

Article

NADES-in-Oil Emulsions Enriched with Essential Oils for Cosmetic Application

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Abstract: This research aims to explore the potential benefits of natural deep eutectic solvents (NADES) in formulating translucent NADES-in-oil emulsions (TEs) containing essential oils (EOs) for cosmetic applications. The TEs investigated in this study are based on previous formulations, consisting of 50 wt.% egg phosphatidylcholine (EPC), 20 wt.% ethanol, 20 wt.% olive oil (OLO), thyme oil (TO), or oregano oil (OrO), and 10 wt.% NADES made from a 1:1 or 1:4 ratio of lactic acid and glycerol (LA). These emulsions exhibit high antioxidant activity, attributed to the terpenes present in the essential oils, such as thymol in TO and carvacrol in OrO. The TEs containing TO and OrO demonstrated a more fluid consistency, along with a more appealing texture and fragrance compared to the OLO control. Additionally, these emulsions exhibited the ability to permeate pig skin, as well as significant antifungal and antibacterial activity, and low toxicity in the *Galleria mellonella* larval model. Overall, the findings expand the potential applications of NADES, particularly in the development of translucent emulsions with EO for treating microbial skin and nail infections.

Keywords: natural deep eutectic solvents (NADES); translucent NADES-in-oil emulsions; essential oils; cosmetics

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1. Introduction

Deep eutectic solvents (DES) are a type of solvents formed by the combination of two or more components that became liquid at room temperature. These solvents have unique properties such as low vapor pressure, high conductivity, and tunable viscosity. DES have gained attention in various industries due to their eco-friendly nature and potential applications [1–7]. The special properties of DES have made them increasingly popular in the cosmetics industry over the years. They are a choice for sustainable cosmetic products

because they are biodegradable and environmentally friendly [8]. However, when selecting DES for the development of a cosmetic product, certain factors must be considered. Despite their promising properties, the use of NADES in cosmetics presents certain challenges, including their relatively low stability in some formulations, potential incompatibility with other cosmetic ingredients, and limited regulatory frameworks that govern their use [8–10]. Overcoming these barriers requires further research into the long-term efficacy and safety of NADES-based products. Therefore, it is necessary to examine the long-term efficacy and reliability of DES in specific applications, as well as its potential incompatibility with certain cosmetic ingredients [8,11].

The use of the natural version of DES (NADES), in which all constituents are of natural origin, could make the use of these solvents in the cosmetic industry even more appealing, particularly in products bearing the sustainable seal. Currently, there are reported studies that address the use of NADES in lotions, creams, serums, emulsions, shampoos, among others [8,11]. Vasyliet et al. [12] proposed NADES composed of choline chloride: 1,2-propanediol or lactic acid as promising solvents for extracting antioxidant bioactive compounds from tomato pomace. This confirmed their application in cosmetic formulations. This study led to the development of a natural oil-in-water emulsion formulation that includes the direct application of NADES containing the natural extract [12]. Another study proposed the removal of cork extractives with NADES and the direct application of the extracts (containing NADES) in cosmetic formulations. The formulations produced showed high antioxidant activity and did not demonstrate toxic effects on keratinocyte cells [13].

Recently, we designed a study addressing the use of NADES (with or without extract) to create the first translucent NADES-in-oil emulsions (TEs) proposed for cosmetic purposes [14]. The NADES used in this study were composed of lactic acid and glycerol (LA:GLY) in ratios 1:1 and 1:4. TEs were prepared by simply mixing 50 wt.% Egg-phosphatidylcholine (EPC), 20 wt.% ethanol, 20 wt.% olive oil, and 10 wt.% LA:GLY (with or without extract). These TEs demonstrated remarkable antimicrobial capacity, which, coupled with their low toxicity, enhances their potential for pharmaceutical and cosmetic applications. The development of new TEs offers distinct advantages over traditional emulsions in cosmetic applications. These emulsions provide improved stability, enhanced incorporation of bioactive compounds, and superior antimicrobial and antioxidant properties, making them a promising candidate for eco-friendly cosmetic formulations [14]. Despite their potential, challenges remain in the long-term stability of NADES-based formulations, their compatibility with other ingredients, and the need for further regulatory approvals in cosmetic applications. Thus, understanding and overcoming these barriers is essential for their widespread adoption [8,10].

In the current study, we relied on previously published TEs composition as a starting point and replaced olive oil with essential oils from *Thymus vulgaris* and *Origanum vulgare* aiming to mainly increment their antimicrobial effect. The essential oils were selected due to their potent antimicrobial and antioxidant properties, as well as their high content of phenolic compounds, such as thymol and carvacrol, which have been shown to effectively combat skin-related pathogens [15]. The characterization of essential oils was carried out using LC-MS (liquid chromatography–mass spectrometry) and GC-MS (gas chromatography–mass spectrometry) techniques, alongside the assessment of phenolic and flavonoid content, chelating power, and antioxidant activity. This study also evaluates the viscosity, texture, and organoleptic properties of the produced TEs. Additionally, the permeation levels in porcine skin, as well as the anti-elastase and anti-tyrosinase activities of the TEs, were investigated as potential cosmetic and pharmaceutical candidates. The antimicrobial properties of both essential oils and TEs were assessed against a range of microbial strains commonly associated with skin and nail

diseases. Finally, the toxicity of TEs was analyzed using an in vivo larval model (*Galleria mellonella*). The primary objective of this study was to develop and characterize translucent NADES-in-oil emulsions (TEs) incorporating thyme and oregano essential oils, with a focus on their antimicrobial, antioxidant, and transdermal permeation properties, to assess their potential as sustainable cosmetic and pharmaceutical candidates.

2. Materials and Methods

2.1. Materials

The following chemicals and materials were used in the study: DL-Lactic acid (80%), ethanol (99.8%), dimethyl sulfoxide (DMSO) (99.9%), glycerol (99%), sodium dodecyl sulfate (SDS) (99%), Folin–Ciocalteu reagent, sodium nitrate (98%), sodium carbonate (99%), anhydrous sodium hydroxide (99%), quercetin (99%), gallic acid (99%), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (99%), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (99%), Trolox (99%), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (98%), potassium persulfate (98%), hydrochloric acid (37%), iron (II) chloride (98%), iron (III) chloride (98%), EDTA (98%), Ferrozine (98%), kojic acid (99%), and L- α -phosphatidylcholine egg yolk (EPC) (95%) were obtained from Merck (Germany) or TCI Chemicals (Europe) and used without further purification. Additional materials included olive oil Pingo Doce (Pingo Doce – Distribuição Alimentar, S.A., Lisboa, Portugal), essential oils from *Thymus vulgaris* and *Origanum vulgare* (Cantinho das Aromáticas®, Vila Nova de Gaia, Portugal), coconut oil (dōTERRA®, Pleasant Grove, UT, USA), and various microbiological culture media including trypticase soy broth (TSB), trypticase soy agar (TSA), nutrient broth (NB), and nutrient agar (NA) (VWR, Radnor, PA, USA), Müeller–Hinton broth (MHB) (CondaLab, Spain), and potato dextrose agar (PDA) (Frilabo, Maia, Portugal). Bacterial strains used in the study, including *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 35984), *Escherichia coli* (ATCC 25922), and *Klebsiella pneumoniae* (ATCC 4352), were sourced from the American Type Culture Collection (ATCC, USA). Fungal strain *Trichophyton mentagrophytes* (MUM 0808) was obtained from the Micoteca da Universidade do Minho (MUM, Braga, Portugal). *Galleria mellonella* larvae were raised in darkness on a diet of pollen grains and beeswax, reaching approximately 250 mg in weight.

2.2. Methods

2.2.1. NADES Preparation

NADES DL-lactic acid:glycerol was prepared in molar proportions of 1:1 and 1:4 according to the method described by Freitas et al. [14,16]. The NADES were prepared by vigorously mixing the constituents at temperatures between 25 °C and 100 °C. Within one hour, a clear solution was formed. The eutectic mixture was then stored at room temperature for further use.

2.2.2. Translucent NADES-in-Oil Emulsions (TEs) Preparation

TEs were prepared at room temperature (20 °C) by combining 50 wt.% of EPC with 20 wt.% ethanol until a thick liquid was obtained (around 5 min). Then, 20 wt.% of either olive oil, thyme oil, or oregano oil was incorporated and stirred until the mixture achieved a uniform texture (around 5 min). Lastly, 10 wt.% of NADES was added and mixed for 5 min until the TE was fully formed (Table 1) [14].

Table 1. Essential oils and translucent emulsion (TE) abbreviations.

Essential Oil	Abbreviation	TE-Oil
Olive oil	OIO	TE-OIO
Thyme oil	TO	TE-TO
Oregano oil	OrO	TE-OrO
Thyme:oregano oil (50:50 wt.%)	TO:OrO	TE-TO:OrO

2.2.3. Total Phenolic Content

The total phenolic content in oils and TE was measured using the Folin–Ciocalteu reagent, optimized for a 96-well plate format [13,14,17]. Briefly, 2 µL of the sample (1 mg/mL in DMSO) was mixed with 10 µL of Folin–Ciocalteu reagent, followed by 120 µL of distilled water and agitation. After one minute, 40 µL of 15% Na₂CO₃ and 28 µL of water were added, and absorbance at 750 nm was recorded with a microplate reader (BioTek, SYNERGY H1M2). The total phenolic content was calculated using a gallic acid calibration curve (0.001–1 mg/mL) and expressed as mg GAE per gram of sample.

2.2.4. Total Flavonoid Content (TFC)

The TFC of oils and TEs was measured using a method adapted for a 96-well plate, following Zhishen et al. [14,18]. To each well, 10 µL of sample, 80 µL of water, and 3 µL of 10% NaNO₂ were added and incubated for 5 min. Then, 3 µL of 20% AlCl₃, 40 µL of 1 M NaOH, and 64 µL of water were added. Absorbance was measured at 510 nm using a BioTek SYNERGY H1M2 microplate reader (Agilent, Santa Clara, CA, USA). TFC was quantified using a quercetin standard curve and expressed as mg QE per gram of sample.

2.2.5. Antioxidant Activity

DPPH Assay

The free radical scavenging activity of oils and TEs was measured using the DPPH assay adapted for a 96-well plate [13,14,17]. To 10 µL of sample (0.001–1 mg/mL in DMSO), 140 µL of DPPH solution (400 µM in ethanol) was added, and absorbance at 515 nm was monitored for 60 min. Trolox served as a standard, and DMSO was used as a blank. The DPPH reduction percentage (*DPPHr*) was calculated according to Equation (1), where *Ar* denotes the absorbance recorded at steady state, and *Ai* signifies the initial absorbance measured at time *t* = 0 min.

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

The EC₅₀ value was determined from the DPPH curve, the *TEC*₅₀ denotes the duration required to achieve a steady-state discoloration at the EC₅₀ concentration, and antiradical efficiency (*AE*) was calculated as:

$$AE = \frac{1}{EC_{50} \times TE_{C50}}. \quad (2)$$

The antioxidant activity was expressed as Trolox equivalents (*TE*) per gram of sample:

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

ABTS Assay

The ABTS^{•+} clearance assay was conducted as per Re et al. [19], with some modifications [14]. A solution of 7.4 mM ABTS and 2.6 mM potassium persulfate was prepared and left to react in the dark for 12–16 h. The ABTS^{•+} solution was diluted to an absorbance of 0.7 ± 0.1 at 734 nm. Samples (0.01–1.0 mg/mL in DMSO) were mixed with 140 µL of ABTS^{•+} solution and incubated for 6 min in the dark. Absorbance was measured at 734 nm. The inhibition rate was calculated as:

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

where A_0 and A_i are the absorbances of the control and sample, respectively. Trolox was used as the standard.

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was used to assess the reducing power of oils and TEs [14,17]. The FRAP reagent was prepared by mixing FeCl_3 , TPTZ, and acetate buffer in a 10:1:1 ratio. To 10 µL of sample (0.01–1 mg/mL), 290 µL of the reagent was added. After 10 min at 37 °C in the dark, the absorbance at 593 nm was measured using a BioTek SYNERGY H1M2 microplate reader. Results were expressed as mg Fe^{2+} per gram of sample.

2.2.6. Chelating Power

The ferrous ion chelation of oils and TEs was measured using a modified method from Bounatirou et al. [14,20,21]. Samples (0.1–1 mg/mL) were mixed with 2 mM FeCl_2 and 5 mM ferrozine, and after 10 min, absorbance at 562 nm was recorded using a BioTek SYNERGY H1M2 microplate reader. Ethanol was used as the negative control, and EDTA as the positive control. Chelating capacity was calculated using the ABTS method and expressed as EDTA equivalents per volume of sample, with triplicate measurements.

2.2.7. Tyrosinase Inhibition Assay

Tyrosinase inhibition by oils and TEs was measured following a modified Di Petrillo et al. [14,22] method. To 30 µL of tyrosinase (1000 U/mL), 70 µL of sample (5–100 µg/mL in DMSO) and 110 µL of 2 mM L-tyrosine were added and incubated for 30 min. Absorbance at 492 nm was recorded using a BioTek SYNERGY H1M2 microplate reader. Kojic acid was used as a positive control. Inhibition was calculated as:

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

where A and B represent the absorbance without and with the sample, respectively.

2.2.8. Elastase Inhibition Assay

Elastase inhibition by oils and TEs was assessed following a modified method from Ndlovu et al. [14,23]. To 25 µL of elastase (0.3 U/mL), 50 µL of sample (0.25–250 µg/mL in 100 mM HEPES buffer, pH 7.5, with 2.5% DMSO) was added and incubated for 20 min. Then, 50 µL of N-Methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (1 mM) was added, and the absorbance at 405 nm was measured after 40 min at 25 °C. Kojic acid was used as a positive control. The IC_{50} value was calculated by plotting elastase inhibition against concentration.

2.2.9. Identification of Compounds Present in Essential Oils

Gas Chromatography–Mass Spectrometry (GC-MS)

The essential oils were derivatized prior to GC-MS analysis using transesterification with pyridine and BSTFA. A 100 µL sample (1 mg/mL in pyridine) was combined with

100 µL of BSTFA and incubated at 60 °C for 15–25 min. The prepared sample was then injected into the GC-MS system. GC-MS analysis was performed on a SCIION SQ1/436 GC (Bruker, Billerica, MA, USA) with a Rxi-5Sil MS column (Restek; 30 m, 0.25 mm ID, 0.25 µm film thickness) under the following conditions: oven temperature held at 70 °C for 1 min, then ramped at 5 °C/min to 260 °C, followed by an instantaneous increase to 300 °C. Injector and detector temperatures were set at 280 °C and 270 °C, respectively. Helium was used as the carrier gas at 1.0 mL/min. Data were collected in electron impact (EI) mode at 70 eV over a mass range of 35–600 *m/z*, with a scan time of 250 ms. Compound identification was based on mass spectra from Wiley and NIST/EPA/NIH libraries and published spectra. The relative abundance of compounds was determined from the total ion chromatogram [16].

Liquid Chromatography–Mass Spectrometry (LC-MS)

The compounds in the oils were identified using a Thermo Scientific™ Vanquish™ Flex UHPLC system connected to a Thermo Scientific™ Orbitrap Exploris™ 120 high-resolution mass spectrometer (Thermo Scientific™, Waltham, MA, USA). Data analysis was performed with Xcalibur 4.5 software. A YMC-Triart C18 UHPLC column (YMC, Lawrence, MA, USA) (150 × 2.1 mm, 3 µm) was used, protected by a guard column of the same material. The approach was previously documented [14,24], albeit with certain variations. The mobile phases were water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The gradient system ran at a flow rate of 0.35 mL/min with the following parameters: 0 min, 95% A; 5.4 min, 85% A; 7.9 min, 80% A; 9 min, 80% A; 12.6 min, 70% A; 16.2 min, 50% A; 18 min, 5% A; 19.8 min, 95% A; 22 min, 95% A. The column was set at 30 °C, the autosampler at 15 °C, and the injection volume was 10 µL. A Heated Electrospray Ionization (HESI) probe was used for ionization, with the ion source set at 350 °C, capillary temperature at 325 °C, and electrospray voltage at 3.5 kV (positive mode) or 2.5 kV (negative mode). Sheath and auxiliary gases were set at 50 and 10, respectively. Data were collected in Full-scan mode and data-dependent MS2 (dd-MS2). The Q-Orbitrap was calibrated weekly for 3 ppm mass accuracy, with MS2 spectra generated by fragmentation using normalized collision energy (NCE) at 30%, 60%, and 90%. The resolution was set to 30,000 FWHM for full scans and 15,000 for MS2.

2.2.10. Characterization and Stability of TEs

TEs Organoleptic Properties

The organoleptic properties (pH, odor, appearance, and color) of the TE were evaluated at 25 °C for a period of 120 days. Periodic visual inspections were conducted to assess these qualities.

TEs Accelerated Stability

The accelerated stability of the TE was assessed using a centrifuge test of 5 g of material at 3000 rpm over 5 days, during which the temperature fluctuated between −20 °C and 25 °C, changing every 24 h, commencing at −20 °C. Upon completion of centrifugation, the TE were analyzed for phase separation.

TEs Size, Dispersity, and Charge

The size and surface charge, expressed as zeta potential, along with the dispersity of the TE, were assessed using dynamic light scattering (DLS) equipment NANO ZS Malvern Zetasizer (Malvern Panalytical Ltd., Malvern, UK) at 25 °C. For size measurement, PDI and zeta potential 5 wt.% TE were dispersed in ethanol. Six independent measurements

were performed for each sample. Malvern dispersion technology software (DTS) was used with multiple narrow modes (high resolution) data processing.

TEs Texture Evaluation

The mechanical consistency of the TE was measured using back extrusion with a texture analyzer (STABLE MICRO SYSTEMS, Model: TA-HDPlus, Godalming, UK) [14]. A 3 mL sample was placed in the probe (A/BE Back Extrusion Ring (90° cone)), and firmness and spreadability were assessed at 10 mm/s speed, 60 mm depth, and 10 g contact force. The probe returned to its original position after each measurement.

TEs Viscoelastic Properties

The viscoelastic properties of the TEs were evaluated using a Discovery HR1 rheometer (TA Instruments, New Castle, DE, USA) with a 40 mm parallel plate [14]. Shear viscosity was measured at temperatures from 20 to 60 °C, using three flow ramps: 0.1 to 300 1/s, held at 300 1/s, and 300 to 0.1 1/s.

Thermogravimetric Analysis (TGA)

TGA was conducted on TEs (8–10 mg) using a TGA 4000 (Perkin Elmer, Waltham, MA, USA) with 13.0 Pyris software. Samples were heated from 25 to 600 °C at 10 °C/min in a nitrogen atmosphere (20 mL/min). Weight loss and its derivative were plotted against temperature, with calibration using Curie temperatures of reference materials [14,25].

2.2.11. Skin Permeation Studies

An ex vivo permeation study was conducted through full-thickness pig skin using a Franz diffusion cell (9 mm orifice diameter, V-Series Stirrers for Franz Cells, PermeGear, Hellertown, PA, USA) [13,14]. The receptor compartment contained 5 mL of 16 mM SDS solution, agitated with a magnetic stirrer at 37 ± 1 °C. Approximately 0.5 mL of TE was applied to the skin. Samples from the receptor solution were collected at 1 to 8 h intervals and replaced with fresh SDS solution. The samples were analyzed spectrally between 230 and 750 nm, with maximum absorption at 270 nm. The concentration in the receptor compartment was determined from the absorbance at λ_{max} . Permeation percentage was calculated using:

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

where C_{fe} is the concentration in the receptor and C_e is the initial concentration in the donor (0.1 g/g). Permeation studies of TEs in pig skin were performed in six independent experiments to ensure statistical robustness and reliability of the results.

2.2.12. Antimicrobial Activity

The antibacterial activity of the oils and TEs was evaluated against Gram-positive bacteria *S. aureus* and *S. epidermidis*, as well as Gram-negative bacteria *E. coli* and *K. pneumoniae*. Zone of inhibition (ZOI) assays, based on the Kirby–Bauer method [26], were performed to measure the antibiotic effectiveness of each sample. The bacterial suspension was prepared to a concentration of 1×10^7 CFU/mL in TSB (*S. aureus*, *S. epidermidis*, and *E. coli*) or NB (*K. pneumoniae*), and plated on TSA (*S. aureus*, *S. epidermidis*, and *E. coli*) and NA (*K. pneumoniae*) plates. 50 µL of each sample were placed in a 6 mm cavity at the center of the agar plates, which were incubated at 37 °C for 24 h. After incubation, the resulting ZOI, indicated by clear areas of agar where bacteria growth was inhibited, was photographed and measured. The experiment was performed in triplicate and repeated at least three times for each sample.

The quantitative antibacterial activity was evaluated using the microdilution method, adapted from CLSI and EUCAST standards [27]. Bacterial inocula were prepared in TSB (*S. aureus*, *S. epidermidis*, and *E. coli*) and NB (*K. pneumoniae*), respectively, and incubated overnight at 37 °C with shaking at 120 rpm. In a 96-well plate, 50 µL of each sample was mixed with 50 µL of bacterial suspensions (1×10^6 CFU/mL) in MHB. TEs were used at 100 wt.%, while oils were applied at a 20 wt.% solution at pH 5, consistent with the final formulation concentration. Controls included bacterial suspensions without the agent and culture medium. Absorbance at 600 nm was measured before (0 h) and after 24 h of incubation at 37 °C. Serial dilutions of the bacterial suspensions were plated on TSA or NA and incubated overnight at 37 °C. Viable bacterial colonies were counted, and the results were expressed as a percentage compared to the control suspensions.

The antifungal activity of the oils and TEs was tested against *T. mentagrophytes*. The fungus was initially cultured on 2 mL of sloping PDA and incubated at room temperature for 7 days. After adding 3 mL of PBS, the cell density was adjusted to an optical density (OD) of 1.0 at 620 nm. The prepared fungal suspension was then spread onto PDA plates, and 50 µL of each sample was placed in a 6 mm well at the center of the plates. The plates were incubated at 37 °C for 4 days. Zones of inhibition (ZoI) were measured, with translucent areas around the wells indicating fungal growth inhibition. All experiments were performed in triplicate with at least three independent trials.

2.2.13. *Galleria mellonella* Assays

The health and melanization indices of *G. mellonella* by oils and TEs were evaluated following the method proposed by Freitas et al. [14]. Briefly, the effect of oils and TEs on the health index of *G. mellonella* was evaluated by applying 5 µL of each sample (oils at 20 wt.% in coconut oil and TEs at 100 wt.%) to the dorsal surface of larvae. A control group received an equivalent volume of water. Ten larvae per condition were used, and the larvae were kept in the dark at 37 °C for 24 h, with the treatment repeated daily for 72 h. The health index was based on larval activity, cocoon production, melanization, and survival rate [28]. The impact of TEs on melanization was assessed by selecting larvae with high melanization and applying the TE solutions every 24 h for 72 h. The study followed ethical guidelines, focusing on minimizing animal use and ensuring statistically meaningful results [29–31].

2.2.14. Statistical Analysis

Statistical analysis was performed to compare the results among the different TEs. All analytical methods used in this study were performed at least in triplicate. Data were expressed as the mean $\pm$ standard deviation (SD) for each formulation group. For comparison of means, one-way analysis of variance (ANOVA) was used, followed by a post hoc Tukey's test to determine significant differences between the formulations. A significance level of $p < 0.05$, $p < 0.01$, $p < 0.001$, or $p < 0.0001$ was considered statistically significant. All statistical analyses were performed using Origin 2018.

3. Results and Discussion

3.1. TE Preparation and Chemical Composition

In the present investigation, translucent NADES-in-oil emulsions (TEs) containing EOs were developed employing NADES composed of lactic acid and glycerol (LA:GLY) in 1:1 and 1:4 ratios, with a pH 5. This formulation has been previously reported; however, in this study, olive oil was replaced by essential oils from *Thymus vulgaris* and *Origanum vulgare*. The olive oil formulation was also prepared as a control. Similarly to previous

research, these TEs can be produced on a large scale and in quantities that can meet industrial demands [14]. Table 1 provides the abbreviations employed in this study.

The characterization of the essential oils and their respective TEs is crucial, as it helps identify the properties that make them suitable for cosmetic applications. To achieve this, measurements of phenolic content, flavonoid content, chelating power, and antioxidant activity were obtained (Table 2).

As expected, both the oils and their respective TEs exhibited relatively low levels of phenolics and flavonoids. However, a notable difference was observed between the oils: olive oil (OIO) showed significantly lower phenolic and flavonoid contents compared to thyme (TO) and oregano (OrO) essential oils. This trend remained consistent throughout the TEs, where emulsions containing olive oil demonstrated lower phenolic and flavonoid levels than those containing thyme or oregano oils.

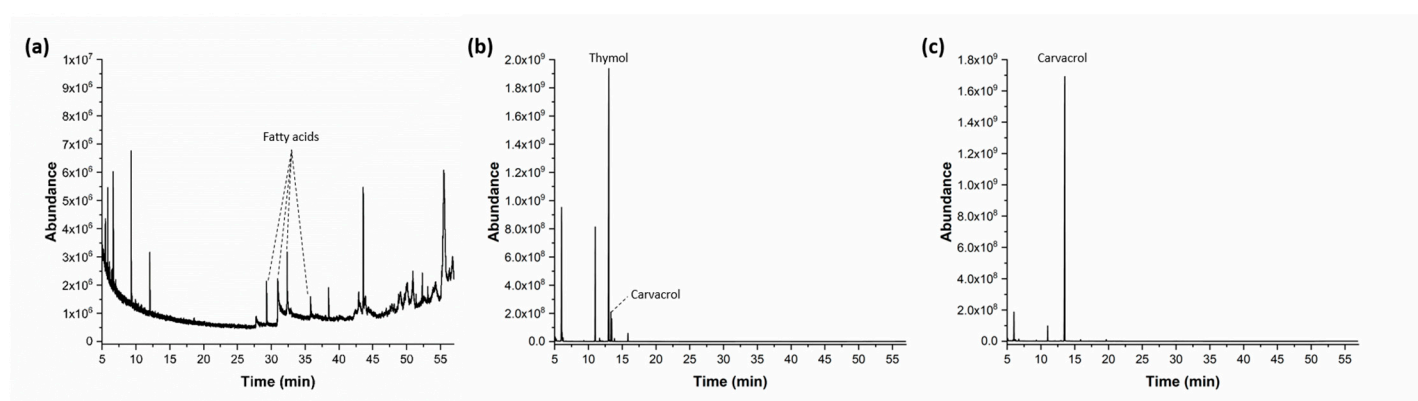
The chelating capacity of the oils, which reflects their ability to bind metal ions that can cause damage to the skin and nails, was also evaluated (Table 2) [13,17,32,33]. According to previous research, NADES with a higher lactic acid concentration (1:1 ratio) have a greater chelating capacity because the molecule can form a bidentate bond, resulting in the formation of Iron (II) lactate. The chelating impact from LA:GLY 1:4 is the lowest because glycerol's ability to chelate iron is lowered. This evidence explains why TEs with a 1:1 NADES ratio has higher chelating power than those with a 1:4 ratio. The chelating power of olive, thyme, and oregano oils is comparable, with values ranging from 25.2 to 28.6 mgEDTA/g_{extract}.

The antioxidant properties of oils and essential oils (EOs) were assessed to predict their potential use in anti-aging and nail protection products. Antioxidant capacity was measured using three methods: DPPH (1,1-diphenyl-2-picrylhydrazyl), FRAP (Ferric Reducing Antioxidant Power), and ABTS (2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid). These assays offer complementary insights into the antioxidant potential of the oils, with each method targeting different mechanisms of antioxidant action. As seen in Table 2, thyme and oregano essential oils exhibited significantly higher antioxidant activity, with values up to five times greater than that of olive oil. The antioxidant activity of emulsions containing thyme and oregano oils (TE-TO, TE-OrO, and TO:OrO) was notably enhanced, following a clear trend: TE-OIO < TE-TO < TE-TO:OrO < TE-OrO. This trend demonstrates that emulsions containing higher concentrations of thyme and oregano oils are more effective at neutralizing free radicals and reducing oxidative stress, positioning them as promising candidates for antioxidant-rich cosmetic formulations. When comparing these TEs with other cosmetic solutions reported in the literature, it is evident that in certain cases they have comparable or superior antioxidant capabilities [8,14,34]. The robust antioxidant capacity of these TEs, demonstrated through their ability to scavenge free radicals and reduce oxidative stress, is particularly relevant for anti-aging applications, as oxidative damage is a major contributor to the breakdown of collagen, elastin, and other structural components of the skin. This capacity may help protect skin cells from premature aging, reduce the appearance of fine lines and wrinkles, and improve overall skin elasticity, making these emulsions valuable candidates for anti-aging cosmetic formulations.

Table 2. Characterization of essential oils and TEs by evaluating phenolic content, flavonoid content, chelating power, and antioxidant activity (DPPH, FRAP, and ABTS assays).

	LA:GLY Ratio (LA:GLY)	Phenolic Content (mgGA/g)	Flavonoids Content (mgQ/g)	Chelating Power (mgEDTA/g)	DPPH (mgTE/g)	FRAP (mgFe ²⁺ /g)	ABTS (mgTE/g)
Oil							
OIO	---	5.2 ± 1.2	3.6 ± 0.9	28.6 ± 1.0	44.8 ± 1.5	27.8 ± 2.5	94.2 ± 2.1
TO	---	23.8 ± 1.0	20.7 ± 1.0	26.9 ± 1.5	179.5 ± 1.1	291.8 ± 2.9	507.7 ± 1.9
OrO	---	26.4 ± 0.5	11.4 ± 1.2	25.2 ± 1.4	196.3 ± 1.3	372.1 ± 2.0	515.2 ± 2.6
TO:OrO	---	29.2 ± 0.8	17.0 ± 0.8	27.0 ± 1.0	183.3 ± 1.2	374.3 ± 2.2	509.2 ± 2.3
TE							
TE-OIO	1:1	16.0 ± 2.0	1.1 ± 0.6	29.4 ± 0.5	58.8 ± 2.2	23.1 ± 2.0	105.5 ± 2.1
	1:4	17.0 ± 1.3	1.3 ± 0.8	12.6 ± 0.9	58.2 ± 2.1	25.1 ± 1.8	105.6 ± 1.8
TE-TO	1:1	21.1 ± 1.2	13.7 ± 1.1	22.0 ± 1.0	123.8 ± 1.5	30.3 ± 1.5	172.0 ± 1.6
	1:4	22.0 ± 0.9	13.9 ± 1.0	15.2 ± 1.1	120.5 ± 1.2	36.2 ± 1.6	172.4 ± 1.6
TE-OrO	1:1	22.1 ± 1.0	9.1 ± 0.4	20.3 ± 0.9	153.6 ± 1.8	32.1 ± 1.0	226.8 ± 1.7
	1:4	24.0 ± 0.8	9.9 ± 0.7	14.7 ± 0.7	135.2 ± 1.5	49.8 ± 1.7	225.4 ± 1.4
TE-TO:OrO	1:1	21.4 ± 0.9	11.2 ± 0.6	25.6 ± 1.1	137.8 ± 1.0	34.1 ± 1.1	209.2 ± 1.3
	1:4	28.1 ± 1.6	11.9 ± 0.5	16.3 ± 1.3	142.0 ± 2.0	41.3 ± 1.3	213.5 ± 1.5

In the current study, HPLC-MS (high-performance liquid chromatography–mass spectrometry) and GC-MS (gas Chromatography–mass spectrometry) techniques were employed to identify compounds in olive oil (OIO), thyme oil (TO), and in oregano oil (OrO) (Figure 1, and SI, S2, Table S1–S3). TO and OrO oils were found to contain a wide variety of terpenic compounds, which contributed to their relatively low phenolic and flavonoid content. Thyme oil was primarily composed of thymol, while oregano oil was predominantly made up of carvacrol. Both thymol and carvacrol are compounds known for their high antioxidant capacity, which supports the findings presented above [17]. OIO contains primarily fatty acids and their derivatives, contributing to the low phenolic and flavonoid content, as well as to the lower antioxidant capacity evaluated.

**Figure 1.** GC-MS chromatograms of olive oil (a), thyme oil (b), and oregano oil (c).

3.2. Physico-Chemical Properties of TEs

In this study, the pH, odor, color, and particle size of TE formulations were evaluated (Figure 2b). As mentioned previously, the pH of the initial LA:GLY was adjusted to 5 before incorporation into TEs. This minor adjustment allowed all TEs produced to have a final pH of 5.6. The emulsions are generally yellowish in color, with thyme and oregano essential oils exhibiting a lighter shade. Regarding odor, TE-OIO has an egg-like scent

(EPC), TE-TO has the aroma of thyme, TE-OrO has a distinct oregano fragrance, and TE-TO:OrO has a mixed scent of both thyme and oregano.

The stability of TEs at room temperature for up to six months was assessed in terms of their organoleptic properties, including color, odor, and overall appearance. Following exposure to a temperature cycling protocol (alternating hot and cold conditions over a 5-day period) and subsequent centrifugation, no significant changes were observed in these properties. This suggests that the TEs exhibit a high degree of resilience to both thermal fluctuations and mechanical stress, maintaining their organoleptic characteristics throughout the storage and treatment conditions. These findings imply that TEs are stable under the tested conditions, which may have implications for their storage and handling in practical applications.

When analyzing TE dispersions in water, the DLS evaluation only shows particle agglomerates, as observed previously for emulsions containing extracts [14]. Once these new TEs are diluted in ethanol (5 wt.%), nanoparticles smaller than 600 nm become detectable. The observed nanoparticle sizes align with those reported in the literature for emulsions containing similar extracts, supporting the reproducibility of the current formulation [14]. For TEs formulated with NADES in a 1:1 ratio, our findings indicate that the TE-TO 1:1 formulation produces smaller nanoparticles, approximately 470 nm in size, whereas the TE-TO:OrO 1:1 formulation results in slightly larger particles, around 500 nm. In contrast, for TEs prepared with NADES in a 1:4 ratio, the TE-TO 1:4 formulation yields smaller nanoparticles, approximately 300 nm, while the TE-OrO 1:4 formulation generates larger particles, approximately 600 nm in size. Additionally, it is evident that the TEs composed of thyme oil consistently produced nanoparticles with smaller sizes across both NADES ratios. This observation suggests that thyme oil may play a significant role in modulating the particle size, potentially due to its unique chemical composition, which could influence the interaction dynamics and self-assembly processes within the formulation.

Emulsion particle size distribution is specified by the polydispersity index (PDI). A PDI value closer to 0 indicates extremely homogeneous solutions and closer to 1 indicates extremely heterogeneous solutions [13]. In this regard, the PDI values of the TEs shown here range between 0.1 and 0.6, showing that the TEs exhibit an acceptable level of uniformity, though there is some degree of variability in the particle sizes (Figure 2c). This degree of heterogeneity may not be problematic for many applications but could require optimization if a more uniform dispersion is critical for specific purposes, such as in drug delivery or other precise formulations [14].

Zeta potential (ζ) is a crucial stability characteristic in particle dispersion, affecting resistance to aggregation when the charge modulus is greater than ± 30 mV [13]. In the case of TEs, they have surface charge levels between 0 and +6 mV (Figure 2d). The lack of a high charge may limit the long-term stability of the dispersion, particularly in the absence of stabilizing agents. The weak electrostatic repulsion between particles could make the system prone to clumping or settling over time, which may need to be addressed with additional stabilizing formulations or by modifying the surface characteristics of the TEs. Although the surface charge is low, the results suggest that the TEs remain stable for at least six months, as the characterization was conducted after this shelf-life period.

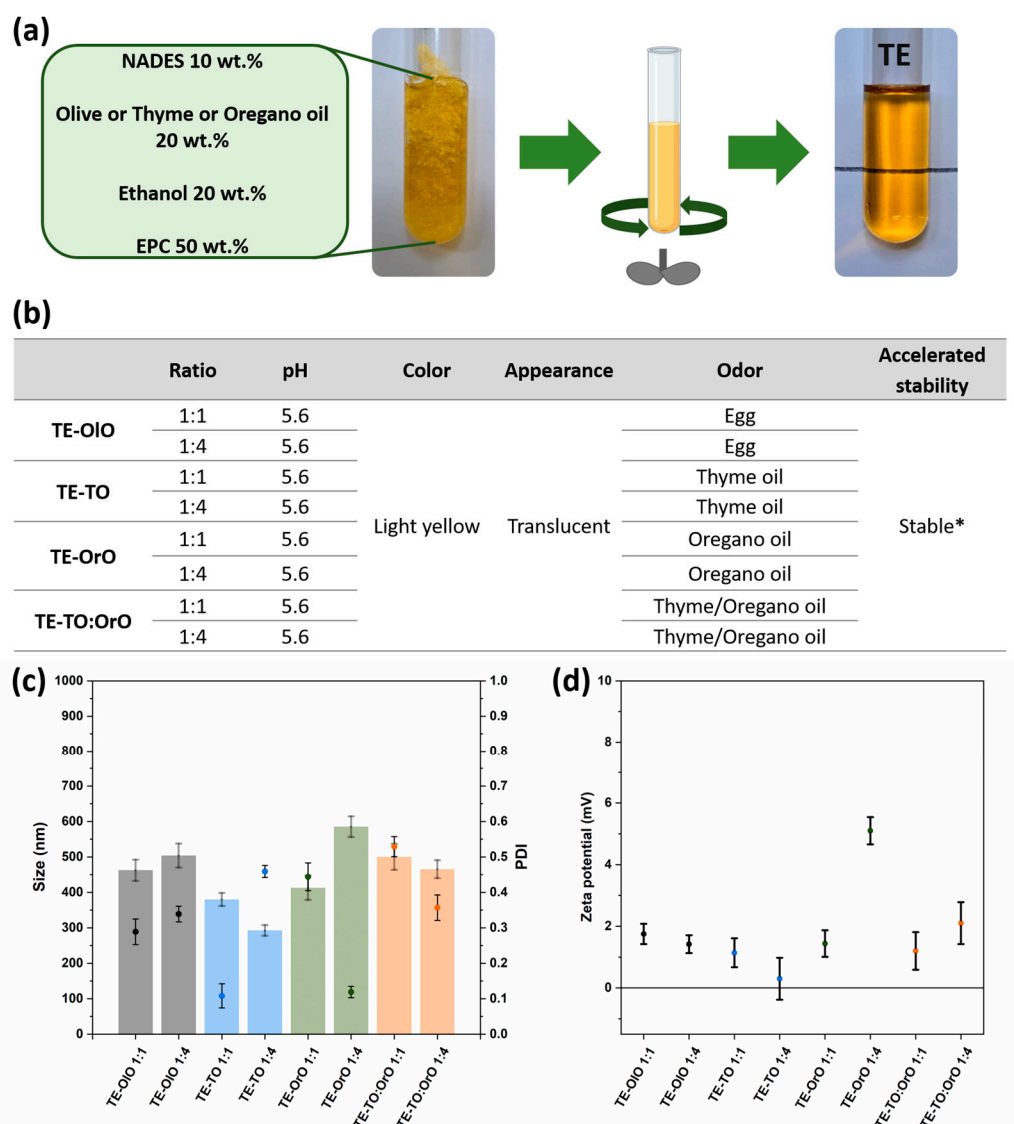


Figure 2. TEs preparation (a) and organoleptic properties (b), size ((c)—bar chart), polydispersity (PDI) ((c)—dot chart) and zeta potential (d). For size measurement, PDI and zeta potential 5 wt.% TE were dispersed in ethanol. Note: TEs can be stored at room temperature for up to 6 months without losing their organoleptic properties. * The color, odor, and overall appearance were unchanged following the temperature cycle (hot and cold for 5 days) and centrifugation.

The shear viscosity was evaluated to determine the fluidity of a TEs during spreading. Low shear viscosity TEs have been formulated for direct application to nails and skin to treat microbial infections, making them easily spread and effective in penetrating affected areas. Figures 3a,b illustrate the shear viscosity results over a range of temperatures. Evidently, in both NADES ratios (1:1 and 4:1), the TEs composed of oregano oil (TE-OrO and TE-TO:OrO) present reduced viscosity in relation to the control (TE-OIO). TE-TO behave differently from the other samples that show a progressive decrease in viscosity with increasing temperature. The TE-TO sample presents deformation of the viscosity-temperature relationship between 30 and 50 °C, which is evident when composed of LA:GLY 1:4. This graphic profile is associated with the non-Newtonian fluid properties of emulsions such as creams or lotions.

The firmness of the samples was evaluated in a texturometer and the data revealed consistency and spreadability of the cosmetic formulations (Figure 3c,d). Firmness and spreadability are interconnected, influencing user perception and product effectiveness.

The firmness and spreadability results of TEs with essential oil (TE-TO, TE-OrO, and TE-TO:OrO) require less applied force to flow, resulting in more fluid emulsions than the control (TE-OIO).

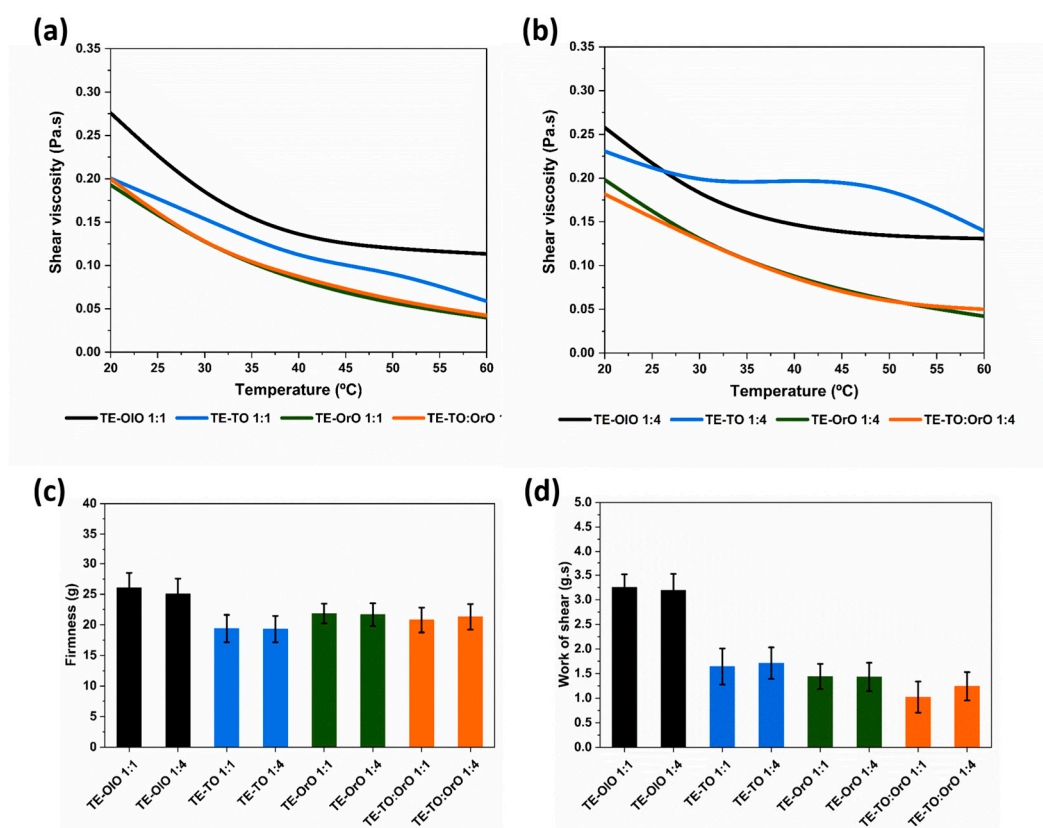


Figure 3. Shear viscosity (Pa.s) vs. temperature (a,b), and firmness (g) (c), and work of shear (g.s) (d) of all the produced TE.

The thermal stability of TEs was evaluated using the Thermogravimetric Analysis (TGA) technique (SI, S1 Figure S1). TEs containing thyme and oregano essential oils (TE-TO, TE-OrO, and TE-TO:OrO) exhibited a higher initial degradation rate compared to the control TE (TE-OIO). While this result is not ideal, it was anticipated due to the higher volatility of thyme and oregano oils compared to olive oil. However, at room temperature, (25 °C), all the produced TEs remained thermally stable, with visible mass loss only occurring at temperatures above 50 °C.

3.3. Transdermal Permeation of TEs

Transdermal permeation of cosmetic products is crucial to their effectiveness in treating topical nails and skin infections. Figure 4 shows the results of TEs permeation through porcine skin. The data suggest that TE-OIO (control) has lower skin permeation than TE-TO, TE-OrO, and TE-TO:OrO. It is worth mentioning that TEs composed of TO, OrO, and TO:OrO have similar skin permeation capacity, with TE-TO being slightly higher. According to previous research, these permeation results can be attributed to compounds found in essential oils (phenolic compounds, terpenes, fatty acids, among others) that have an affinity for the skin [13,14,16]. The permeation results obtained are consistent with previous research, and despite being $\leq 5\%$, they are adequate for the primary goal of these topical agents, which are intended to treat fungal and bacterial infections on nails and skin surface [14].

In this work, we also verified the promising potential of essential oils and TEs to inhibit tyrosinase and elastase, two enzymes associated with skin aging and loss of elasticity (Figure 4b,c, and SI, S3 Table S4). These findings were promising, as all TEs produced had IC_{50} values only four times higher than the kojic acid positive control for tyrosinase. For elastase inhibition, the values obtained with kojic acid were similar to those achieved with the TEs. Notably, the TE combined with thyme oil (TO), oregano oil (OrO), and the TO:OrO essential oil blend showed lower IC_{50} values for elastase inhibition compared to the TE-OIO control. This suggests that the specific composition of essential oils (TO, OrO, and their combination) may enhance the activity of the TEs, leading to improved efficacy and reduced IC_{50} values. These findings are highly promising and suggest that TEs could potentially help reduce skin aging and loss of elasticity.

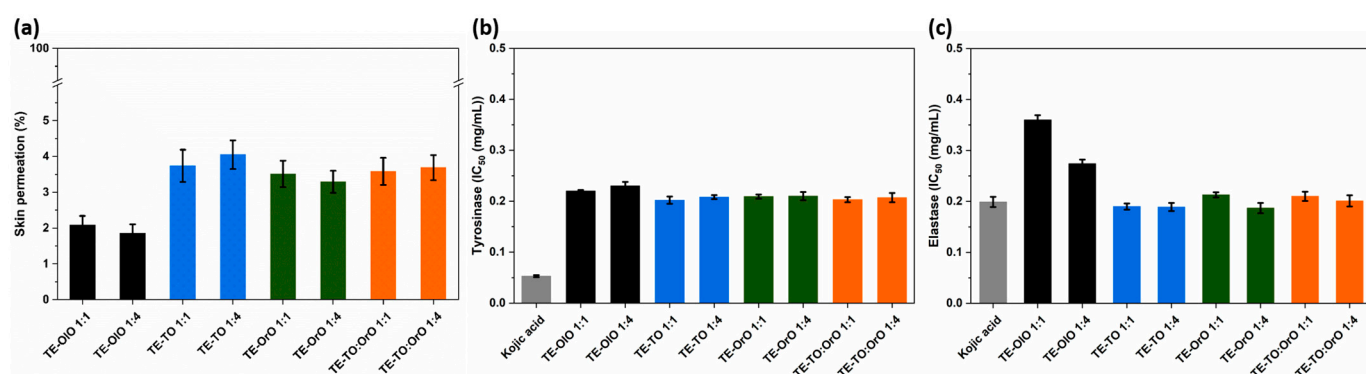


Figure 4. Evaluation of transdermal skin permeation (a), tyrosinase inhibition (b), and elastase inhibition (c) of all TEs produced.

3.4. Antimicrobial Activity of the Essential Oils and TEs

Antimicrobial tests were conducted using pure translucent emulsions (TEs), with the primary aim of assessing their potential for the direct prevention and treatment of fungal and bacterial infections of the skin and nails (Tables 3 and 4). The zone of inhibition (ZoI) and minimum inhibitory concentration (MIC) tests demonstrated the high antibacterial efficacy of thyme oil (TO) and oregano oil (OrO) in comparison to the control, olive oil (OIO). Previous studies demonstrated that TO and OrO oils exhibited MIC values below 1 mg/mL against certain tested microorganisms [35,36]. In the present study, TO and OrO oils displayed MIC values ranging from 36 to 8 μ g/mL for TO and 41 to 11 μ g/mL for OrO, depending on the microorganism tested. The results also revealed that TEs containing essential oils exhibited significantly greater antibacterial capacity than those containing olive oil alone (TE-OIO), highlighting the enhanced antimicrobial potential provided by the essential oils.

The antibacterial activity observed in the TEs followed a similar pattern, where emulsions containing essential oils specifically, TE-TO, TE-OrO, and the combined TE-TO:OrO, demonstrated stronger antibacterial effects when compared to the control formulation (TE-OIO). This trend was especially noticeable for Gram-negative bacteria, such as *Staphylococcus aureus* and *Staphylococcus epidermidis*, which showed a more pronounced inhibition, whereas Gram-positive bacteria like *Escherichia coli* and *Klebsiella pneumoniae* displayed comparatively weaker inhibition. These findings suggest that the essential oils have a preferential inhibitory effect on Gram-negative bacteria, which is consistent with the known mechanisms of action of terpenoid compounds, such as thymol (from TO) and carvacrol (from OrO), which disrupt bacterial cell membranes.

Regarding fungal inhibition, the TEs containing essential oils, TO, OrO, and the combined formulation TE-TO:OrO, showed significantly larger inhibition halos against *Trichophyton mentagrophytes* compared to the pure essential oils. This suggests that the

emulsion formulation enhances the antifungal efficacy of the oils, likely due to a slower diffusion of the oils into the agar medium, as the emulsion components may act to retard the rapid evaporation of volatile compounds. Since the tests were conducted at 37 °C for five days, with the fungus growing during this period, the more rapid evaporation of the essential oils in their pure form (20 wt.%) may have reduced their effective exposure to the fungal cells, thus limiting their inhibitory action. In contrast, the emulsions allowed for more sustained contact, resulting in greater inhibition.

The combination of thyme oil and oregano oil in the TE-TO:OrO formulation exhibited superior antimicrobial activity, further supporting the hypothesis of a synergistic effect between the two oils. Both oils contain high levels of thymol and carvacrol, which are well-documented for their antimicrobial properties, and the combined formulation seems to leverage the complementary effects of these compounds, enhancing the overall antimicrobial activity. The higher antimicrobial potency of the TEs, especially in comparison to the pure oils, suggests that the emulsion system improves the delivery and efficacy of these essential oils, making them more effective for therapeutic use.

As previously mentioned, essential oils like thyme oil and oregano oil are rich in terpenes, such as thymol and carvacrol, which are known to exhibit significant antimicrobial properties. These compounds are widely used in commercial pharmacocosmetic formulations for their ability to combat a variety of microorganisms, including bacteria and fungi [17]. The incorporation of TO and OrO into TEs was intended to further enhance their antimicrobial capacity and surpass the findings of previous studies. The results of the current study support this goal, demonstrating that the use of emulsions enriched with essential oils offers a more efficient and sustained antimicrobial effect compared to the use of the oils in their pure form.

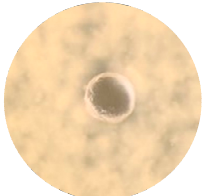
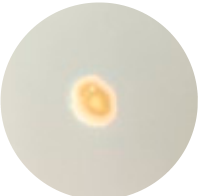
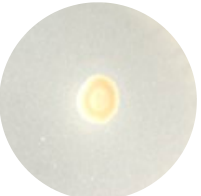
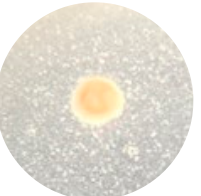
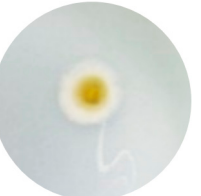
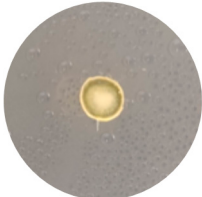
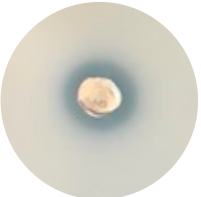
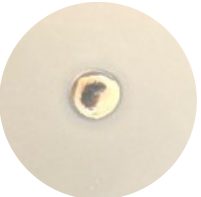
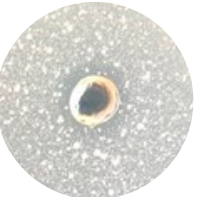
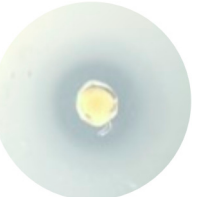
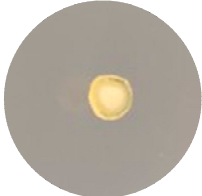
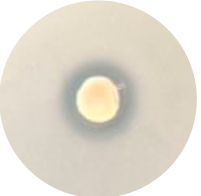
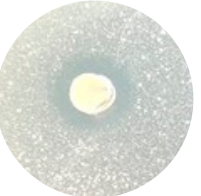
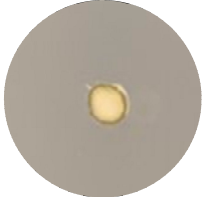
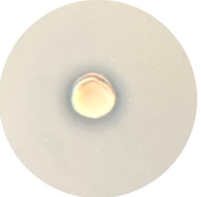
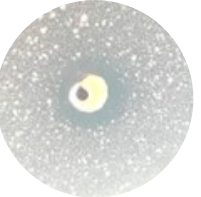
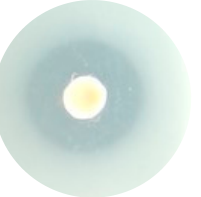
Table 3. The evaluation of antimicrobial activity. The ZoI test was performed on the essential oils and TE samples against the fungus *T. mentagrophytes* and the bacteria *S. aureus*, *S. epidermidis*, *E. coli*, and *K. pneumoniae*. TE was used at 100% and essential oil was used in a 20 wt.% solution at pH 5.6 to match the concentration present in the final formulation (TE). The diameter of the holes ($\varnothing = 6$ mm) was included. The MIC test was performed in the essential oil samples against the *S. aureus*, *S. epidermidis*, *E. coli*, and *K. pneumoniae* (results highlighted in green). The relative inhibitory (RI) of TE (50 vol.%) against the bacteria *S. aureus*, *S. epidermidis*, *E. coli*, and *K. pneumoniae* results (highlighted in blue). The ZoI and MIC results of the essential oils revealed statistically significant differences ($p \leq 0.0001$) between the TO, OrO, and TO:OrO oils and the control (OIO). The ZoI and RI results of the TE-extracts revealed statistically significant differences ($p \leq 0.0001$) with the respective TE-controls. The activity scale proposed by Rota et al. was used, which reports weak activity with ZoI (halo) ≤ 12 mm (^a), moderate activity with ZoI ranging between >12 and <20 mm (^b) and strong activity with ZoI zone ≥ 20 mm (^c) [37,38].

		Fungi			Bacteria					
		<i>T. mentagrophytes</i>		<i>S. aureus</i>	<i>S. epidermidis</i>		<i>E. coli</i>		<i>K. pneumoniae</i>	
NADES Ratio (LA:GLY)				MIC	MIC		MIC		MIC	
		ZoI (mm)	ZoI (mm)	(µg/mL)	ZoI (mm)	(µg/mL)	ZoI (mm)	(µg/mL)	ZoI (mm)	(µg/mL)
				RI (%)		RI (%)		RI (%)		RI (%)
EO										
OIO	---	6.5 ± 0.6 ^a	6.0 ± 0.0 ^a	>1000.0	6.0 ± 0.0 ^a	>1000.0	6.0 ± 0.0 ^a	>1000.0	6.0 ± 0.0 ^a	>1000.0
TO	---	43.8 ± 0.5 ^c	38.3 ± 0.9 ^c	36.9 ± 1.1	26.3 ± 1.1 ^c	34.3 ± 2.2	31.7 ± 0.9 ^c	12.3 ± 1.8	45.0 ± 1.5 ^c	7.8 ± 2.1
OrO	---	55.0 ± 1.0 ^c	25.0 ± 1.0 ^c	41.1 ± 2.2	28.0 ± 0.8 ^c	40.9 ± 2.5	40.8 ± 1.4 ^c	17.2 ± 1.2	45.0 ± 1.9 ^c	10.6 ± 1.5
TO:OrO	---	51.5 ± 1.1 ^c	27.0 ± 0.5 ^c	42.3 ± 3.0	40.0 ± 1.0 ^c	35.3 ± 1.1	40.0 ± 1.1 ^c	16.3 ± 1.1	45.0 ± 1.2 ^c	9.9 ± 1.2

TE										
TE-OIO	1:1	6.5 ± 0.4 ^a	6.0 ± 0.0 ^a	**	6.0 ± 0.0 ^a	**	6.0 ± 0.0 ^a	**	6.0 ± 0.0 ^a	**
	1:4	7.0 ± 0.5 ^a	6.0 ± 0.0 ^a	**	6.0 ± 0.0 ^a	**	6.0 ± 0.0 ^a	**	6.0 ± 0.0 ^a	**
TE-TO	1:1	90.0 ± 0.0 _{c*}	12.3 ± 0.2 ^b	100 ***	8.3 ± 0.4 ^a	100 ***	9.3 ± 1.1 ^a	100 ***	13.0 ± 0.3 ^b	100 ***
	1:4	90.0 ± 0.0 _{c*}	8.0 ± 0.6 ^a	100 ***	8.0 ± 0.3 ^a	100 ***	10.0 ± 0.8 ^a	100 ***	15.0 ± 0.7 ^b	100 ***
TE-OrO	1:1	90.0 ± 0.0 _{c*}	15.0 ± 1.7 ^b	100 ***	9.7 ± 0.9 ^a	100 ***	12.7 ± 0.5 ^b	100 ***	19.3 ± 1.0 ^b	100 ***
	1:4	90.0 ± 0.0 _{c*}	11.0 ± 0.6 ^a	100 ***	11.0 ± 0.5 ^a	100 ***	13.0 ± 0.5 ^b	100 ***	18.7 ± 1.1 ^b	100 ***
TE-TO:OrO	1:1	90.0 ± 0.0 _{c*}	12.0 ± 0.3 ^a	100 ***	9.0 ± 0.7 ^a	100 ***	12.3 ± 0.8 ^b	100 ***	19.0 ± 0.8 ^b	100 ***
	1:4	90.0 ± 0.0 _{c*}	9.0 ± 0.5 ^a	100 ***	9.0 ± 0.3 ^a	100 ***	11.3 ± 0.9 ^a	100 ***	18.3 ± 0.6 ^b	100 ***

* Total inhibition on 90 mm plate; ** Control sample RI test; *** Inhibition in relation to control (TE-OIO) RI test.

Table 4. Photographic representation of ZoI obtained after antimicrobial assay with TE with NADES 1:1 for the fungus *T. mentagrophytes* and the bacteria *S. aureus*, *S. epidermidis*, *E. coli*, and *K. pneumoniae*. The diameter of the holes (Ø = 6 mm) was included.

	<i>T. mentagrophytes</i>	<i>S. aureus</i>	<i>S. epidermidis</i>	<i>E. coli</i>	<i>K. pneumoniae</i>
TE-OIO	 6.5 mm	 6 mm	 6 mm	 6 mm	 6 mm
TE-TO	 Total	 12.3 mm	 8.3 mm	 9.3 mm	 13.0 mm
TE-OrO	 Total	 15.0 mm	 9.7 mm	 12.7 mm	 19.3 mm
TE-TO:OrO	 Total	 11.0 mm	 9.0 mm	 12.3 mm	 19.0 mm

3.5. Effect of NADES-Extracts and TEs on the Health Index of *Galleria mellonella* and on the Melanization Phenomenon

The larval health index is an important tool for assessing the overall health of *G. mellonella*, taking into account factors such as larval activity, cocoon formation, melanization, and survival [28]. The reaction of *G. mellonella* to specific cosmetic products or compounds provides an indication of its toxicity [14,39]. Assessment of larval health is critical for ensuring the reliability and safety of proposed TEs for future cosmetic applications. Figure 5a shows the health index of *G. mellonella* in the presence of the different TEs produced. The larval health index was found to be lower in TEs containing essential oils (TE-TO, TE-OrO, and TE-TO:OrO), suggesting a level of toxicity in compared to the control (TE-OIO). However, despite the reduced larval health indices for TE-TO, TE-OrO, and TE-TO:OrO, the results remain acceptable, as they are still above 6.

The melanization status of *G. mellonella* is an important indicator of their health, as high melanization indicates compromised health, while reduced melanization suggests a strengthened immune system and improved overall health. Figure 5b demonstrates that the melanization state improved over time for all TEs. Notably, the TEs containing essential oils (TO, OrO, and TO:OrO) showed slightly lower levels of melanization compared to the control (OLO), suggesting that OLO has a more protective effect against melanization in larvae.

Several studies have investigated the health index of *Galleria mellonella* larvae in response to various essential oils, including thyme and oregano oils [40,41]. All these studies identified the toxicity of these oils for this larval model, suggesting the need for a reduced dosage. In this study, a 20 wt.% concentration of oil was selected to ensure effective microbial inhibition while maintaining the health index within acceptable limits for the use of these new cosmetic solutions.

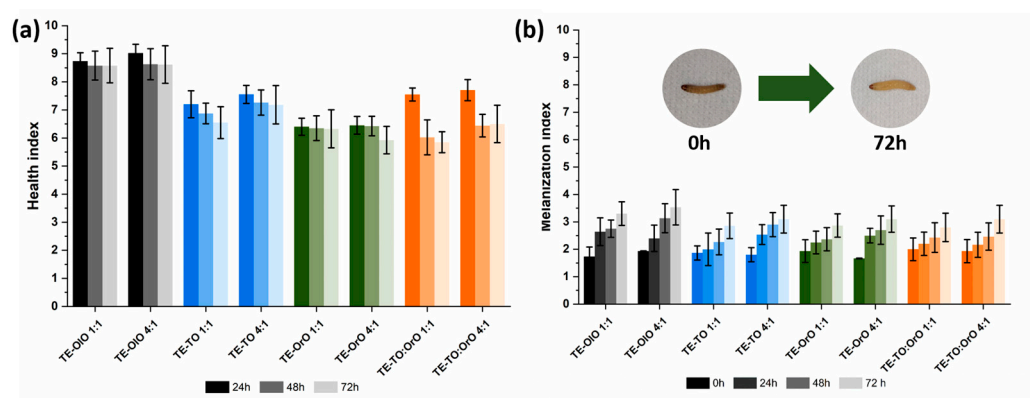


Figure 5. The *G. mellonella* health index assay (a) and melanization index assay (b) for all the produced TE. The results of the larval health index (a) in the presence of TE-extracts at 72 h revealed statistically significant differences ($p \leq 0.0001$) with the respective TE-controls. The melanization index results of the larvae (b) in the presence of TEs from 0 h to 72 h revealed statistically significant differences ($p \leq 0.05$) within the same sample group. No statistically significant differences were observed between the TE-extracts and their respective TE-controls after 72 h of exposure.

4. Conclusions

This study successfully developed NADES-in-oil emulsions (TEs) designed for pharmaco-cosmetic applications, leveraging the unique properties of natural deep eutectic solvents (NADES) and essential oils. The novelty of this work lies in the substitution of traditional olive oil with essential oils from *Thymus vulgaris* (thyme) and *Origanum vulgare* (oregano), combined with the use of NADES composed of lactic acid

and glycerol at pH 5. The resulting emulsions demonstrated significant antioxidant activity, driven by the terpene compounds thymol and carvacrol, which are abundant in thyme and oregano oils. These findings suggest that essential oils not only enhance the antioxidant potential of the emulsions but also provide additional antimicrobial benefits.

The TEs displayed favorable properties for topical applications, including moderate skin penetration and significant antifungal activity against *Trichophyton mentagrophytes* and antibacterial efficacy against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Klebsiella pneumoniae*. The use of the *Galleria mellonella* larval model confirmed the low toxicity of the emulsions, while also highlighting their potential to promote skin health by improving larval melanization. These results underscore the potential of TEs as a safe and effective option for the treatment and prevention of fungal and bacterial skin infections.

The implications of this work extend beyond the immediate scope of topical therapeutic applications, suggesting that NADES-based emulsions enriched with essential oils could serve as a promising platform for the development of advanced pharmaco-cosmetic formulations. These emulsions may offer a more sustainable and potent alternative to conventional skincare products, benefiting from the synergy between natural ingredients and the stability provided by the NADES matrix.

Moving forward, future research should focus on optimizing the formulation parameters to further enhance the efficacy and stability of the TEs. Additionally, clinical studies are needed to confirm the safety and effectiveness of these emulsions in human skin applications. Investigating the synergistic interactions between different essential oils and exploring the use of other natural solvents could provide valuable insights into expanding the range of therapeutic applications for NADES-based emulsions in dermatology and beyond.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pr13020374/s1>, Figure S1: TGA degradation steps (a) and TGA thermograms (b) and c) of all the produced TEs.; Table S1: Compounds identified in olive oil by GC-MS.; Table S2: Compounds identified in thyme oil by GC-MS.; Table S3: Compounds identified in oregano oil by GC-MS.; Table S4: Evaluation of the inhibition of tyrosinase and elastase by essential oils and respective TE.

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