effect of the solvent. In TC hydrolate, since no solvent was used, ultrapure water was used at the maximum tested concentration to mimic the dilution of the culture medium caused by the hydrolate, at the highest tested concentration (50%). Negative (Control) and positive (SDS 2%) controls for cytotoxicity were also included. If the effect on cellular viability was above 70%, the correspondent concentration was considered non cytotoxic. The presented data correspond to the means ± SD of three independent assays and are represented as percentage (%) of Control. Statistical analysis: One-way ANOVA; *p < 0.05 was considered a significant reduction.

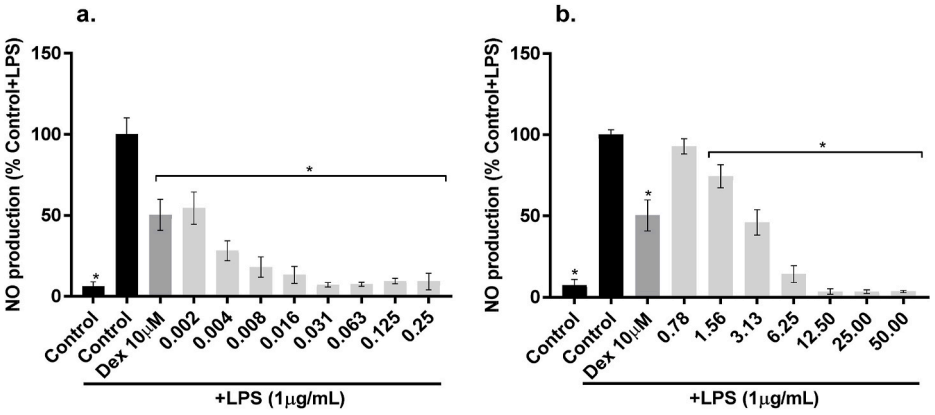


**Fig. 6.** Effect of TC essential oil (a) and hydrolate (b) on macrophage NO production upon an inflammatory stimulus. Cells were plated and exposed to DMEM medium or increasing concentrations of essential oil or hydrolates for 24h, in the presence of an inflammatory stimulus [LPS (1 μg/mL)]. An anti-inflammatory drug (Dexamethasone at 10 μM) was included as positive control. Griess assay was performed to assess levels of NO stable metabolites (nitrites) in the cell supernatants. Data correspond to the means ± SD and are represented as % of control cells exposed to LPS (Control + LPS). A control without LPS was also included to evaluate basal NO production (Control). Statistical analysis: one-way ANOVA *p < 0.05 was considered a significant reduction.

essential oil ($IC_{50}$ = 0.071 ± 0.002%; AAI = 0.0363 ± 0.0017). Both plant preparations also showed significantly lower (p-value < 0.05) antioxidant activity when compared to ascorbic acid, a very strong antioxidant used as standard ($IC_{50}$ = 2.89 ± 0.41 μg $mL^{-1}$; AAI = 8.58 ± 1.52).

### 3.7. *Daphnia magna* acute toxicity

TC essential oil caused immobilisation to *D. magna* in a dose -dependent manner. The calculated $EC_{50}$ at 48h was 32.05 $mg.L^{-1}$ with a 95% confidence interval between 29.82 and 35.21 $mg.L^{-1}$. The hydrolate caused no observable effects to *D. magna* after 48h of exposure, up to the highest concentration tested (2000 $mg.L^{-1}$).

According to the classification system by the Globally Harmonized

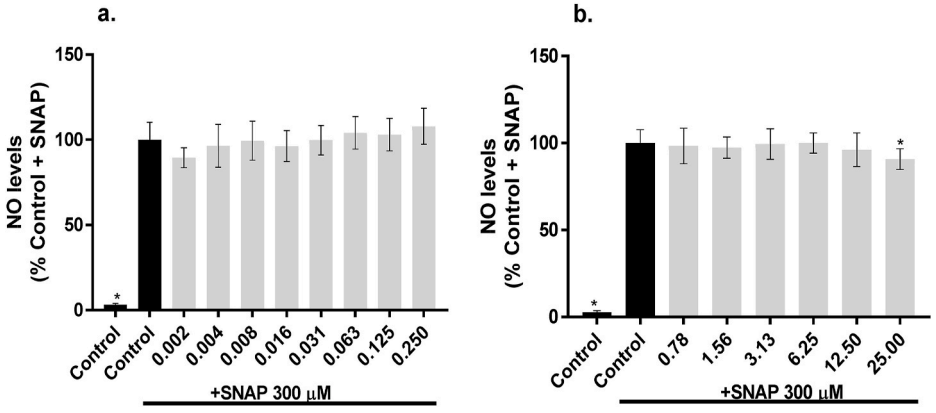


**Fig. 7.** NO scavenging capacity of TC essential oil (a) and hydrolate (b). Results are expressed as a percentage of NO release triggered by SNAP. Data correspond to the means ± SD of three independent experiments and are represented as % of DMEM medium with SNAP (Control + SNAP). A Control without SNAP was included to address basal nitrite values in the DMEM culture medium (Control). Statistical analysis One-away ANOVA; *p < 0.05 was considered a significant reduction. Scavenging activity could not be determined in TC hydrolate at the highest concentration tested (50%) due to an interference of SNAP with the plant preparation.

**Table 4**
Antioxidant properties of TC preparations.

| Parameter | TC essential oil (%) | TC hydrolate (%) | Ascorbic acid (μg/mL) |
|---|---|---|---|
| $IC_{50}$ | 0.071 ± 0.002* | 20.08 ± 1.25* | 2.89 ± 0.41 |
| AAI | 0.0363 ± 0.0017* | 0.00013 ± 0.00001* | 8.58 ± 1.52 |
| Antioxidant activity | Poor | Poor | Very Strong |

Results are expressed as mean ± SD; * indicates a significant result when compared with Ascorbic acid (p-value < 0.05).

System of Classification and Labelling of Chemicals (GHS) of the United Nations, and considering the obtained results, the essential oil from TC can be classified under the acute 3 category ($EC_{50}$(48h) between 10 and 100 $mg.L^{-1}$). The hydrolate can be classified as not toxic according to the same classification system (Nations, 2017).

### 3.8. Summary of the obtained bioactivities

Table 5 represents an integrative summary of the obtained bioactivities by comparing the obtained $MIC_{50}/E_{50}/IC_{50}$ values, for anti-acne related assays.

Overall, TC essential oil presented a more potent profile, when compared with TC hydrolate, with lower $IC_{50}/EC_{50}$ values. Regarding cellular viability, TC essential oil presented a higher cytotoxicity when compared with hydrolate, presenting $EC_{50}$ values of 0.01% and 16.90%, respectively. Still both plant preparations were able to reduce 50% of NO production at nontoxic concentrations since $EC_{50}$ for NO production were 5–6 times lower than the correspondent $EC_{50}$ for cytotoxicity for both plant preparations. Regarding their anti-oxidant capacity, TC essential oil was also more potent that hydrolate, with both $IC_{50}$ values being higher than their biocompatibility concentration range.

Regarding antimicrobial activity, TC essential oil presented a similar activity against acne related bacteria, with a slightly higher $MIC_{50}$ for *S. aureus*. For TC hydrolate $MIC_{50}$ value were not determined for *S. aureus* and *S. epidermidis* as they were out the tested concentration range. Regarding the potential to modulate virulence of *C. acnes* by impairing biofilm adhesion and disrupting mature biofilms, TC essential oil presented much lower $EC_{50}$ values when compared with hydrolate for biofilm adhesion (0.022% *vs* 19.26%) and biofilm disruption (0.059% *vs* 19.92%). Still, for TC hydrolate both biofilm related $EC_{50}$ values were lower than *C. acnes* $MIC_{50}$, presenting an anti-biofilm potential even at sub inhibitory concentrations. For both plant preparations, antimicrobial related $EC_{50}$ were lower than cellular cytotoxicity $EC_{50}$. Still this difference was less pronounced for TC hydrolate.

## 4. Discussion

With this work, we aimed to support the anti-acne potential of TC, a thyme species majorly known for its application in food industry. Specifically, we intended to study the potential not only of the essential oil, but also of the by-product of its production - hydrolate – a product that is typically discarded, to be used as bioactive ingredients for dermatological applications, as it been studied for other thyme species (Caverzan et al., 2020).

We first characterized both plant preparations regarding their main volatile compounds. As disclosed in the results section, the main compounds present in TC essential oil were geraniol, followed by 1,8-cineole and thymol. Several studies have described geraniol as the main component of TC essential oils from plants with different origins, with relative percentages ranging from 36.4% to 39.2% (Duman and Özcan, 2017; Horváth et al., 2006; Sacchetti et al., 2005) to values as high as

**Table 5**
Summary of the $IC_{50}/EC_{50}$ values obtained for anti-acne related assays.

| | TC essential oil | | TC hydrolate | |
|---|---|---|---|---|
| | $EC_{50}$ (% v/v) | 95% CI | $EC_{50}$ (% v/v) | 95% CI |
| *Anti-inflammatory activity* | | | | |
| Cellular viability | 0.010 | 0.009 to 0.011 | 16.90 | 15.6 to 18.29 |
| NO production | 0.0019 | 0.0017 to 0.0021 | 2.76 | 2.60 to 2.93 |
| *Radical* Scavenging *activities* | | | | |
| NO Scavenging potential | – | – | – | – |
| DPPH• scavenging potential | 0.071 ± 0.002 | – | 20.08 ± 1.25 | – |
| *Antimicrobial activity* | | | | |
| *S. aureus* | 0.070 | 0.066 to 0.075 | – | – |
| *S. epidermidis* | 0.027 | 0.025 to 0.029 | – | – |
| *C. acnes* | 0.029 | 0.021 to 0.032 | 24.45 | 22.18 to 26.85 |
| Biofilm adhesion | 0.022 | 0.019 to 0.024 | 19.26 | 16.94 to 21.84 |
| Biofilm disruption | 0.059 | 0.049 to 0.071 | 19.92 | 16.72 to 25.1 |

60% (Stahl-Biskup and Holthuijzen, 1995). The relative amount in the studied sample was slightly lower from the ones reported in the literature, fact that can be due to numerous factors, including edaphoclimatic conditions, vegetative development and genetic structure (Toncer et al., 2017). Also, since TC is considered to be an interspecific hybrid from parental species *T. vulgaris* and *T. pulegioides, geraniol chemotype* (Stahl-Biskup and Holthuijzen, 1995), the essential oil accumulation and its chemical composition could also depend from the genetic expression of the parental species (Ložienė et al., 2021).

Hydrolates, also known as hydrosols or aromatic waters, are obtained from the condensation of the steam used in the distillation for the preparation of the essential oil. They are very diluted aqueous solutions of the most hydrophilic volatile components isolated during the distillation. Comparing to the essential oils, hydrolates are typically unvalued and insufficiently studied products, mainly due to their lower content in organic, and probably, bioactive molecules. However, these distillation by-products can and must be valorised as ingredients for specific products, as cosmetics.

Here both plant preparations were tested regarding their ability to control bacterial growth, specifically against acne related bacteria *S. aureus*, *S. epidermidis* and *C. acnes*, the last being described as one of the hallmarks for acne vulgaris progression, and the likeliest pathogen in *acnes vulgaris* (Lee et al., 2019). Recent metagenomics' studies have uncovered possible different roles of *C. acnes* in skin microbiome, acting either as a commensal or a pathogen, depending of the strain and, more importantly, of the balance between strains from different phylogenetic clades and/or different ribotypes (RT). In this study, we used *C. acnes* DSM 1897, a collection type strain primarily isolated from an acne lesion in human facial skin, and classified as belonging to the clade IA-1 (RT1), being strains from this phylogenetic clade typically associated with both acne-free individuals and acne patients (Barnard et al., 2016; Liu et al., 2014). Still, the use of this strain is well established in the literature as a model to study the anti-acne potential of different extracts or ingredients.

Few essential oils have been studied for their anti-acne applications, and only tea tree oil has a commercial application. Still some thyme species' essential oils, specifically *T. quinquecostatus* and *T. vulgaris* had shown some anti-acne potential by targeting acne related bacteria (Sinha et al., 2014). Additionally, Yuangang Zu et al. (2010) reported activity of *T. vulgaris* against *C. acnes*, with 24h MIC values of 0.016% (Zu et al., 2010). To our knowledge, no reports have made specifically for TC essential oil. Still, by similarly affecting growth of acne related bacteria, it presents an interesting potential, as targeting only *C. acnes* can lead to over proliferation of *S. aureus* and/or *S. epidermidis*, triggering inflammatory flare-up or leading to unbalanced skin homeostasis and risk of nosocomial infections (Claudel et al., 2019). TC hydrolate did not shown a marked antibacterial activity, with visual MICs obtained only against *C. acnes*, still at very high concentrations. This marked difference in their bioactivity may result from the low concentrations volatile organic compounds in the hydrolate, those that the antibacterial activity of the essential oils is usually assigned (Šilha et al., 2020). In fact, geraniol which is the main volatile compound in the TC essential oil sample, has a reported antibacterial activity against gram-positive and gram-negative bacteria (Guimarães et al., n.d.). Interestingly 1, 8-cineole, which was the main volatile compound of TC hydrolate did not show antibacterial properties (Guimarães et al., n.d.). This can also contribute for the week antimicrobial activity of the hydrolate.

Both plant preparations presented the ability to modulate not only biofilm adhesion, but also to disrupt preformed biofilms. The presence of a bacterial biofilm in *acne vulgaris* reduces the effectiveness of the antimicrobial agents, increasing their tolerance level, thus leading to longer treatment periods with low success rates (Linfante et al., n.d.). In our study, TC essential oil was able to prevent biofilm adhesion and to disrupt preformed biofilms, although in less extent, at sub-inhibit concentrations. Interestingly, TC hydrolate was more effective in disrupting preformed biofilms than in preventing its adhesion, suggesting a different mechanism when compared with TC essential oil. By comparing our results with the control included to discard the effect of the dilution caused by the aqueous phase of the hydrolate, it appears that water itself was able to disrupt the biofilm in a similar manner than TC hydrolate. This could suggest a mechanical effect of the watery fraction of the hydrolate. Still, adhesion was impaired with TC hydrolate, thus valuing the ability of this plant preparation in repressing bacterial virulence associated with acne.

Other important hallmark of *acne vulgaris* progression is the presence of an inflammatory condition (Portugal et al., 2007). In this study, we used LPS-stimulated mouse macrophages (RAW 264.7) as an *in vitro* model of acute inflammation. Specifically, we studied the ability of TC essential oil and hydrolate to impair NO production, by quantifying NO stable metabolites as a screening for their anti-inflammatory potential. TC essential oil was able to markedly reduce NO production leading to comparable results with dexamethasone even at the lowest tested concentration. TC hydrolate was more modest when compared with the EO, still comparable results with dexamethasone were obtained. To further investigate if these low levels, observed after treatment, were due the direct scavenging potential of both plant preparations, a reaction between a NO donor was performed. As neither plant preparations were able to directly scavenge NO, we excluded this hypothesis to justify the reduction of nitrite levels in LPS-stimulated macrophages. Thus, a possible effect in the expression of inducible form of nitric oxide synthase (that catalyses the synthesis of NO) may be involved. To the best of our knowledge, no similar studies were performed with TC plant preparations. However, similar profiles were obtained for other *Thymus* species as *T. albicans, T. carnosus, T. camphoratus* and *T. zygis* (Roxo et al., 2020)(Zuzarte et al., 2018). Additionally, the anti-inflammatory effect of geraniol, the main component present in TC essential oil, was already reported in the literature. De Cássia Da Silveira E Sá et al. (2013) reviewed the anti-inflammatory activity of monoterpenes, as geraniol, and reported a reduction on NO and $PGE_2$ production in LPS-stimulated RAW 264.7 cells, after exposure to geraniol, in a dose-dependent manner (De Cássia Da Silveira E Sá et al., 2013). A reduction of protein and mRNA expression levels of iNOS, after geraniol exposure was also reported in the same study (De Cássia Da Silveira E Sá et al., 2013). This described anti-inflammatory activity of the main compound present in our TC plant preparation could justify the anti-inflammatory potential here described.

Oxidative stress is also indirectly related to acne pathogenesis. As sebum is produced through impaired follicular walls of sebaceous glands that contains reactive oxygen species, these free radicals are reported to be accountable for the incidence of irritation during acne disorder (Pereira et al., 2013b). In order to further investigate about the ability of both plant preparations to scavenge free radicals, we performed an additional study, namely the DPPH scavenging assay. Contrarily to the results obtained with NO scavenging assay, both plant preparations were able to scavenge DPPH•, with TC essential oil presenting a much lower $IC_{50}$ when compared with hydrolate. Still both preparations presented poor antioxidant capacity when compared with ascorbic acid (very strong antioxidant). Despite several studies have been performed to assess *Thymus* species anti-oxidant capacity using the DPPH model, no direct comparisons can be drawn due to the lack of standardization of this method among different studies. However, the antioxidant effect of geraniol, one of the main components in our TC preparations, was already described using DPPH• scavenging (Hyang-Sook et al., 2000; Prasad and Muralidhara, 2017), still with poor antioxidant capacity when compared other terpenoids as eugenol or carvacrol, the first presenting comparable results with ascorbic acid (Hierro et al., 2004). The reported low antioxidant capacity of geraniol, a relevant component of our plant preparations, specifically regarding the essential oil, could justify their poor antioxidant potential here described.

Finally, we classified the studied preparations regarding their potential for environmental hazard, using the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) from the United

Nations. This unified system provides a basis for globally uniform physical, environmental, health and safety information on potential hazardous chemicals, through the harmonisation of the criteria for their classification and labelling. Accordingly, the substances are ranked in four different categories in which Acute 1 corresponds to the most toxic. As disclosed in the Results section, TC essential oil can be classified under the acute 3 category and the hydrolate can be classified as not toxic. As discussed before, as hydrolates are by-products of the distillation process to obtain essential oils, they usually contain fewer amounts of compounds and these tend to be of more hydrophilic nature when compared to ones present in the essential oil. These differences have been reported in other studies (Moukhles et al., 2019). To our best knowledge, there are no available studies regarding the acute effects of these plant preparations on *Daphnia magna*. There is, although, information regarding the acute toxicity of one of their main compounds, geraniol, in the European Chemicals Agency (ECHA) database. According to reported data, geraniol is acutely harmful to the fish *Danio rerio* [$EC_{50}$(96h)= 22.0 mg.$L^{-1}$], to *Daphnia magna* [$EC_{50}$(48h) for immobilisation of 10.8 mg.$L^{-1}$], and to the microalgae *Desmodesmus subspicatus* [$EC_{50}$(72h)= 13.9 mg.$L^{-1}$ for the growth rate; and a No Observed Effect Concentration (NOEC) of 1.0 mg.$L^{-1}$]. Singulani et al. (2018) have also reported toxicity of geraniol (malformations and coagulation) to zebrafish (*Danio rerio)* embryos. They reported that high concentrations of the compound (500 and 1000 mg.$L^{-1}$) caused mortality of all embryos at 5 h post-fertilisation, with an $LC_{50}$ value of 31.3 mg.$L^{-1}$ (Singulani et al., 2018). Based on the discussed information, as geraniol can have acutely harmful effects on different aquatic systems' organisms, ranging from primary producers to consumers, precautions should be taken to avoid accidental or intentional water bodies' contamination. Despite this information, since essential oils are complex mixtures of compounds (Falleh et al., 2020), the observed toxicity cannot be attributed only to the presence of geraniol as several different compounds were also quantified. This is also applicable to TC hydrolate. Still, since in hydrolates these compounds are present in very small amounts, this could account for the lack of observable effects in our *Daphnia magna* experiments.

## 5. Conclusions

In an integrative manner, comparing the overall interest in both TC preparations for their potential to be included as active ingredients with anti-acne applications, TC essential oil presented a more pronounced effect when compared with hydrolate. However, the concentrations required to obtain a direct antimicrobial and anti-biofilm activities were proven cytotoxic, using our model. Further studies using more robust models, as *in vitro* three-dimensional cellular models, are necessary to confirm cytotoxicity at concentrations required to produce an antimicrobial effect. Despite these results, the concentrations required to obtain an anti-inflammatory activity were lower than the cytotoxic threshold. The essential oil was also proven hazardous for aquatic organisms, so caution must be taken to avoid accidental contamination during its production.

When considering TC hydrolate, and despite its antimicrobial activity was less pronounced, it proved to be more biocompatible when compared with essential oil. Additionally, it was still able to impair *C. acnes* growth forms at concentrations closer to biocompatible ones. Regarding its anti-inflammatory potential, TC hydrolate was able to reduce NO production at much lower concentrations than the ones required to produce cytotoxicity.

Also, despite TC preparations have shown potential to impair bacterial growth, particularly *C. acnes*, on both planktonic and biofilm forms, it would also be interest to study the effect of the preparations on different strains of *C. acnes*, belonging to different clades or ribotypes, in order to further understand about the activity against virulent *versus* commensal *C. acnes* strains.

Overall, our results support the anti-acne value of different TC preparations for different applications. TC hydrolate by presenting higher biocompatibility, an anti-inflammatory potential and the ability to modulate *C. acnes* virulence can be advantageous to be included in a product for everyday application to promote skin health of acne-prone skin. On the other hand, essential oil by presenting a marked antimicrobial, anti-biofilm and anti-inflammatory activities, still with some cytotoxicity, may be better suited for application in acute flare-ups, for short treatment periods.

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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. **Carlos Cavaleiro:** Investigation, Formal analysis, Writing – review & editing. **Lígia Salgueiro:** Investigation, Formal analysis, Writing – review & editing. **Rita Palmeira-de-Oliveira:** Methodology, Writing – review & editing. **Celso Ferraz:** Investigation, Writing – original draft. **Susana Coelho:** Investigation, Writing – original draft. **M. Ramiro Pastorinho:** Writing – review & editing, Formal analysis, Supervision. **Ana Catarina Sousa:** Writing – review & editing, Formal analysis, Supervision. **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.

## Abbreviations

| | |
|---|---|
| EO | Essential oil |
| TC | *Thymus x citriodorus* |
| GHS | Globally Harmonized System of Classification and Labelling of Chemicals |
| MIC | Minimum inhibitory concentration |
| MLC | Minimum lethal concentration |
| NO | Nitric oxide |
| ATCC | American-Type Culture Collection |
| DSM | Deutsche Sammlung von Mikroorganismen und Zellkulturen |
| TSA | Tryptic soy agar |
| BHI | Brain Heart Infusion broth |
| DMEM | Dulbecco's Modified Eagle's Medium |
| FBS | Fetal Bovine Serum |
| MTT | 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide |
| SDS | Sodium dodecyl sulfate |

| | |
|---|---|
| LPS | Lipopolysaccharide |
| DPPH | 2,2-Diphenyl-1-picrylhydrazyl |
| OECD | Organisation for Economic Co-operation and Development |
| GHS | Globally Harmonized System of Classification and Labelling of Chemicals |
| ASTM | American Society for Testing and Materials |
| DMSO | Dimethyl sulfoxide. |

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2021.114935.

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