



Developing translucent emulsions using sustainable NADES-based extracts

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ABSTRACT

In this work, we explored natural deep eutectic solvents (NADES) for developing translucent NADES-in-oil emulsions (TEs) for cosmetic applications. NADES, composed of lactic acid and glycerol (1:1 and 1:4 ratios), were selected for their natural origin, non-toxicity, and compatibility with living organisms. These green solvents facilitated the extraction of bioactive compounds from *Lavandula angustifolia*, *Corymbia citriodora*, *Thymus vulgaris*, *Quercus suber*, *Allium sativum*, and *Origanum vulgare*. The resulting NADES-extracts were characterized using LC-MS for compound identification and incorporated into TEs formulations. The content of phenolics, flavonoids, chelating power, and the antioxidant activity were assessed for both NADES-extracts and TEs. The produced TEs (50 wt% egg L- α -phosphatidylcholine (EPC), 20 wt% ethanol, 20 wt% olive oil, and 10 wt% NADES/NADES-extracts) effectively hindered the activity of enzymes responsible for skin melanin regulation and aging, namely tyrosinase ($IC_{50} \approx 0.18$ mg/mL for eucalyptus) and elastase ($IC_{50} \approx 0.23$ mg/mL for thyme). Gram-negative bacteria, particularly *K. pneumoniae*, were found to be more susceptible to NADES-extracts and TE-extracts, resulting in larger zones of inhibition. The study reported no toxic effects in the larval model, with a noticeable decrease (2-fold) in melanization in *G. mellonella* larvae after TE application. Cytotoxicity results indicated that TEs could be tolerated in skin cells at concentrations up to 5 %. Transdermal testing showed TEs permeation into the skin of 1–5 %, suggesting their use for cosmetic purposes without significant cellular damage. This study highlights the potential of NADES combined with natural extracts as sustainable, non-toxic ingredients in cosmetic formulations, paving the way for innovative skin regeneration products.

1. Introduction

Water-in-oil (W/O) emulsions have gained interest due to their practical applications in a wide range of fields. However, their long-term stability remains a challenge in colloid research. The scientific community has been searching for innovative solutions for the preparation of oil-in-water (O/W) and water-in-oil (W/O) emulsions containing active compounds for application in cosmetic and pharmaceutical formulations. In 2002, Nazzari et al. suggested a semi-solid, self-emulsified drug-delivery system based on eutectic phenomena that can overcome some of the drawbacks of conventional emulsified systems, namely limited solubility and irreversible precipitation of the active drug in the

medium [1,2]. In this sense, deep eutectic solvents (DES) and their natural versions (NADES) appeared as agents for the preparation of emulsions due to their great capacity to dissolve bioactive natural compounds [1]. These solvents (DES and NADES) are an evolution of ionic liquids (IL) and result from the combination of two or more compounds that become liquids at room temperature [3–5]. NADES' properties such as polarity, viscosity, biodegradability, electrical conductivity and thermal stability, can be altered by changing one of its components and molar ratios, as well as by addition of a co-solvent [5,6]. These alterations confer them capacity to dissolve natural or synthetic chemicals that exhibit low solubility in water [6]. The dissolution capacity of NADES resulted in its regular application in the

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extraction of natural compounds of varying polarities, including alkaloids [7], triterpene saponins [8], lignin [9], and phlorotannins [10], among others.

Aromatic compounds and phytochemicals that are beneficial to human health can be found in large quantities in medicinal and aromatic plants. Recent research indicates that NADES can effectively co-extract components from plant sources. When ingested or applied topically, most NADES extracts from plants and algae present no health risks. NADES are especially desirable for cosmetic applications because of this safety feature [5,7–11]. In this context, phenolic antioxidants from *Lavandula angustifolia* flowers can be extracted via NADES based on choline chloride for application in food and pharmaceutical industries [12]. Wild thyme herbal powder has been efficiently used as a raw material for the recovery of polyphenolic antioxidants using NADES extraction. Pavlic et al. demonstrated that NADES composed of L-proline and glycerol can improve the yield of polyphenols and the antioxidant activity of extracts of wild thyme (*Thymus serpyllum* L.) [13]. Recently, NADES were also identified as promising alternatives for extracting value-added compounds from biomass. Hydrophobic DES based on menthol and thymol were used by Silva et al for the extraction of triterpenic acids from *E. globulus* bark [14]. Garlic extracts dissolved in NADES evidenced promising synergistic effect as a natural antibiotic, as was recently discovered by Moufok et al. [15]. Several bioactive compounds present in cork (*Quercus suber*) can be also extracted with NADES composed of lactic acid and glycerol using different extraction processes (sealed systems, ultrasound and enflourage) [16,17].

The separation of NADES from extracts may be problematic due to the strong network of hydrogen interactions generated [18]. This problem may be overcome by using separation methods like liquid/liquid extraction and steam distillation, using solvents such as water, ethanol, ethyl acetate, among others [19,20]. However, extracts containing NADES can be applied directly in cosmetic and pharmaceutical formulations, without compromising the toxicity and performance of the final formulations. Their application in these types of formulations will always depend on their stability and compatibility at a biological level [17].

The application of oil/NADES emulsions can provide a surfactant-free route and reduce the use of harmful organic solvents for the preparation of drug-loaded nano/microparticles and microcapsules [21]. NADES (menthol: dodecanoic acid) in water (30: 70 wt%) emulsions stabilized with partially oxidized cellulose nanoparticles offer great potential for non-aqueous reaction systems, chemical extraction, and controlled drug-release [22]. O/W emulsions based on NADES (choline chloride: (poly-)carboxylic acids: water) containing phenolic extracts from olive pomace were recently developed by Pontes et al.. The findings show that the extract containing NADES can be used without additional purification and that it can also be employed as an aqueous phase in the production of O/W emulsions [23].

The present study investigated two potential applications of NADES: their effectiveness as extraction solvents and their suitability as the primary component of a transparent emulsion. Herein, NADES were used to extract natural compounds from *Lavandula angustifolia* flower, *Corymbia citriodora* leaves, *Thymus vulgaris* leaves, *Quercus suber* cork, *Allium sativum*, and *Origanum vulgare* leaves. NADES composed of DL-lactic acid and glycerol in the proportions 1:1 and 1:4 were used for assisted extraction of bioactives from the natural sources using a mechanical impeller. The chemical composition of the extracts containing NADES (NADES-extracts) was analyzed by Liquid chromatography-mass spectrometry (LC-MS). Additionally, the NADES-extracts were characterized in terms of antioxidant capacity, total phenolic content, total flavonoids, and chelating power. The NADES-extracts were applied without purification in the development and optimization of NADES-in-oil translucent emulsion (TE) for application in pharmaceutical/cosmetic formulations. The organoleptic properties, namely pH, odor, color, particle size, and surface charge, of the developed emulsions were evaluated as well as the levels of permeation on pig skin,

aiming to predict their potential applicability on cosmetic treatments, like skin and nails disorders. NADES-extracts and TEs were tested for anti-elastase and anti-tyrosinase activity as cosmetic and pharmaceutical candidates. The antimicrobial properties of NADES-extracts and TE against a range of microbial strains (Bacteria: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Klebsiella pneumoniae*; Fungus: *Trichophyton mentagrophytes*), involved in several skin and nails disorders, were evaluated. The toxicity of the NADES-extracts and of the TE were analyzed through cell viability using skin keratinocytes, as well as through an *in vivo* larval model (*Galleria mellonella*).

2. Experimental section

2.1. Materials

DL-Lactic acid (80 %), glycerol (99 %), ethanol (99.8 %), dimethyl sulfoxide (DMSO) (99.9 %) sodium dodecyl sulphate (SDS) (99 %), Folin-Ciocalteu reagent, sodium carbonate (99 %), sodium nitrate (98 %), sodium hydroxide anhydrous (99 %), gallic acid (99 %), quercetin (99 %), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (99 %), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (99 %), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (99 %), potassium persulfate (98 %), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (98 %), hydrochloric acid (37 %), iron (III) chloride (98 %), iron (II) chloride (98 %), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate (Ferrozine) (98 %), Ethylenediaminetetraacetic acid (EDTA) (98 %), Tyrosinase from mushroom (lyophilized powder, ≥ 1000 unit/mg solid), L-tyrosine (98 %), Elastase from porcine pancreas (lyophilized powder, ≥ 4 unit/mg solid), N-Methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (98 %), kojic acid (99 %), L- α -phosphatidylcholine egg yolk (EPC) (95 %), and colorimetric Xpert Blue Cell Viability assay were purchased from Merck (Germany) or TCI chemicals (Europe) and used without further purification. Other materials: olive oil (Pingo Doce), *Origanum vulgare* L. leaves (Margão; Batch number: L52918235 B), and ground garlic cloves (Margão; *Allium sativum*; Batch number: L53157788 B) were purchased from Pingo Doce – Distribuição Alimentar, S.A. (Portugal); *Lavandula angustifolia* flower (Batch number: LAGLK2J), *Corymbia citriodora* leaves (Batch number: ELCA1J), and *Thymus vulgaris* leaves (GGN: 4056186025678 (GLOBAL.A.P. Product Certificate Number: 00079-VVKHT-0002); Batch number: TVA1H) were purchased from Cantinho das Aromáticas® (Portugal); cork powder from *Quercus suber* was supplied by Cork Supply Portugal, S.A. (grain size of 0.5–1 mm) (Portugal); trypticase soy broth (TSB), trypticase soy agar (TSA), nutrient broth (NB), and nutrient agar (NA) were acquired from VWR (USA); Müller-Hinton broth (MHB) was obtained from CondaLab (Spain); and potato dextrose agar (PDA) was purchased from Frilabo (Portugal). All tested bacteria were supplied from American Type Culture Collection (ATCC, USA): *Staphylococcus aureus* (S. aureus; ATCC 6538; grown in TSB/TSA); *Staphylococcus epidermidis* (S. epidermidis; ATCC 35984; grown in TSB/TSA); *Escherichia coli* (E. coli; ATCC 25922; grown in TSB/TSA); and *Klebsiella pneumoniae* (K. pneumoniae; ATCC 4352; grown in NA/NB). Fungi were supplied from Micoteca da Universidade do Minho (MUM, Portugal): *Trichophyton mentagrophytes* (T. mentagrophytes; MUM 0808; grown in PDA). *Galleria mellonella* larvae were reared on a pollen grain and bee wax diet at 25 °C in darkness and used in a final stage of development with a weight of approximately 250 mg. Human keratinocytes cell line (HaCaT) – DKFZ HaCaT adherent cell line (immortalized human keratinocytes) was obtained from Cyton (previously known as CLS) (Germany), and provided by DKFZ (Germany).1 Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were bought from PAN BIOTECH (Germany). Antibiotic-Antimycotic Solution, trypsin-EDTA solution, dimethyl sulfoxide (DMSO) Cell Culture Grade and Xpert Blue Cell Viability Assay were obtained from GRISP (Portugal).

2.2. Methods

2.2.1. NADES formation

The production of DL-lactic acid:glycerol NADES in the molar proportions of 1:1 and 1:4 was carried out according to the method described by Freitas et al. [16].

2.2.2. Extraction procedure

The natural sources of *Lavandula angustifolia* flower, *Corymbia citriodora* leaves, *Thymus vulgaris* leaves, and *Origanum vulgare* leaves were initially ground in a mill until a powder was obtained. The natural sources powders (10 g for *Quercus suber* cork and 20 g for other natural sources) were placed in a small reactor with 100 mL of extraction solvent (NADES 1:1 or 1:4), and the extraction process was carried out at 70 °C for 1.5 h, under mechanical stirring using an impeller. At the end of the extraction, the remaining material was removed by filtration and washed with water to remove the solvent adsorbed. Finally, the water content present in the extract containing the eutectic mixture (NADES-extract) was removed by evaporation in a rotary evaporator and by freeze-drying. The NADES-extract was obtained for further application. The extraction yields were obtained by calculating the weight loss between the initial natural source weight and its weight after extraction [16,17].

2.2.3. Translucent emulsions (TEs) preparation

TEs were prepared by mixing EPC 50 wt% with ethanol 20 wt% at room temperature until a thick liquid was formed. Next, 20 wt% of olive oil was added and stirred until a uniformly textured liquid was formed. Finally, 10 wt% NADES (with or without extract) was added and stirred until a TE was formed (Table 1).

2.2.4. Determination of the total phenolic content

The total phenolic content was measure using the Folin-Ciocalteu reagent with a procedure adapted to the quantities used in a 96-well plate [13,17]. First, 2 µL of sample (NADES, NADES-extracts and TE at [C] = 1 mg/mL in DMSO) was added to 10 µL of Folin-Ciocalteu reagent, followed by addition of 120 µL of distilled water and agitation in thermoblock. After 1 min, 40 µL of 15 wt% Na₂CO₃ and 28 µL of distilled water were added, and the absorbance was measured at 750 nm using a microplate reader (BioTek, SYNERGY H1M2, Agilent Technologies, USA), and compared to a control with just distilled water. The total phenolic content was determined using a calibration curve with gallic acid as the standard (0.001–1 mg/mL), and the results were represented as mg of gallic acid equivalent/g of extract (GAE mg/g).

2.2.5. Determination of the total flavonoid content (TFC)

The total flavonoid content (TFC) was determined as previously described Zhishen et al.[24] with the protocol optimized to the quantities used in a 96-well plate. Initially, 10 µL of sample, 80 µL of water and 3 µL of 10 wt% NaNO₂ were mixed and left for 5 min at room temperature under shaking. After this incubation time, 3 µL of 20 wt%

AlCl₃, 40 µL of 1 M NaOH and 64 µL of distilled water were added. The absorbance was measured at 510 nm using a microplate reader (BioTek, SYNERGY H1M2, Agilent Technologies, USA). Quantification was performed against a quercetin calibration curve and the results were expressed in mgQE/g of extract. Assays were done in triplicate.

2.2.6. Antioxidant activity

The antioxidant activity was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay, and the ferric reducing antioxidant power (FRAP) assay. All procedures were adapted for a 96-well plate [13,17,25].

2.2.6.1. 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The free radical scavenging activity of NADES, NADES-extracts, and TE was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay adapted for a 96-well plate [13,17]. To 10 µL of sample dissolved in DMSO at a range of concentrations (0.001 – 10.0 mg/mL), 140 µL of DPPH stock solution (400 µM, 100 wt% ethanol) was added. The absorbance was measured continuously at 515 nm for 60 min with a microplate reader (BioTek, SYNERGY H1M2, Agilent Technologies, USA) to ensure that the reaction was complete (plateau state). DMSO was used as a blank, and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard to generate a calibration curve of DPPH reduction and as a chemical reference in comparison to the antioxidant capabilities of the extracts. All measurements were made in triplicate. The results were expressed in terms of the percentage of reduced DPPH at steady state (DPPH_r) were calculated followings the Eq. (1) in which *A_r* was the absorbance registered at steady state, and *A_i* was the initial absorbance registered at time *t* = 0 min.

$$DPPH_r (\%) = \frac{A_i - A_r}{A_i} \times 100 \quad (1)$$

The *EC*₅₀ was obtained from the percentage of reduced DPPH curve and represented the concentration needed to reduce the DPPH discoloration by 50 %. Antiradical efficiency (*AE*) was then determined according to the Eq. (2), in which *EC*₅₀ was the concentration required to reduce the coloration of DPPH in 50 %, and *TEC*₅₀ was the time needed to reach a discoloration steady state at *EC*₅₀ concentration.

$$AE = \frac{1}{EC_{50} \times TEC_{50}} \quad (2)$$

The antioxidant activity of the NADES, NADES-extracts, and TE was also expressed in terms of Trolox equivalents (*TE*) per 1 g of sample (mg TE/g extract), as follows:

$$TE = \frac{EC_{50} \text{ Trolox}}{EC_{50} \text{ Sample}} \quad (3)$$

2.2.6.2. 2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay. The ABTS^{•+} clearance assay was performed according to Re et al. [25] with some modifications. First, a solution was prepared by mixing 7.4 mM ABTS and 2.6 mM potassium persulfate solutions in a 1:1 v/v ratio and allowed to react for 12 to 16 h at room temperature in the dark. Then, the ABTS^{•+} mixture was diluted with 100 wt% ethanol to reach an absorbance of 0.7 ± 0.1 at 734 nm. Samples were prepared at different concentrations (0.01–10.0 mg/mL in DMSO), and 10 mL were reacted with the ABTS^{•+} mixture (140 µL) and left in the dark for 6 min. The remaining amount of ABTS^{•+} was measured spectrophotometrically at 734 nm (BioTek, SYNERGY H1M2, Agilent Technologies, USA). 10 µL of DMSO was used as a control sample, while trolox was used as the standard antioxidant in this work. All experiments were conducted in triplicate and the ABTS^{•+} scavenging activity was calculated using Eq. (4), where *A_i* is the absorbance of the test sample at 734 nm, and *A₀* is the absorbance of the control sample at 734 nm.

Table 1

Natural sources, NADES-extracts (extracts containing NADES), and translucent emulsions (TE) abbreviations.

Natural source	Abbreviation	NADES-extract	TE-extract
<i>Lavandula angustifolia</i> flower(Lavender flower)	LF	NADES-LF	TE-LF
<i>Corymbia citriodora</i> leaves(Eucalyptus citriodora leaves)	EL	NADES-EL	TE-EL
<i>Thymus vulgaris</i> leaves(Thyme leaves)	TL	NADES-TL	TE-TL
<i>Quercus suber</i> cork(Cork powder)	CP	NADES-CP	TE-CP
<i>Allium sativum</i> cloves(Garlic cloves)	GC	NADES-GC	TE-GC
<i>Origanum vulgare</i> leaves(Oregano leaves)	OL	NADES-OL	TE-OL

$$\text{Inhibition rate (\%)} = \frac{A_0 - A_i}{A_0} \times 100 \quad (4)$$

2.2.6.3. Ferric reducing antioxidant power (FRAP) assay. The FRAP assay was used to determine the reducing power of the NADES, NADES-extracts, and TEs [13,17]. Firstly, the FRAP reagent was prepared in acetate buffer (300 mM, pH 3.6) by combining 20 mmol/L FeCl_3 solution, 10 mmol/L TPTZ in 40 mmol/L HCl in a 10:1:1 v/v/v ratio. To 10 μL of sample solutions, prepared in a series of different concentrations (0.01 – 10.0 mg/mL) was added 290 μL of FRAP reagent. The absorbance's at 593 nm were measured after 10 min in the dark at 37 °C with a microplate reader (BioTek, SYNERGY H1M2, Agilent Technologies, USA). All samples were prepared in triplicate and mean reducing power values were provided as mg of Fe^{2+} per g of sample.

2.2.7. Chelating power

The degree of chelation of ferrous ions was evaluated according to Bounatirou et al. [26,27] with some modifications to be performed directly in 96 well plate. Briefly, solutions of the samples (0.1–10.0 mg/mL in final concentration) were incubated at 1:1 v/v with 2 mM FeCl_2 ethanolic solution. The reaction was started by adding the same volume of a 5 mM ferrozine solution, and after 10 min, the absorbance at 562 nm was measured with a microplate reader (BioTek, SYNERGY H1M2, Agilent Technologies, USA). The negative control sample was the ethanolic solution. The same process used in ABTS was used to calculate the percentage of chelating ability. In triplicate experiments, EDTA was employed as a positive control, and the results were expressed as the quantity of EDTA equivalents per volume of sample.

2.2.8. Inhibition of tyrosinase

Inhibition of tyrosinase activity was evaluated according to Di Pettillo et al. [28] with some modifications. First, 30 μL of tyrosinase at 1000 U/mL (50 mM potassium phosphate buffer at pH 6.5) was added to 70 μL of NADES, NADES-extracts, and TE dissolved in DMSO at concentrations ranging from 5 to 100 $\mu\text{g/mL}$. Then, 110 μL of 2 mM L-tyrosine (50 mM potassium phosphate buffer at pH 6.5) were added and incubated at room temperature for 30 min before measuring the absorbance at 492 nm with a microplate reader (BioTek, SYNERGY H1M2, Agilent Technologies, USA). All experiments were conducted in triplicate and the inhibition of tyrosinase activity was calculated using Eq. (5), where A represents absorbance at 492 nm without the test sample and B represents absorbance at 492 nm with the test sample.

$$\text{Tyrosinase inhibition (\%)} = \frac{A - B}{A} \times 100 \quad (5)$$

2.2.9. Inhibition of elastase

The extracts were examined using a modified version of the procedure reported by Ndlovu et al. [29]. Initially, 25 μL of elastase enzyme solution (0.3 U/mL) is added to 50 μL of NADES, NADES-extracts, and TEs in a range of concentrations (0.25–250 $\mu\text{g/mL}$) prepared in 100 mM HEPES buffer at pH 7.5, containing no more than 2.5 % DMSO. The mixture was then incubated at room temperature for 20 min with 125 μL of HEPES buffer. After that, 50 μL of N-Methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (1 mM) were added, and the enzymatic activity was measured at 405 nm during substrate addition and after 40 min of incubation at 25 °C with a microplate reader (BioTek, SYNERGY H1M2, Agilent Technologies, USA). As a positive control, kojic acid at 0.25–250 $\mu\text{g/mL}$ was used. Experiments were performed in triplicate, and enzymatic activity was determined as a percentage of velocities in relation to the assay using buffer without any inhibitor for each concentration. The IC_{50} value was calculated by interpolating the % of elastase inhibition vs. concentration.

2.2.10. Identification of compounds by liquid chromatography–mass spectrometry (LC-MS)

The different compounds present on the obtained extracts were identified using a Thermo Scientific™ Vanquish™ Flex UHPLC (ultra-high-performance liquid chromatography) system, which was connected to a Thermo Scientific™ Orbitrap Exploris™ 120 high resolution accurate mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The instrument control and data processing were performed using Xcalibur 4.5 software, developed by Thermo Fisher Scientific. The UHPLC column employed in this study was a YMC-Triart C18 column with dimensions of 150 × 2.1 mm (inner diameter) and a particle size of 3 μm . To ensure the integrity and longevity of the UHPLC column, it was safeguarded with a guard column made of the same material, with an inner diameter of 2.1 mm. The method was previously reported [30], but with some modifications. The mobile phases were A (water containing 0.1 wt% formic acid) and B (acetonitrile containing 0.1 wt% formic acid). Using the linear gradient system (t in min; % A), the flow rate was 0.35 mL/min: 0 min, 95 %; 5.4 min, 85 %; 7.9 min, 80 %; 9 min, 80 %; 12.6 min, 70 %; 16.2 min, 50 %; 18 min, 5 %; 19.8 min, 95 %; and 22 min, 95 %. The column temperature was set to 30 °C, the autosampler temperature to 15 °C, and the injection volume was set to 10 μL . A heated electrospray ionization (HESI) probe was attached to the ion source (Thermo Scientific™ OptaMax™ NG ion source). The Q-Orbitrap's external mass calibration was performed once a week to achieve a working mass accuracy of 3 ppm. A Heated Electrospray Ionization (HESI) source was installed on the Orbitrap Exploris 120 mass spectrometer. The optimal HESI temperature was 350 °C, the capillary temperature was 325 °C, and the electrospray voltage was 3.5 kV for positive mode and 2.5 kV for negative mode. Sheath and auxiliary gas were each 50 and 10. All qualitative data were collected using Full-scan mode and data-dependent MS2 (dd-MS2). The Q-Orbitrap performs data dependent scans in full MS/dd-MS2 for confirmation purposes. If the targeted compounds were found, the quadrupole-selected precursor ions were delivered to the Q-Orbitrap mass spectrometer's HCD collision cell. To produce product ion spectra, they were fragmented with normalized collision energy (NCE). Full scan analysis with 30,000 FWHM mass resolution and target mass list for MS2 with 15,000 resolution and NCE 30, 60, and 90 %.

2.2.11. Characterization and stability of TEs

2.2.11.1. TEs organoleptic properties. The organoleptic properties (pH, odor, appearance, and color) of the TE were assessed at 25 °C for 120 days. Visual inspection over time was performed to evaluate these properties.

2.2.11.2. Accelerated stability of TEs. Accelerated stability of the TE was performed by a centrifugation test of 5g of sample at 3000 rpm after 5 days, in which the temperature varies between –20 °C and 25 °C (changed every 24 h and starting at –20 °C). At the end of the centrifugation, the TE were examined for phase separation.

2.2.11.3. TEs size, dispersity, and charge. The size and the surface charge, expressed as zeta potential, and the dispersity of the TE was evaluated in a Malvern Zetasizer NS (Malvern Instruments, UK) at 25 °C.

2.2.11.4. Texture evaluation of TEs. The mechanical consistency of TE was evaluated by back extrusion (firmness and work of shear) using a texture analyzer from STABLE MICRO SYSTEMS (Model: TA-HDPlus). The mechanical properties were determined following the guidelines outlined in Moisturizing cream – SPREADABILITY protocol provided by TAHDPlus software, for the comparison of the consistencies of moisturizing creams by back extrusion. For these tests, 3 mL of the TE were placed in the probe (A/BE Back Extrusion Ring (90° cone)). Firmness and spreading measurements were taken at 10 mm s^{–1} test speed, 60

mm depth, and 10 g contact force, before returning to the position above the sample's surface.

2.2.11.5. TEs viscoelastic properties. Viscoelastic properties of TE were performed using a rheometer Discovery HR1 (TA Instruments). Shear viscosity was measured using the 40 mm parallel plate (Peltier plate Steel – 107318, TA Instruments, USA) in a temperature range of 20–60 °C. To measure the shear viscosity, three flow ramps were performed, the first from 0.1 to 300 1/s, the second from 300 to 300 1/s, and the third from 0.1 to 300 1/s.

2.2.11.6. Thermogravimetric analysis (TGA). Thermogravimetric analysis (TGA) was carried out using a TGA 4000 (Perkin Elmer, Waltham, MA, US) and the Pyris software (version 13). NADES (8–10 mg) were examined from 25 to 600 °C at 10 °C/min under a nitrogen environment (flow rate: 20 mL/min), and the weight loss, in percentage, and its derivative were plotted against temperature. The temperature was calibrated using Curie temperatures of reference materials such as alumel, nickel, and perkalloy under the same conditions as the tested samples [31].

2.2.12. Skin permeation studies

The method used for skin permeation studies was based on previously reported work with some modifications [17]. A horizontal Franz diffusion cell with a 9 mm orifice diameter (V-Series Stirrers for Franz Cells, PermeGear, Hellertown, PA, USA) was used to conduct an *ex vivo* permeation study through full thickness pig skin. The receptor compartment was filled with 5 mL of 16 mM of SDS solution, which was constantly stirred with a small magnetic bar and a thermostat at 37 ± 1 °C. The amount of TE applied to the skin surface was approximately 0.5 mL. At regular intervals of 1, 2, 3, 4, 5, 6, 7, and 8, samples of the receiving solution were collected. Each sample removed was replaced with fresh SDS solution. To evaluate the transdermal permeation, a trace spectrum of the samples collected in the receptor compartment was measured between 230 and 750 nm, with points taken every 5 nm with a microplate reader (BioTek, SYNERGY H1M2, Agilent Technologies, USA). By observing the spectra obtained, it was concluded that the λ_{max} of absorption was 270 nm. The absorbance values acquired at this wavelength were used to determine the concentration of the samples in the receiving compartment. The TE's permeation (%) was calculated using Eq. (6), where C_{fe} was the concentration of TE at the receptor and C_e was the initial concentration of TE at the donor (0.1 g/g).

$$\text{Permeation (\%)} = \frac{C_{fe}}{C_e} \times 100 \quad (6)$$

2.2.13. Antimicrobial activity

The antibacterial activity of the NADES, NADES-extracts, and TEs was evaluated against Gram-positive bacteria *S. aureus* and *S. epidermidis*, and Gram-negative bacteria *E. coli* and *K. pneumoniae*.

The zone of inhibition (ZOI) tests, adapted from the Kirby–Bauer test [32], were carried out to determine the antimicrobial activity of each sample. The size of the halo was used as a qualitative measure of sample antibacterial activity. Initially, the bacterial suspension was adjusted to a concentration of 1×10^7 colony forming units (CFUs)/mL in TSB (NB for *K. pneumoniae*) and incorporated into TSA and NA (for *K. pneumoniae*) Petri dishes. Then, 50 μ L of each sample were placed on a cavity ($\varnothing \approx 6$ mm) in the middle of the agar plate and incubated for an additional 24 h at 37 °C. Following the incubation period, the halo (zone of transparent agar indicating no microorganism growth, there is the ZOI) formed around the sample was photographed to document the results. All experiments were carried out in triplicate for each sample and repeated at least three times.

The quantitative inhibitory assay was determined using the microdilution method adapted from the standard published by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee

on Antimicrobial Susceptibility Testing (EUCAST) [33]. Bacteria inoculums were prepared in TSB (*S. aureus* and *S. epidermidis*, and *E. coli*) or NB (*K. pneumoniae*) and incubated overnight at 37 °C and 120 rpm. In a 96-well plate, 50 μ L of the samples were combined with 50 μ L of bacterial suspensions prepared at 1×10^6 CFU/mL in MHB. TE was used at 100 wt% and NADES (with/without extract) was used in a 10 wt% solution at pH 5 to match the concentration present in the final formulation (TE). Controls were agent-free bacterial suspensions and culture media. Absorbance readings at a wavelength of 600 nm (EZ READ 2000 Microplate Reader, Biochrom, UK) were taken before (0 h) and after incubating the plate for 24 h at 37 °C and 120 rpm. Aliquots of the bacterial suspensions were collected and serially diluted in PBS (10^1 – 10^5), plated on TSA or NA (for *K. pneumoniae*), and further incubated for 24 h at 37 °C. Colonies of surviving bacteria were counted, and the results were expressed as % by comparing the control suspensions.

The antifungal activity of the NADES, NADES-extracts, and TEs was also evaluated and against the *T. mentagrophytes* fungus. For that, initially, *T. mentagrophytes* was seeded on 2 mL of sloped PDA at room temperature for 7 days. Then 3 mL of PBS were added, shaken, and the cell density adjusted to an optical density (OD) of 1.0 at 620 nm. The samples were then spread on PDA plates using a swab. Then, 50 μ L of each sample were placed in a well ($\varnothing \approx 6$ mm) made at the centre of the agar plates and incubated at 37 °C for 4 days. To document the results, the halos (zone of translucent agar showing absence of fungal growth, there is the ZOI) were measured. All experiments were carried out in triplicate for each sample and at least three independent assays.

2.2.14. Galleria mellonella assays

The effect of NADES, NADES-extracts, and TE on the health index of *G. mellonella* was evaluated according to Araújo et al. [34]. For that, approximately 5 μ L of the NADES (10 wt% in distilled water), NADES-extracts (10 wt% in water), and TE (100 wt%) were applied to the back of each larva (with a set of 10 larvae per condition). As a control, the same volume of water was applied to one set of larvae. After application, the larvae were placed on Petri dishes and kept in the dark at 37 °C for 24 h. The process was repeated another three days until a total of 72 h. The health index of *G. mellonella* was assessed using four key parameters: larval activity, cocoon formation, melanization, and survival rate. The effect of TE on the reduction of larval melanization was also evaluated. Larvae with a high melanization score (darker larvae) were selected from a population of larvae and the TE solutions were applied to the back of each larvae four times for a total of 72 h. The *G. mellonella* model did not require ethical approval for the experiments. Nevertheless, the experiments were conducted with consideration for power reduction and sample sizes were determined to be sufficient to detect statistically meaningful effects (Cohen's d) [35–37].

2.2.15. Cytotoxicity assay

The cytotoxic potential of the TEs was evaluated using a human cell line HaCaT (skin keratinocytes) [17]. The viability of cells incubated with the extracts was assessed using the Xpert Blue Cell Viability Assay. Cells were maintained in DMEM (37 °C, 5 % CO₂) and culture media was renewed every 2–3 days. The day before starting the assays, keratinocytes were plated in 96-well plates at a density of 7500 cells/well. Cells were exposed to different concentrations of TEs extracts (1 wt% and 5 wt %) and the viability was evaluated after 24 of exposure. Cells incubated with fresh culture media and cells incubated with 30 vol% DMSO were used as life control and death control, respectively. A control with 1 vol % DMSO was also performed to evaluate the amount of DMSO used to assist the availability of the TEs to cells when in culture media. Based on the guidelines provided by the OECD [38], a substance is classified as irritating if its average relative viability falls below 50 % of the average viability observed in the negative control group, within 15–60 min of exposure [17].

3. Results and discussion

3.1. NADES-extracts preparation

In this work, NADES made by DL-lactic acid (LA) and glycerol (GLY) in two different molar ratios, namely 1:1 and 1:4, were employed as solvents for extracting natural compounds from various natural sources. Recent studies have shown that LA:GLY NADES is significantly more effective at extracting bioactive compounds from plants than traditional solvents like water or dioxane [16,17]. Its high extraction capacity, combined with proven compatibility in cosmetic and pharmaceutical applications, positions this NADES as an ideal ingredient for the formulation of translucent emulsions (TE) [16,39]. The extraction method employing mechanical stirring in a reactor was chosen for its ease of scalability, making it suitable for future industrial applications. Compared to previously published results, the extraction temperature used is relatively mild, since many extraction processes, such as those involving supercritical liquids, Soxhlet extraction, or other reflux systems, often exceed 70 °C [40]. The NADES-extracts produced, rich in bioactives typical of the natural sources studied, were then used in the development of potential cosmetic formulations.

The results reported good extraction yields when using both LA:GLY ratios (1:1 and 1:4) (Table 2). Generally, the extraction yields were comparable, with a slight tendency towards higher yields observed for the 1:1 ratio. Notably, upon investigation of various natural sources, garlic exhibited a significantly high extraction yield, reaching approximately 90 % with both LA:GLY ratios. In contrast, the extraction yields from other natural sources investigated (LF, EL, TL, and OL) were lower, ranging from 45 % to 65 %. Upon visual inspection of the remaining material, it was observed that the solid matrix of the natural sources, such as the plant stem, was still present. In a previous work, we described the extraction of natural chemicals from cork powder using different NADES, including NADES LA:GLY (1:1), which yielded

satisfactory extraction results. However, in this study, we were able to enhance the extraction efficiency of this solvent by around 5 %. This improvement can be ascribed to the implementation of a mechanical impeller, marking a difference from previous methodologies that relied on magnetic or ultrasound-assisted stirring techniques at comparable or elevated temperatures. It is noteworthy that the concentration of NADES-extracts obtained from cork powder is comparatively lower than that obtained from other sources due to the lower extraction yield. Furthermore, the proportion of raw material used, due to the density of the powder, was 10 g per 100 mL of NADES, while for the other natural sources studied was 20 g per 100 mL. The low extraction efficiency observed for cork may be attributed to its significant hydrophobic nature, as well as to its composition rich in fatty acids, phenolic chemicals and terpenoids [16,17].

3.2. Nades-extracts characterization

The characterization of NADES-extracts is particularly important since it allows to infer about the features that make them suitable for direct use in cosmetic formulations. For that, the quantification of phenolic content, flavonoid content, chelating power, and antioxidant activity was conducted (Table 2). The important role of natural phenolic compounds lies in their wide range of actions, which include antioxidant, anti-inflammatory, radical-scavenging, and antibacterial capabilities, among others [13,17,20,41]. Table 2 presents the estimated phenolic composition of NADES-extracts, quantified in mg of gallic acid per g of extract. Phenolic compound levels obtained were comparable for both LA:GLY ratios, 1:1 and 1:4, being slightly higher when using the 1:1 ratio. The NADES-GC sample displayed the lowest concentration of phenolics, consistent with previous studies that suggest their scarcity in garlic cloves. The use of natural sources consisting of leaves (EL, TL, and OL) led to the generation of NADES-extracts, which exhibited elevated levels of phenolic chemicals. The NADES-CP extract, made up of cork,

Table 2
Characterization of NADES-extracts by evaluating phenolic content, flavonoid content, chelating power, and antioxidant activity (DPPH, FRAP and ABTS assays). Extraction conditions: 20 g of natural source in 100 mL of NADES, at 70 °C for 1.5 h. NADES in the respective proportion was used as control (pure NADES) and the data obtained was subtracted to the respective NADES-extract sample. All the measurements were performed in triplicate. The DPPH results are expressed both as TE and IC₅₀.

NADES-extract	NADES ratio (LA:GLY)	Extraction yield (%)	Extracts conc. (mg/mL)	Phenolic content (mgGA/g)	Flavonoids content (mgQ/g)	Chelating Power (mgEDTA/g)	DPPH		FRAP (mgFe ²⁺ /g)	ABTS (mgTE/g)
							mgTE/g	IC ₅₀ (mg/mL)**		
NADES-LF	1:1	48.5	97.1	28.6 ± 1.0	13.2 ± 1.1	14.2 ± 0.1	89.0 ± 3.1	3.9 ± 0.3	21.4 ± 0.7	97.9 ± 2.1
	1:4	50.4	100.8	25.6 ± 0.8	12.9 ± 1.3	9.7 ± 0.2	84.1 ± 2.5	4.1 ± 0.3	21.5 ± 0.5	97.0 ± 1.9
NADES-EL	1:1	51.5	102.9	44.3 ± 0.9	13.9 ± 0.9	10.0 ± 0.2	206.5 ± 1.0	1.7 ± 0.1	128.8 ± 1.1	191.3 ± 1.4
	1:4	47.5	95.0	45.4 ± 0.2	12.2 ± 1.0	8.0 ± 0.2	209.9 ± 2.1	1.6 ± 0.2	127.0 ± 0.8	182.5 ± 1.0
NADES-TL	1:1	63.3	126.5	38.8 ± 0.6	17.7 ± 0.5	10.9 ± 0.4	138.4 ± 2.3	2.5 ± 0.2	81.2 ± 0.9	103.6 ± 1.6
	1:4	56.2	112.3	35.9 ± 0.5	17.0 ± 0.5	9.4 ± 0.3	136.9 ± 3.3	2.5 ± 0.3	76.8 ± 1.7	104.9 ± 1.7
NADES-CP*	1:1	32.3	32.3	25.0 ± 1.1	15.2 ± 0.3	12.4 ± 0.4	198.8 ± 0.5	1.7 ± 0.1	115.0 ± 1.2	181.8 ± 0.8
	1:4	30.4	30.4	27.0 ± 0.8	14.4 ± 0.6	9.2 ± 0.6	149.2 ± 4.1	2.3 ± 0.4	110.1 ± 2.0	179.7 ± 0.8
NADES-GC	1:1	90.6	181.2	7.5 ± 0.5	11.9 ± 0.8	12.6 ± 0.5	101.3 ± 1.6	3.4 ± 0.2	27.8 ± 1.0	100.7 ± 1.0
	1:4	90.0	180.0	8.4 ± 0.4	11.6 ± 0.9	14.0 ± 0.1	92.3 ± 0.5	3.7 ± 0.1	26.4 ± 0.7	106.4 ± 1.1
NADES-OL	1:1	59.8	119.6	42.4 ± 0.7	13.7 ± 0.9	14.7 ± 0.3	170.1 ± 1.9	2.0 ± 0.2	68.4 ± 0.3	116.6 ± 0.5
	1:4	54.2	108.5	40.0 ± 0.6	13.2 ± 1.0	12.6 ± 0.6	153.6 ± 3.7	2.2 ± 0.4	71.1 ± 0.6	116.8 ± 0.2

* 10 g of cork powder in 100 mL of NADES.

** Trolox IC₅₀ = 0.344 mg/mL.

presented a considerable concentration of phenolic compounds (25.0 mg_{GA}/g_{extract} for 1:1 and 27.0 mg_{GA}/g_{extract} for 1:4). The NADES-LF presented a phenolic concentration comparable to that obtained for cork extract, but with twice the amount of the original natural source used for extraction.

Flavonoids are a class of polyphenols found in a variety of botanical sources which exhibit a wide range of biological effects according to their distinct chemical composition. The medicinal potential of flavonoids has been extensively investigated due to their antioxidant, anti-inflammatory, antiviral and anticarcinogenic activities. Currently, there is significant research to explore the therapeutic advantages of flavonoids in various dermatological diseases, due to their proven ability to absorb UV radiation [13,17,20,41]. The flavonoid content results presented in Table 2, show that NADES-TL exhibits the highest concentration, whereas NADES-GC shows the lowest. The low flavonoid content observed for NADES-GC may be directly related with its low content of phenolics.

The chelating capability of a natural extract (Table 2) is measured by its ability to capture metal ions that might damage the skin and nails, hence facilitating their elimination from the body [13,17,20,41]. The initial chelating capability of NADES have been determined as 21.95 mg_{EDTA}/g_{Extract} for the 1:1 ratio and 10.02 mg_{EDTA}/g_{Extract} for the 1:4 ratio, as shown in the Supplementary information (SI, S1 Table S.1). NADES with a higher lactic acid concentration (1:1 ratio) tend to exhibit a greater chelating capacity due to the molecule's ability to create a bidentate bond, resulting in the formation of Iron (II) lactate. The chelating impact of NADES (1:4) is the lowest since glycerol's ability to chelate iron is reduced. Upon excluding the influence of the control (NADES 1:1 or 1:4), the results revealed a modest rise of the chelating power by NADES-extracts ranging from 8.0 to 14.2 mg_{EDTA}/g_{Extract}.

Throughout history, natural compounds have been conventionally employed in the field of skincare. There is an ongoing search for novel natural bioactive chemicals with the potential to improve skin health and provide protection against harmful factors such as UV radiation and free radicals. The presence of free radicals has shown significant impact on the degeneration of the skin's defense and restoration mechanisms, eventually leading to considerable damage and an accelerated aging process. Plants contain natural compounds with antioxidant properties and ability to destroy free radicals, much like the ones explored in this work [13,17,20,41–43].

The antioxidant capabilities of NADES-extracts were evaluated aiming to predict their ability to be applied in anti-aging and nail protector products. For this, chemical-based approaches such as DPPH and ABTS radical scavenging assays were used, as well as a FRAP assay (Table 2). The NADES tested exhibited an initial antioxidant capacity of 51.5 mg_{TE}/g_{Extract} for the 1:1 ratio and 51.2 mg_{TE}/g_{Extract} for the 1:4 ratio, evaluated by the DPPH method. By FRAP method, the initial antioxidant capacity of NADES is 13.5 mg_{Fe2+}/g_{Extract} for the 1:1 ratio and 16.8 mg_{Fe2+}/g_{Extract} for the 1:4 ratio (SI, S1 Table S.1). The ABTS method revealed an initial antioxidant capacity of 107.6 mg_{TE}/g_{Extract} NADES 1:1 and 110.5 mg_{TE}/g_{Extract} for NADES 1:4 (SI, S1 Table S.1). The remarkable antioxidant activity of pure NADES is most likely due to the presence of hydroxyl (—OH) groups in glycerol, as well as hydroxyl (—OH) and carboxyl (—COOH) groups in lactic acid.

Upon analyzing the control data, it is evident that there are no substantial fluctuations in the antioxidant capacity between the ratios of 1:1 and 1:4 (Table 2). The antioxidant capacity of the final extracts (NADES-extracts) can be attributed to the presence of NADES, which is additionally boosted by the inclusion of chemicals isolated from diverse natural sources. Based on these data, it is important to highlight that NADES-EL exhibited the highest level of antioxidant activity compared to the other NADES-Extracts examined. This result is consistent with previous studies, as lemon eucalyptus oil is known to have high antioxidant power [44]. We were able to obtain a lavender extract (NADES-LF) with an antioxidant capacity comparable to those reported in the literature describing the use of a less environmentally friendly method

[45]. As expected, the antioxidant capacity of NADES-TL and NADES-OL is comparable, which is consistent with previous findings [46]. Garlic extract (NADES-GC) has significant antioxidant capacity, with values comparable to those reported [47]. As observed, despite having the lowest concentration of extractives, NADES-CP has great antioxidant capacity. Cork is described in the literature as an endless source of antioxidant compounds, being a natural source that the cosmetic industry exerts considerable attention [48].

The current study allowed the identification of the compounds found in the NADES-extracts (SI, S2 Tables S.3–S.8). The extracts obtained contained a range of polarity compounds, from the most polar (sugars) to the most non-polar (fatty acids). The findings agree with previous research that demonstrated the wide variety of substances that can be extracted using this type of NADES, composed by lactic acid and glycerol [16]. The utilization of NADES in a 1:1 ratio consistently resulted in a higher number of detected compounds from each natural source under study. Conversely, a higher glycerol content in NADES (ratio 1:4) resulted in a decrease in the amount of extracted compounds. Throughout the process of individualizing the NADES-extracts, the presence of terpenes commonly found in lavender samples, such as lily-aldehyde and linoleyl alcohol, was observed in NADES-LF. The eucalyptus extract, so-called NADES-EL, was found to include eucalyptol, eugenol, cumene, and 2-bornene. Thymol, the primary constituent found in thyme oil, was identified in the NADES-TL samples. The cork extract, so-called NADES-CP, comprises phenolic compounds derived from lignin, such as coumaric and cinnamic acid, along with suberinic acids like suberic and sebacic acid, and terpenes like cumene. The garlic extract (NADES-GC) contains some typical identified sulfur compounds, including diallyldisulfide and S-allylcysteine. NADES-OL is comprised of carvacrol and thymol, potent antibacterial compounds that are frequently found in oregano extracts.

3.3. New method for translucent emulsions (TEs) preparation

After the successful extraction of bioactives from different natural sources, it was imperative to develop a new method to include the NADES-extracts into a TE. One of the novel aspects of the current study lies in the formulation of translucent oil-based emulsions containing NADES-extracts, specifically designed for cosmetic applications. The TE are composed of 50 wt% of egg L- α -phosphatidylcholine (EPC), 20 wt% ethanol, 20 wt% olive oil, and 10 wt% NADES, with or without extract. (Fig. 1a). The developed technology is attractive for future applications due to the affordability, ease of production, and biocompatibility of all the TE components used. Fig. 1b displays the different proportions of the individual components in the formulations that result in TE.

The study restricted the concentration of NADES to 10 wt% due to limitations in the use of lactic acid in cosmetic formulations [39]. Moreover, increasing the ethanol concentration beyond 30 wt% can potentially cause significant skin dehydration in formulations designed for topical use [39]. The presence of high levels of ethanol can also lead to decreased stability of the TE due to evaporation. 1,2-Hexanediol has the potential to replace ethanol, however its use in the cosmetics sector is limited to a maximum concentration of 10 wt% due to current requirements. The goal of surpassing a 20 wt% proportion of olive oil was not achieved due to the prevalence of opaque emulsions and, in some cases, separation of phases. The formation of TE formulations is only possible if EPC ratio remains within the specified range of 50 wt% to 60 wt%. Replacing EPC by the botanical alternative sodium lecithin resulted in extremely viscous TE, which limited its usefulness in cosmetic compositions. The current work shows that TEs can be successfully prepared using both pure NADES and NADES-extracts. Because NADES-extracts liquor is obtained without the use of sophisticated extraction and purifying techniques, but rather by the simple combination of the ingredients it can be considered environmentally friendly and sustainable. The physicochemical and organoleptic characteristics of each TEs are addressed in Sections S3, S4, and S5 of the SI.

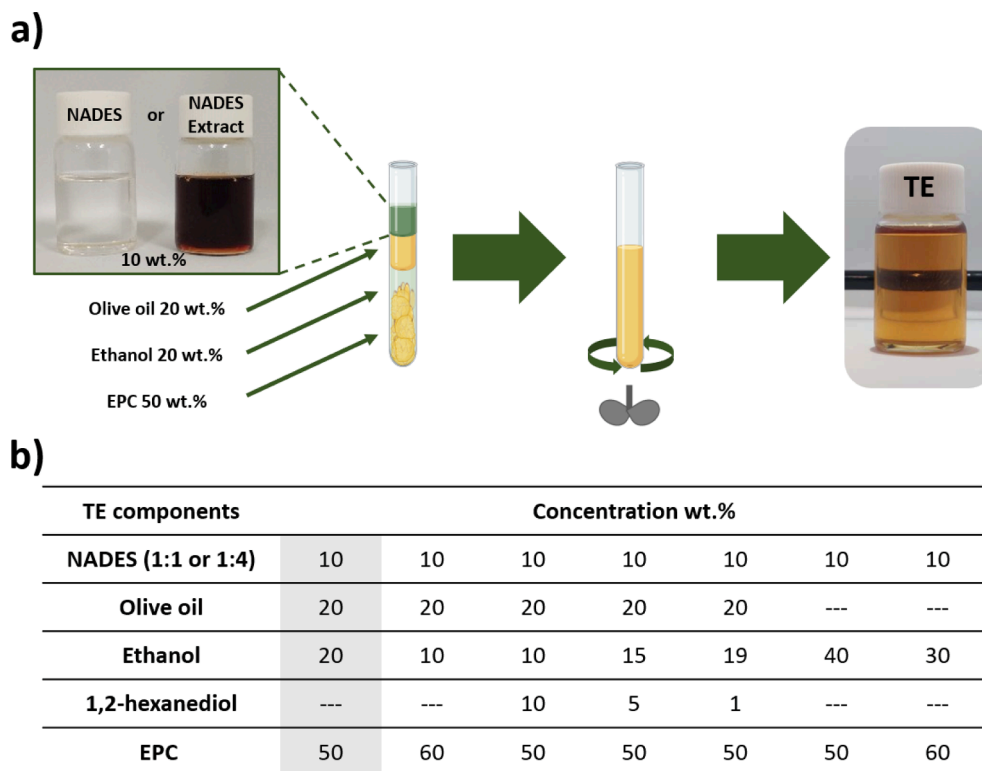


Fig. 1. Scheme of the preparation of TE (a) and the composition of TE tested (b). The column marked in grey is related to the composition chosen for the study.

3.4. Inhibition of tyrosinase and elastase by NADES-extracts and TEs

Tyrosinase is a key enzyme involved in the production of melanin, the pigment responsible for determining the skin tone. Suppression of melanin formation occurs when tyrosinase is inhibited in the skin. The cosmetic industry focuses on regulating skin pigmentation issues by targeting this specific enzyme and creating a range of products to manage skin tone [28,49]. Fig. 2a displays the results of tyrosinase inhibition by the NADES-extracts and TE using kojic acid (KA) as positive control. All samples tested containing a mixture of compounds exhibited higher IC_{50} than those observed for pure KA. It is evident that pure NADES-extracts demonstrate a greater level of tyrosinase inhibition in comparison to their TE counterparts. This probably occurs because TEs contains only 10 wt% of NADES-extract and the remaining components probably have lower tyrosinase inhibitory capacity. Phenolic compounds that are present in NADES-extracts can structurally resemble KA and, as a result, inhibit tyrosinase [13,49]. Among of the several pure extracts tested, it has been observed that NADES-LF shows the strongest inhibition of tyrosinase, whilst NADES-OL displays the lowest value. NADES extracts demonstrate higher IC_{50} values in comparison to pure NADES. Nevertheless, it is crucial to note that the potential of pure NADES as a tyrosinase inhibitor should not be underestimated, given it's the low IC_{50} values. The presence of phenolic compounds and flavonoids in NADES extracts may help to explain their greater inhibitory capacity for tyrosinase than NADES (Control).

Elastase is an enzyme with a crucial role in maintaining the elasticity and firmness of the skin. It monitors the deterioration of elastin, a protein that contributes to the youthful appearance of the skin. Elastase is essential for maintaining skin health, but excessive enzymatic activity can cause the breakdown of elastin fibers, resulting in shapeless skin and the formation of wrinkles. Inhibitors from natural extracts have shown potential in lowering elastase activity and preventing skin aging. These compounds have antioxidant capabilities that can neutralize free radicals, facilitating elastase activation [29,49]. Fig. 2a shows the inhibition of elastase by NADES-extracts and TE-extracts, demonstrating that these

are slightly higher than the KA value. In this specific case, it is observed that the use of TE-extracts provides higher IC_{50} values in terms of elastase inhibition compared to their respective NADES-extracts. These findings may be attributed to the specific composition of the emulsions. The phosphocholine group in EPC may exhibit an affinity for elastase in this specific scenario, as it can potentially mimic the protein elastin [50,51]. Although the IC_{50} shows minimal variation between TE, it is worth highlighting that the TE-TL 1:1 has the greatest inhibitory effect.

3.5. Transdermal permeation of TEs

Transdermal permeation of cosmetic products is related to the capacity of their constituents to penetrate the skin barrier and access the inner dermal layers. The efficacy of skin care products heavily relies on this particular process, as it enables the active constituents to selectively address distinct skin concerns, including but not limited to wrinkles, acne, and hyperpigmentation. Enhancing the transdermal permeation of cosmetic products through the skin can lead to more efficient delivery of their intended benefits, ultimately resulting in enhanced skin health and appearance.

The results of porcine skin permeation by the TE-extracts are presented in Fig. 2b. Based on the obtained data, it can be observed that the TE-control exhibit lower skin permeation compared to the TE-Extract. Among the several TEs investigated, it is shown that those with cork extract (NADES-CP) exhibit a higher capacity of skin penetration. This enhanced penetration can be attributed to the chemicals found in the extract (terpenes, phenolic compounds, fatty acids, among others), which possess a better affinity for the skin [16,17]. Although the relatively modest penetration, the obtained results align with the intended purpose of these topical treatments, which primarily target the management of fungal and bacterial infections in skin.

3.6. Antimicrobial activity of NADES-extracts and TEs

The antimicrobial assays were conducted using the highest possible

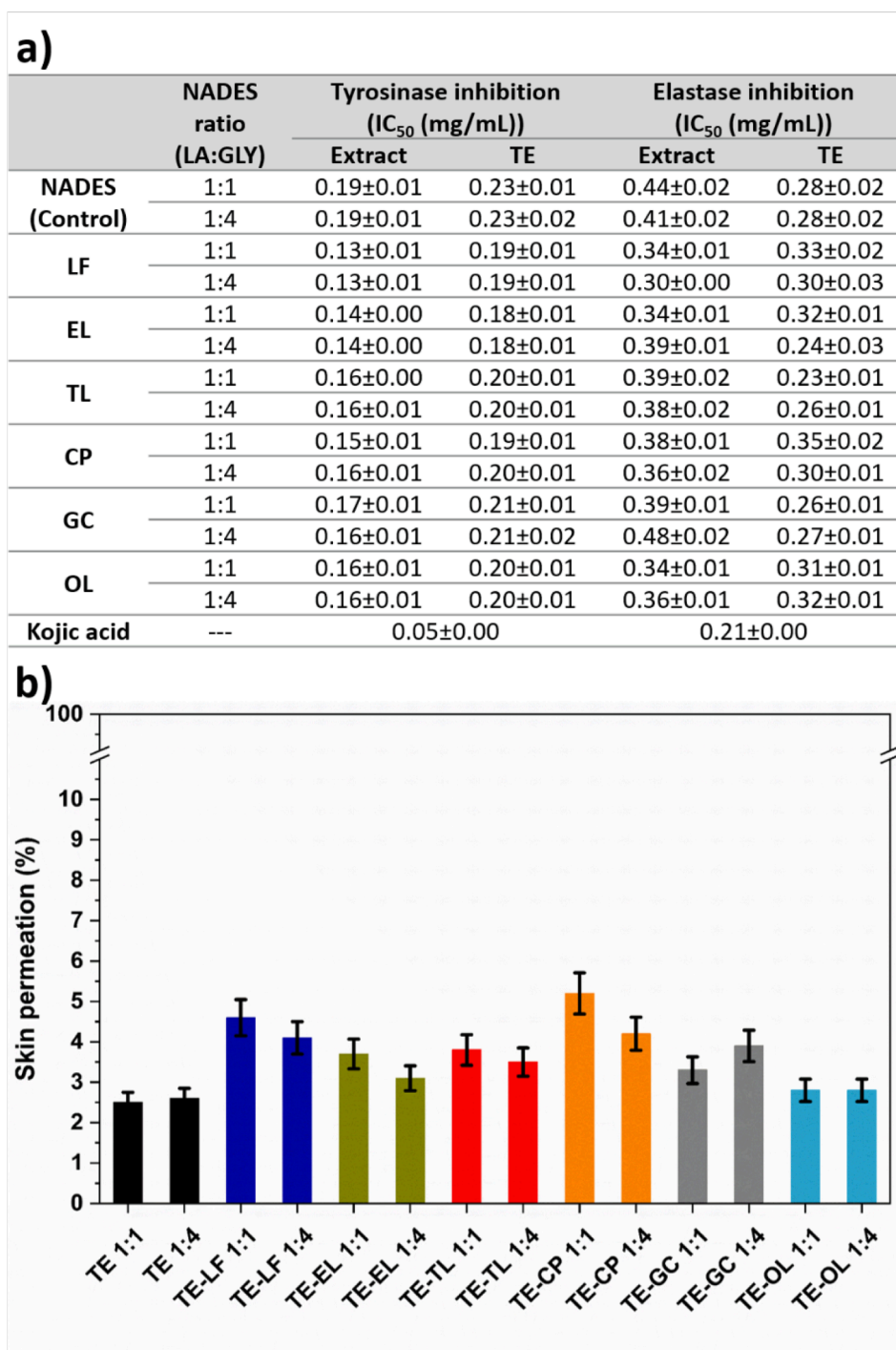


Fig. 2. Evaluation of the inhibition of tyrosinase and elastase by NADES-extracts and respective TE (a), and study of the transdermal skin permeation of all TE produced (b). Note: For NADES and TE, both ratios (1:1 and 1:4) were studied as well as all extracts obtained: lavender (LF), eucalyptus (EL), thyme (TL), cork (CP), garlic (GC), and oregano (OL). All the measurements were performed in triplicate.

concentration of the TE, as well as the highest concentration of NADES (with or without extract) solution to match the final concentration of the extract found in the TE (SI, S6 Table S9 and S10).

The antibacterial activity of the NADES-extracts and TEs was evaluated against Gram-positive (*S. aureus* and *S. epidermidis*) and Gram-negative (*E. coli* and *K. pneumoniae*) bacteria (Fig. 3 and SI, S6 Table S9). These microorganisms are responsible for a range of bacterial infections, which can result in skin infections. In general, NADES-extracts exhibit higher inhibition capacity than TE samples (Fig. 3). In Gram-negative bacteria, they present greater susceptibility to NADES

samples (with/without extract) leading to the formation of larger ZoI. Among the bacteria tested, *K. pneumoniae* shows the highest susceptibility to both NADES-extracts and TE-extracts. The findings regarding the antimicrobial activity of NADES are likely related to the pH (pH 5) of the tested solution and its solvent constituents. For NADES-extracts, the pH influences the antimicrobial activity, being this effect enhanced by the active compounds present in the extracts. A limited diffusion of TEs was seen inside the agar medium during the investigation, resulting in a less prominent ZoI. The ZoI was completely missing in several cases. Antioxidant activity and antimicrobial capacity can be compared, and

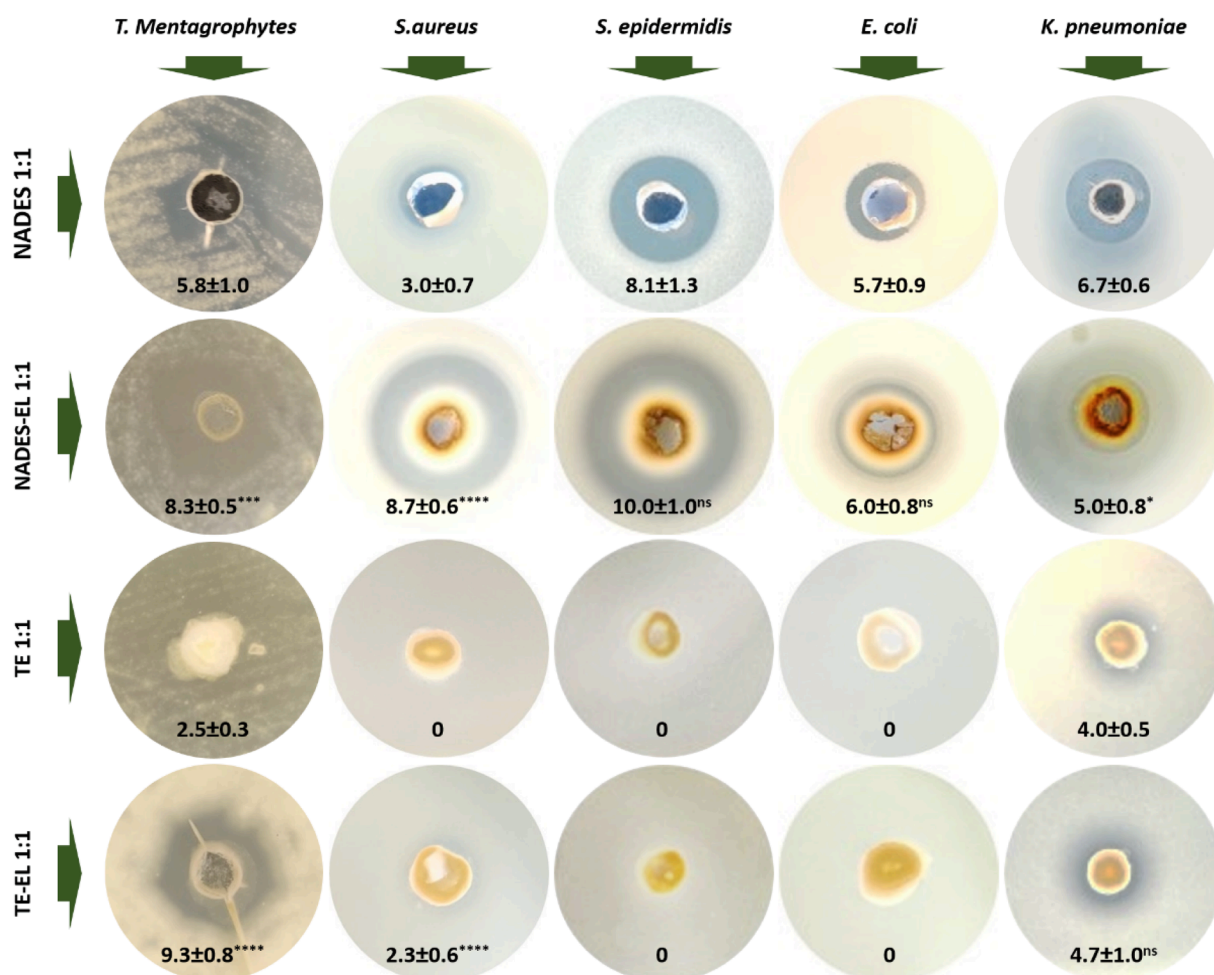


Fig. 3. Photographic representation of ZoI (mm) obtained after antimicrobial assay with NADES (1:1), NADES-EL (1:1), TE (1:1), and TE-EL (1:1) against the fungus *T. mentagrophytes* and the bacteria *S. aureus*, *S. epidermidis*, *E. coli* and *K. pneumoniae*. TE was used at 100 % and NADES (with/without extract) was used in a 10 wt% solution at pH 5 to match the concentration present in the final formulation (TE). The sample hole ($\varnothing \approx 6$ mm) was removed. Note: EL is the NADES-extract obtained from lemon eucalyptus. The statistically significant differences were indicated: * significantly different from the respective control ($p \leq 0.05$); ** significantly different from the respective control ($p \leq 0.01$); *** significantly different from the respective control ($p \leq 0.001$); **** significantly different from the respective control ($p \leq 0.0001$); ns = no statistically significant differences with the respective control.

the results show that NADES-EL has the highest antioxidant capacity and, consequently, the highest antimicrobial capacity. In contrast, NADES-GC displays a lowest antibacterial potential resulting from its relatively reduced antioxidant capacity.

The present study aimed to evaluate the antifungal ability of NADES-extracts and TE-extracts against *T. mentagrophytes*, since this specific fungus can lead to the development of skin and nail pathologies. The results of the growth inhibition halo produced by the formulations against *T. mentagrophytes* are presented in Table S9 (SI, S6) and Fig. 3. An inhibitory halo is visible in samples containing NADES (with and without extract) and TEs (with and without extract). The halo in NADES-extracts and TE-extracts is higher compared with the halo of the corresponding control samples (NADES and TE-control). The extract obtained from garlic cloves exhibited the most significant inhibitory halo, thus reinforcing its known antifungal activity. The antifungal efficacy of garlic extracts (NADES-GC) may be attributed to the presence of sulphur compounds within its composition, leading to favorable effects.

The colony count results offer insights into the bacterial inhibitory capacity of NADES, with/without extract and of TE-extracts (SI, S6 Table S9). Bacterial growth was clearly inhibited in all samples studied, with variable degrees of inhibition reported. The amount of inhibition in the control group (NADES-control and TE-control) was lower in several cases. The data on bacterial growth inhibition lend support to the

hypothesis that some NADES-extracts and TE-extracts have the potential to become antibacterial agents in future applications.

The antimicrobial activity of the extracts studied could be attributed to potential synergistic effect between NADES and the natural source components present in NADES-extracts. LC-MS allowed the identification of terpenes known for their antimicrobial properties, lily aldehyde and linoleyl alcohol, which are present in NADES-LF [52]. NADES-EL contained terpene compounds such as eucalyptol, eugenol, cumene, and 2-bornene, which may be responsible for the extract's larger ZoI results [14]. Thymol and carvacrol are antimicrobials that are widely used in antimicrobial preparations and have been found in NADES extracts of thyme and oregano [13]. Cork has been shown to have a high content of phenolic compounds and their derivatives, which inhibits microbial growth [16,17]. Garlic extract (NADES-GC) had the lowest antibacterial activity, which was to be expected given that this natural source is known for its antifungal properties. NADES-GC has antifungal activity due to the presence of sulphur compounds such as diallyldisulfide and S-allylcysteine [15].

3.7. Effect of NADES-extracts and TEs on the health index of *Galleria mellonella* and on the melanization phenomenon

The larval health index is an essential tool for evaluating the general

health state of *G. mellonella*, as it considers several factors such as larval activity, cocoon formation, melanization, and survival [34]. Through the assessment of larval health index, useful information can be obtained regarding the vulnerability of *G. mellonella* to a specific cosmetic product or compound. *Galleria mellonella* is extensively employed in the field of toxicology due to its resemblance to mammals in terms of immune response and vulnerability to infections. The assessment of the health of *G. mellonella* larvae is of crucial significance to guarantee the reliability and safety of TEs proposed in this work for cosmetic application. All tested TE and NADES (with or without Extract) demonstrated no toxicity effects on the larval model with similar health index values in all conditions (Fig. 4a and SI, S7, Fig. S4).

The assessment of the melanization status holds significance in the evaluation of the well-being of *G. mellonella* larvae. The larval model's high degree of melanization acts as an indicator of the larva's compromised health condition. Essentially, a reduced level of melanization indicates a heightened immune system and improved overall health of the larvae. As shown in Fig. 4b and c, the current research also examined the reduction in the melanization state of *G. mellonella* larvae upon application of TE. It is evident that there is a decrease in the level of melanization from 0 h to 72 h across all TE samples, with a particularly notable decrease observed in the case of TE-extracts. The findings of this study demonstrate that the TE synthesized in this research possesses the ability to mitigate the melanization process in *G. mellonella* larvae. This observation suggests that these cosmetic formulations may have potential as anti-melanises.

The cytotoxicity findings were promising, since several TEs may be tolerated in skin cells at concentrations as high as 5 % (SI, S8). Transdermal tests revealed that the permeation of TE in the skin was 1–5 %, and at this concentration, we were able to determine that some of them

can be used for cosmetic purposes without causing significant cellular damage.

4. Conclusions

In this study, we demonstrated the remarkable efficacy of Natural Deep Eutectic Solvents (NADES), specifically formed by DL-lactic acid (LA) and glycerol (GLY) in ratios of 1:1 and 1:4. Our focus was on extracting bioactive compounds from diverse plant sources, including *Lavandula angustifolia* flowers, *Corymbia citriodora* leaves, *Thymus vulgaris* leaves, *Quercus suber* cork, *Allium sativum* cloves, and *Origanum vulgare* leaves. High extraction yields were achieved using a method that involved a reactor with mechanical stirring at 70 °C. The various NADES-extracts obtained were characterized by evaluating their chemical composition using HPLC-MS, which confirmed the presence of active compounds associated with each natural source. The phenolic and flavonoid contents, chelating power, and antioxidant activity of the different NADES-extracts were also evaluated. These properties were then related to their antimicrobial effect and their inhibitory capacity on enzymes present in the skin.

For the first time, NADES-in-oil emulsions (TEs) have been innovatively developed specifically for cosmetic applications. These emulsions comprised 50 wt% EPC, 20 wt% ethanol, 20 wt% olive oil, and 10 wt% NADES, with or without extract. Importantly, all components in the proposed method are cost-effective, easily produced, and biocompatible in the utilized quantities. The potential of NADES, NADES-extracts, and TE to inhibit tyrosinase and elastase, two enzymes associated with skin aging and loss of elasticity, is promising.

Significantly, all NADES, NADES-extracts, and TEs demonstrated notable antifungal activity against *T. mentagrophytes* and antibacterial

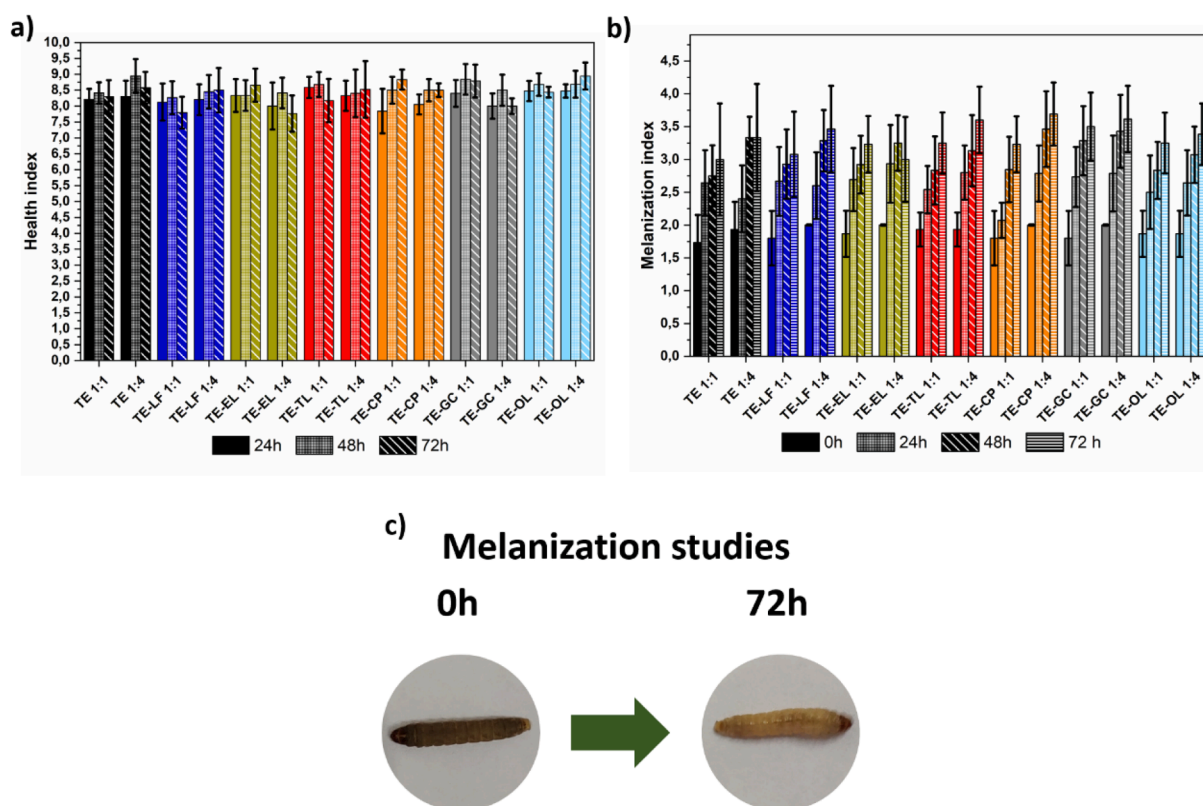


Fig. 4. The *G. mellonella* health index assay for all TE (a)) and *G. mellonella* melanization index for all TE (b) and c)). Note: For TE, both ratios (1:1 and 1:4) were studied as well as all extracts obtained: lavender (LF), eucalyptus (EL), thyme (TL), cork (CP), garlic (GC), and oregano (OL). The results of the larval health index (a)) in the presence of TE-extracts at 72 h revealed no statistically significant differences respecting to TE-controls. The results of the melanization index of the larvae (b)) in the presence of TEs between 0 h and 72 h revealed statistically significant differences ($p \leq 0.0001$) within the same group of samples. No statistically significant differences were observed after 72 h of exposure between the TE-extracts and the respective TE-controls.

activity against *S. aureus*, *S. epidermidis*, *E. coli*, and *K. pneumoniae*. The assessment of skin keratinocytes cell line and a larval model (*G. mellonella*) confirmed the low toxicity of these novel emulsions. Furthermore, the evaluation of all TEs showed a remarkable enhancement in the melanization condition of the larvae, serving as an indicator of improved larval health.

In summary, NADES were successfully produced and incorporated into TE without the use of harmful compounds, highlighting their significant potential for implementation in the cosmetic industry. This ground-breaking approach not only addresses cosmetic needs but also showcases broader applications, emphasizing the safety and efficacy of NADES-based emulsions.

CRediT authorship contribution statement

David S. Freitas: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Diana Rocha:** Methodology. **Joana Santos:** Validation, Methodology. **Jennifer Noro:** Validation, Methodology. **Tânia D. Tavares:** Visualization, Methodology. **Marta O. Teixeira:** Methodology. **Daniela Araújo:** Visualization, Methodology. **Joana Castro:** Validation, Methodology. **Sónia Silva:** Validation, Methodology, Conceptualization. **Helena P. Felgueiras:** Visualization, Validation, Methodology, Conceptualization. **Artur Ribeiro:** Visualization, Validation, Methodology. **Armando Venâncio:** Validation, Methodology. **Artur Cavaco-Paulo:** Visualization, Validation, Methodology. **Carla Silva:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

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.

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Appendix A. Supplementary material

The authors have cited additional references within the Supporting Information [17]. Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molliq.2024.126413>.

Data availability

No data was used for the research described in the article.

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