



Cite this: DOI: 10.1039/d6an00048g

Upcycling lemon peel waste into sustainable bioactive ingredients: chemical characterization and efficacy assessment

Ritamaria Di Lorenzo,^{†a} Ilaria Neri,^{†a,b,c} Raffaele Raimondo,^d Daniela Di Matteo,^d Carlo Irace,^a Maria Grazia Ferraro,^e Mariavittoria Verrillo,^f Teresa Ponticorvo,^a Teresa Di Serio,^a Sonia Laneri ^{*a} and Lucia Grumetto ^{*a,b,c}

The upcycling of agro-industrial by-products represents a sustainable strategy to reduce waste, generating high-value bioactive compounds. In this study, exhausted *Citrus limon* peels, which are residues generated as a by-product of limoncello production, a traditional liqueur of Southern Italy, were investigated as a potential source of bioactive compounds using supercritical CO₂ extraction. The resulting extracts were chemically characterized by gas chromatography coupled with mass spectrometry, revealing a complex phytochemical profile mainly composed of monoterpenes and oxygenated monoterpenes. Two representative extracts (EO-A and EO-B) were selected based on the extraction yield, chemical profile, and safety considerations. Evaluation in human keratinocytes (HaCaT cell line) demonstrated their high biocompatibility, with cell viability exceeding 90% at all tested concentrations. Antioxidant properties were assessed through ABTS and DPPH radical scavenging assays, showing significant activity, particularly for EO-B. Both extracts also exhibited antimicrobial activity against selected Gram-positive and Gram-negative bacterial strains, including *Salmonella typhi* and *Escherichia coli*. The essential oils exhibited inhibitory effects on collagenase and elastase, suppression of melanogenesis, and activity against *Cutibacterium acnes*, highlighting their potential for anti-aging, skin-brightening, and antimicrobial applications. Preliminary clinical evaluations indicated improvements in skin hydration, softness, and elasticity. Overall, these findings support the use of supercritical CO₂ extraction as a green, sustainable, and highly efficient technology, coupled with a well-established and widely recognized analytical technique, for converting lemon peel waste into multifunctional and eco-sustainable cosmetic ingredients.

Received 15th January 2026,
Accepted 8th April 2026

DOI: 10.1039/d6an00048g

rsc.li/analyst

Introduction

The “upcycling” paradigm has gained increasing attention to reuse waste. Unlike traditional recycling, which generally disposes of the original material after use, upcycling focuses on preserving, and in many cases enhancing, the functional qualities of organic by-products through their reuse in high-value applications. Therefore, the concept of upcycling is closely

aligned with the principles of sustainability, contributing to waste reduction, innovation in product design and better environmental management. Beyond its environmental advantages, this approach is now being successfully applied in industrial sectors, particularly where the demand for natural and sustainable ingredients keeps growing.

The food processing industry contributes significantly to the production of organic waste, which is usually discharged into landfills or incinerated, leading to considerable economic and environmental impacts. By-products from the agro-food industry, including peels, seeds, stems, wastewater and pulp, can account for over 40% of the total biomass of some plant foods, such as citrus fruits, papaya, pineapple and asparagus.¹

Among the most promising raw materials, citrus by-products, particularly lemon peels, are abundant, low cost, and rich in valuable phytochemicals.²

Citrus plants (genus *Citrus*, family Rutaceae), also known as agrumes, are some of the world’s major fruit crops with global availability and significantly contributing to the human diet.

^aDepartment of Pharmacy, School of Medicine and Surgery, University of Naples Federico II, Via D. Montesano, 49, I-80131 Naples, Italy. E-mail: grumetto@unina.it

^bConsorzio Interuniversitario INBB, Viale Medaglie d’Oro, 305, I-00136, Rome, Italy

^cNational Laboratory on Endocrine Disruptors, National Institute of Biostructures and Biosystems (INBB), Via dei Carpegna, 19-00165 Roma, Italy

^dMATER Soc. Cons. a r.l., Via Breccia a S. Erasmo 114, 80100 Naples, Italy

^eDepartment of Molecular Medicine and Medical Biotechnologies, School of Medicine and Surgery, University of Naples Federico II, Via S Pansini, 5, 80131 Naples, Italy

^fDepartment of Agricultural Sciences, University of Naples Federico II, Piazza Carlo di Borbone 1, 84055 Portici Naples, Italy

[†]These authors contributed equally to this work.



They are rich in vitamin C, vitamins B (thiamine, pyridoxine, niacin, riboflavin, pantothenic acid, and folate) and phytochemicals, such as carotenoids, flavonoids, and limonoids.³ Citrus processing generates large quantities of agricultural by-products, which can be valuable sources of bioactive compounds and essential oils.⁴ Among them, lemon (*Citrus limon*) residues are particularly attractive due to their abundance and phytocompound richness.

Essential oils (EOs) are composed of many valuable mixtures of hydrocarbons, oxygenated compounds and non-volatile residues, including terpenes, sesquiterpenes, aldehydes, alcohols, esters and sterols. EOs from *Citrus limon* are well known due to their strong antimicrobial, antioxidant and anti-inflammatory properties and have several potential applications, including their use as food additives, preservatives against spoilage, pharmaceuticals and cosmeceuticals.⁵

In Southern Italy, the Campania region stands out for its extensive citrus cultivation. A significant portion of this cultivation is dedicated to limoncello production, a traditional liqueur made by steeping lemon peels in ethanol. Producing just one litre of limoncello requires approximately fifteen lemons, resulting in a substantial amount of exhausted lemon peel waste.

In this scenario, our research aimed to develop an important resource for the circular economy, to create a virtuous cycle of by-product reuse, and to propose the valorisation of wastes derived from limoncello production using supercritical fluid extraction (SFE). EOs obtained from the extraction were characterized for their chemical composition and evaluated for their activity *in vitro* and *in vivo*.

In recent years, green extraction techniques have emerged as sustainable alternatives to conventional methods for isolating phytochemicals (PCs) by reducing or eliminating the use of organic solvents and operating under energy-saving conditions, allowing the selective isolation of thermolabile bioactive molecules.

Accordingly, the present study aimed at valorising exhausted lemon peels as a sustainable source of functional EOs through green extraction technology for potential cosmetic applications. This work presents a novel contribution by demonstrating the valorisation of exhausted lemon peel waste, a matrix that has been largely overlooked compared to fresh citrus by-products. In particular, the application of supercritical fluid extraction and the integration of chemical characterization with both *in vitro* and *in vivo* bioactivity assessment reinforce their relevance within a circular economy framework.

Materials and methods

Citrus limon L. (var. Sfusato Amalfitano) is one of the six Italian lemon cultivars recognized with Protected Geographical Indication (IGP), distinguished by its unique local microclimate and organic farming practices. The spent peels remaining after the alcoholic extraction used to produce the liqueur were supplied by *Distillerie Nastro D'Oro*, a local lemon-liqueur

manufacturer in Naples, Italy and stored at $-20\text{ }^{\circ}\text{C}$ until the extraction procedures were carried out. Frozen samples (18 kg of fresh lemon peel matrix) were subjected to freeze-drying using a Buchi Lyovapor L-300 system and were subsequently ground with a food grinder. EOs from the *Citrus limon* peels were extracted through supercritical CO_2 extraction (SC-CO_2) using a pilot-scale supercritical CO_2 extractor (SCFN-L7®, SepareCo, Turin, Italy) equipped with a 7 L extraction vessel. Food-grade CO_2 was supplied by Nippon Gases (Milan, Italy). The extractor was equipped with PLC (programmable logic controller) and SCADA (supervisory control and data acquisition) systems to ensure accurate process control and reproducibility. The extraction variants were obtained by carefully modulating the operational parameters, such as pressure, temperature, and CO_2 flow, with the aims of optimizing both the yield and the quality of the extracts, investigating the impact of individual operating parameters on yield, and performing the extraction at pressures of 200–300 bar, temperatures of $50\text{ }^{\circ}\text{C}$ – $60\text{ }^{\circ}\text{C}$, and CO_2 flow rates of $10\text{--}20\text{ kg h}^{-1}$. The operating conditions for each variant are reported in Table 1.

Each extraction trial was conducted in duplicate to ensure the reproducibility of the process and the reliability of the results. The EO fractions were recovered at the end of each process, with only one EO fraction for each condition stored in amber glass vials at $4\text{ }^{\circ}\text{C}$ for subsequent analyses.

The yield of extraction was calculated as follows:

$$\text{Yield} = \frac{\text{matrix quantity (g)}}{\text{extract quantity (g)}} \times 100,$$

where matrix quantity refers to grams of matrix weighed analytically and subjected to extraction and extract quantity is the weight of the final collected eluate.

Gas chromatography-mass spectrometry analysis (GC-MS)

An Agilent 6850 Series II gas chromatograph equipped with a single-quadrupole mass selective detector (Agilent 5973 Network) was employed. Ionization was carried out by electron impact (EI) at 70 eV, with the instrument scanning masses between 40 and 350 m/z . Separation was performed on a DB-5 ms capillary column (5% phenyl–95% methylpolysiloxane; $30\text{ m} \times 0.25\text{ mm i.d.}$; $0.25\text{ }\mu\text{m}$ film thickness). Helium served as the carrier gas, at a flow rate of 1.0 mL

Table 1 Operational conditions for the supercritical CO_2 extraction of *Citrus limon* peels

SFE conditions			
Extract	Pressure (bar)	Temperature ($^{\circ}\text{C}$)	Flow (kg h^{-1})
EO-A	200	60	20
EO-B	300	60	10
EO-C	300	50	20
EO-D	300	50	10
EO-E	200	50	10
EO-F	200	60	10



min⁻¹. The injector operated at 280 °C in split mode (9 : 1). A 5 µL aliquot of each EO was introduced into the system using an Agilent 7683 Automatic Liquid Sampler.

The oven temperature program started at 50 °C and was increased to 300 °C at a rate of 10 °C min⁻¹. Identification of the EO constituents relied on matching retention times and mass spectra with entries in the National Institute of Standards and Technology Library (NIST) 2020 MS database. Each sample was injected in triplicate, and the results are reported as the mean relative percentage composition.

Study design

The study was structured as a multi-phase experimental protocol encompassing the supercritical CO₂ extraction of EOs from exhausted lemon peels, preclinical investigations using human skin cell models (HaCaT cells) and cell-free biochemical systems, and a pilot randomized short-term clinical trial. Cell biocompatibility and preliminary assessments of antioxidant and antimicrobial properties were also carried out. Specifically, cell viability was evaluated through the MTT assay on human keratinocytes (HaCaT cell line), while antioxidant potential was determined through ABTS and DPPH radical scavenging assays. Antimicrobial activity was assessed by the disk diffusion assay (DDA) and minimum inhibitory concentration (MIC) methods against representative Gram-positive and Gram-negative bacterial strains.

The clinical phase consisted of a randomized, monocentric, double-blind, placebo-controlled short-term study conducted on healthy human volunteers. The primary endpoint was the assessment of cutaneous tolerability of the cosmetic formulations through a 48 hour occlusive patch test, while secondary outcomes included instrumental evaluations of skin hydration, softness, and elasticity using standardized, non-invasive probes. Skin parameters were assessed at baseline (T_0) and after 1 hour (T_{1h}) following topical application of the tested formulations. This approach is consistent with established cosmetic testing protocols aimed at detecting rapid, instrumentally measurable changes in skin parameters (e.g., hydration and elasticity), while minimizing inter-individual variability and ensuring high sensitivity in capturing early functional responses of the formulations.

HaCaT cell culture

HaCaT cells, an immortalized human keratinocyte cell line obtained from ATCC, were cultured in DMEM (Invitrogen) supplemented with 10% foetal bovine serum (FBS, Cambrex), 2 mM L-glutamine, penicillin (100 U mL⁻¹, Sigma-Aldrich), and streptomycin (100 µg mL⁻¹). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂ and seeded at a density of 2–4 × 10⁴ cells per cm² and allowed to grow to approximately 80%–90% confluence.

Assessment of EO-A and EO-B biocompatibility

The biocompatibility of two selected essential oil, EO-A and EO-B, extracts was assessed through the calculation of a cell survival index derived from the combined evaluation of cell

viability and automatic cell count.⁶ Cells were seeded in 96-well plates at a density of 104 cells per well and allowed to grow for 24 h. Subsequently, the cells were treated with EO-A and EO-B over a concentration range from 0–1000 µg mL⁻¹ for 48 h. DMSO was used as a vehicle to solubilize the extracts. DMSO toxicity was evaluated in control cultures at a concentration ranging from 0.025% to 0.5% v/v to exclude interference with cell viability. The final DMSO concentration in all treatments did not exceed 0.5%. Cell viability was evaluated using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, a colorimetric assay based on the ability of active mitochondria to convert the yellow MTT solution into insoluble purple formazan, evaluating cellular mitochondrial dehydrogenase activity levels. The assay was performed according to the manufacturer's instructions (Sigma-Aldrich, cat. no. 475989). The absorbance was measured at 550 nm using a microplate reader (Thermo Fisher). Cell number was determined using a TC20 automated cell counter (Bio-Rad, Milan, Italy), which provides accurate and reproducible total cell counts cells and a live/dead ratio through trypan blue exclusion assay. The assay was performed according to the manufacturer's instructions (1450021 Bio-Rad).

Antioxidant activity evaluation

Since no single assay can accurately reflect the wide range of antioxidant properties in complex systems such as EOs, their antioxidant activity was assessed using two complementary methods: the DPPH and ABTS assays.

DPPH radical scavenging assay

For free radical scavenging activity, 30 µg of EO-A or EO-B was incubated in 0.4 mL of 0.1 M Tris-HCl buffer (pH 7.5 ± 0.1) and 0.5 mL of 0.3 mM DPPH in the dark at room temperature (20 °C ± 2 °C) for 20 min. The absorbance (A_{sample}) was measured at 517 nm using a PerkinElmer Lambda 25 UV/Vis spectrometer and compared to a reference (A_{control}) consisting of 80% methanol, 0.1 M Tris-HCl buffer, and 0.5 mL of 0.3 mM DPPH, which served to monitor the maximum radical stability.

The scavenging percentage was calculated using the following formula:

$$\% \text{ DPPH scavenging} = (1 - A_{\text{control}}/A_{\text{sample}}) \times 100.$$

To ensure a precise quantitative assessment, results were expressed as IC₅₀ values (the concentration required to inhibit 50% of the DPPH radical). These values were derived from a dose–response calibration curve generated by testing each EO at multiple concentrations. Ascorbic acid was utilized as a positive control to validate the sensitivity of the assay and provide a benchmark for high antioxidant potency.

ABTS radical cation decolorization assay

The ABTS assay was performed by reacting a 0.007 M ABTS solution with 2.45 mM potassium persulfate for 16 h in the



dark to produce the $\text{ABTS}^{+\cdot}$ radical cation. This stock was diluted with ethanol to an absorbance of 0.7 ± 0.02 at 734 nm. For the analysis, 4 mg of each EO (EO-A and EO-B) was dissolved in 2 mL of DMSO solution (5%), and 100 μL of this solution was added to 1.9 mL of the $\text{ABTS}^{+\cdot}$ working solution.

After 2 min of reaction in the dark, the absorbance was measured at 734 nm using a Varioskan multiplate reader. The antioxidant capacity was quantified as Trolox equivalent antioxidant capacity (TEAC). This value was calculated by interpolating the absorbance data into a linear calibration curve of Trolox (a water-soluble vitamin E analogue) prepared in the range of 0.1–100 mg L^{-1} ($R^2 = 0.998$).

Quality control and validation

All experiments were performed in triplicate to ensure reproducibility. To confirm the accuracy of the multiwell system and the stability of the radicals, negative controls (solvents without EOs) and reference standards were processed alongside the samples, ensuring that all data met the required validation criteria for precision and linearity. Since no single assay can accurately reflect the wide range of antioxidant properties in complex systems such as EOs, their antioxidant activity was appraised using two complementary methods: the DPPH and ABTS assays. For the free radical scavenging activity, 30 μg of each EO sample was incubated in 0.4 mL of 0.1 M Tris-HCl buffer (pH 7.5 ± 0.1) and 0.5 mL of 0.3 mM DPPH in the dark at room temperature ($20 \text{ }^\circ\text{C} \pm 2 \text{ }^\circ\text{C}$) for 20 min. The absorbance (A_{sample}) was measured at 517 nm using a PerkinElmer Lambda 25 UV/Vis spectrometer (PerkinElmer Inc., Waltham, MA, USA) and compared to a reference (A_{control}) consisting of 80% methanol, 0.1 M Tris-HCl buffer, and 0.5 mL of 0.3 mM DPPH. The scavenging percentage was calculated as % DPPH scavenging $(1 - A_{\text{sample}}/A_{\text{control}}) \times 100$, and the results were further expressed as IC_{50} values (the concentration required to inhibit 50% of the DPPH radical) derived from a dose-response curve, using ascorbic acid as a positive control. The ABTS assay was performed by reacting a 0.007 M ABTS solution with 2.45 mM potassium persulfate for 16 h in the dark to produce the $\text{ABTS}^{+\cdot}$ radical cation. This stock was diluted with ethanol to an absorbance of 0.7 ± 0.02 at 734 nm. For the analysis, 4 mg of each EO was dissolved in 2 mL of DMSO solution (5%) and 100 μL of this solution were added to 1.9 mL of the $\text{ABTS}^{+\cdot}$ working solution. After 2 min of reaction in the dark, the absorbance was measured at 734 nm using a Varioskan multiplate reader (Diatron MI PLC, Budapest, Hungary). Results were expressed as Trolox equivalent antioxidant capacity (TEAC) based on a linear calibration curve of Trolox in the range of 0.1–100 mg L^{-1} ($R^2 = 0.998$). All experiments were performed in triplicate to ensure reproducibility, and data were validated against reference standards to confirm the accuracy of the multiwell system.

Antibacterial activity evaluation

The antibacterial activity of EO-A and EO-B was evaluated using the disk diffusion assay (DDA) and broth microdilution method (MIC). The bacterial strains used in this work include

Staphylococcus aureus ATCC 6538P, *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC19115, *Salmonella typhi* ATCC14028, *Escherichia coli* ATCC 33780 and *Bacillus cereus* ICE170.

Three independent experiments were performed for each DDA and MIC value.

The DDA was performed according to the National Committee for Clinical Laboratory Standards (NCCLS) standard method, using 30 μg of each tested sample. The inoculum of the colonies was suspended in sterile saline and the inoculum was adjusted to 10^8 CFU mL^{-1} (0.5 McFarland standard), which is equivalent to 50% transmittance at 580 nm (Coleman model 6120, Maywood, IL, USA). Subsequently, 200 μL of the bulk suspension was placed onto the surface of Mueller–Hinton agar. Disks (6.0 mm diameter) were impregnated with 25 μL of a 1.2 mg mL^{-1} solution of each sample and placed on the agar Petri dish and incubated at $37 \text{ }^\circ\text{C}$ for 24 h. Sterile distilled water (25 μL) and ampicillin and clavulanic acid (30 μg) were used the negative control and positive references, respectively. The total diameters were measured by considering the size of the inhibition zones. Each experiment was performed in triplicate. The second antimicrobial assay was performed using the broth microdilution method in Mueller–Hinton broth medium using sterile 96-well polystyrene microtiter plates. The microbial inoculum size used was $1 \times 10^6 \text{ CFU mL}^{-1}$ (NCCLS, 1993). Two-fold serial dilutions of different samples were carried out to obtain concentrations ranging from 10 to 1000 $\mu\text{g mL}^{-1}$. Then, the bacterial cells were inoculated from an overnight culture at a final concentration of about $5 \times 10^5 \text{ CFU mL}^{-1}$ per well and incubated with different samples overnight at $37 \text{ }^\circ\text{C}$. The minimal inhibitory concentration (MIC) values, that is, the lowest concentration of material that inhibited the growth of microorganisms after 24 h of incubation at $37 \text{ }^\circ\text{C}$, were determined by measuring spectrophotometric absorbance at 570 nm using a Varioskan multiplate reader.

Statistical analysis and data processing

To ensure the reliability and reproducibility of the results, all antimicrobial tests (DDA and MIC) were performed in triplicate ($n = 3$) across three independent experimental sessions. Data are expressed as mean \pm standard deviation (SD). Statistical significance between the antibacterial activities of EO-A, EO-B, and the positive controls was evaluated using one-way analysis of variance (ANOVA), followed by Tukey's honest significant difference (HSD) *post-hoc* test to identify specific differences between the groups. All statistical analyses were performed with a significance threshold set at $p < 0.05$ using the XLSTAT software (Addinsoft, Paris, France).

EOs-based topical formulation preparation

Oil-in-water (o/w) emulsions were prepared using the hot emulsification method. According to Table 2, a solution consisting of deionized water (71.78% w/w) and disodium EDTA (0.10% w/w) was heated with magnetic stirring ($200 \pm 25 \text{ rpm}$) from $60 \text{ }^\circ\text{C}$ to $70 \text{ }^\circ\text{C}$ until complete dissolution.



Table 2 Composition of EO-containing o/w emulsions

Phase	INCI name	Function	% w/w
A	Water	Solvent	71.78
A	Disodium EDTA	Chelating agent	0.10
B	Glyceryl stearate SE	Emulsifying	6.00
B	Cetyl stearyl alcohol	Viscosity controlling	3.00
B	Vegetable stearin	Emulsifying	2.00
B	Butylated hydroxytoluene	Antioxidant	0.02
B	Ethylhexyl stearate	Emollient	3.00
B	Isopropyl myristate	Emollient	2.00
B	Dicaprylyl ether	Emollient	1.00
C	<i>Citrus limon</i> (lemon) peel extract	Active ingredient	1.00
C	Caprylic/capric triglyceride	Emollient	4.00
C	Dimethicone	Emollient	1.00
C	Glycerin	Humectant	4.00
C	Phenoxyethanol (and) ethylhexylglycerin	Preservative	1.00
C	Sodium hydroxide	pH modifier	0.10

The oil phase (phase B), composed of glyceryl stearate SE (6.00% w/w), cetyl stearyl alcohol (3.00% w/w), vegetable stearin (2.00% w/w), butylated hydroxytoluene (0.02% w/w), ethylhexyl stearate (3.00% w/w), isopropyl myristate (2.00% w/w), and dicaprylyl ether (1.00% w/w), was heated separately to 75 °C until complete melting of the solid components.

The oil phase was then slowly added to the aqueous phase under high-shear mixing (6000 rpm for 5 min) to obtain a homogeneous emulsion. The system was subsequently cooled under moderate stirring to below 40 °C.

At this stage, phase C, containing the preservative system (phenoxyethanol and ethylhexylglycerin, 1.00% w/w), caprylic/capric triglyceride (4.00% w/w), dimethicone (1.00% w/w), glycerin (4.00% w/w), and the selected essential oil (1.00% w/w), was added under low-shear mixing (2000 rpm for 2 min).

Finally, the emulsion was adjusted to pH 5.5 using a 30% sodium hydroxide solution (0.10% w/w), and the final product was left to stabilize for 24 h before analysis.

Clinical trials

The randomized, double-blind, placebo-controlled efficacy study was conducted in accordance with the quality management system for cosmetic clinical trials (IMQ S.p.A, Milan, Italy) and complied with the UNI EN ISO 9001 standard (European Commission, 2018). The study protocol adhered to the principles of the Declaration of Helsinki, (World Medical Association, 2013) and the COLIPA Guidelines for the Evaluation of the Efficacy of Cosmetic Products (Cosmetics Europe, 2008b). Considering the non-invasive nature of the procedures and the inclusion of healthy adult volunteers, ethical committee approval was not required.⁷ All participants provided written informed consent prior to enrolment.

Forty healthy volunteers (aged 20–70 years), regular users of cosmetic products, were enrolled and randomly assigned to one of the three treatment groups (group 1: topical formula containing 1% w/w EO-A; group 2: topical formula containing 1% w/w EO-B and group 3: placebo). Following a seven-day

wash-out period, participants applied approximately 2 mg of the assigned formulation evenly to their entire facial area for 1 hour. All measurements were performed at baseline (T_0) and after 1 hour ($T_{1\text{h}}$) by the same trained operator, following a 30 minute acclimatization period under controlled environmental conditions (20 °C ± 2 °C; 50% ± 5% relative humidity).

The primary endpoint was the evaluation of cutaneous tolerability, assessed through a 48 hour occlusive patch test performed under medical supervision. The test was conducted on intact skin of the volar forearm to evaluate the potential irritant properties of the formulations, in compliance with EEC Directive 76/768. The Finn Chambers® AQUA patch system (Epitest Ltd, Finland) was used according to standardized procedures.⁸ Reactions were scored based on the morphological criteria established by the International Contact Dermatitis Research Group, with an irritancy threshold set at 1.5 on a 0–3 visual scale. No adverse skin reactions or signs of irritation were observed, confirming the good tolerability profile of both formulations.

The secondary endpoint was the evaluation of short-term effects on skin hydration and mechanical properties. Instrumental assessments included corneometry (skin hydration), skin softness, and skin elasticity, measured using widely recognized, non-invasive devices. Specifically, skin hydration was assessed using a Corneometer® CM 825, skin softness with an Indentometer® IDM 800, and skin elasticity with a Cutometer® MPA 580. The latter was evaluated using the R^2 parameter (gross elasticity), calculated as U_a/U_f , which is considered a robust indicator of the ability of the skin to recover its original shape after deformation and reflects the condition of collagen, elastin, and extracellular matrix components.⁹

Statistical analysis in clinical trials

A total of 40 participants were enrolled in the clinical trial and randomly assigned to two treatment groups, with twenty subjects per group (EO-A and EO-B). This sample size was considered adequate to ensure sufficient statistical power for detecting clinically relevant differences between treatments. Intra-group comparisons, expressed as mean percentage changes from the baseline, were analysed using the paired Student's *t*-test. A *p*-value <0.05 was considered statistically significant. In addition to *p*-values, 95% confidence intervals (CI) were calculated for all primary outcome measures to provide an estimate of the precision and reliability of the observed effects. Effect sizes were also determined using Cohen's *d*, allowing quantitative interpretation of the magnitude of treatment-related changes beyond statistical significance. All statistical analyses were performed using the SAS® software, version 9.4 (SAS Institute Inc., Cary, NC, USA).

Results and discussion

SC-CO₂ and GC-MS analysis

The extraction of EOs from lemon by-products is a critical process where the method chosen directly impacts the chemi-



cal integrity, aroma, and bioactivity of the oil. While conventional methods remain the industrial standard, there is a significant shift toward “green” technologies to improve efficiency and preserve quality.¹⁰ SFE is considered safe and sustainable, offering advantages such as adjustable solvating power, low-temperature operation, and complete removal of solvent residues.¹¹ Besides, carbon dioxide, with a critical point of 31.1 °C and 73.8 bar, is ideal for extracting lipophilic components such as essential oils while preserving their structural integrity. Its non-toxic, inert, and recyclable characteristics further contribute to the environmentally friendly profile of the process.

The extraction process is influenced by several matrix characteristics, such as particle size, shape, surface area, porosity, and moisture, because CO₂ diffusion into the matrix depends on these features; therefore, pretreatment is usually recommended.¹² Indeed, before extraction, the lemon peels were subjected to freeze-drying and then ground. Moreover,

different combinations of operational parameters, particularly pressure and the influence of CO₂ solubility, and consequently the affinity between CO₂ and the bioactive compounds in the matrix, were investigated. This directly impacts the compositional profile of the obtained extracts, as extraction efficiency and compound selectivity depend on these conditions.

The quantitative yields of the extracts were influenced by the operational conditions, as presented in Table 3.

A comparison between extracts C and D demonstrates the critical impact of flow rate on performance. At a constant 300 bar and 50 °C, the lower flow rate of 10 kg h⁻¹ (EO-D) outperformed the 20 kg h⁻¹ flow rate (Extract C), yielding 10.18% versus 8.20%. These findings suggest that the slower passage of the solvent allows for more thorough extraction, making flow rate a key factor in yield optimization. Following SFE optimization, the obtained EOs were characterized by GC-MS to map their chemical profiles. Compound identification was performed by matching mass spectra with the NIST 2020 MS Database. While the use of authentic standards is the gold standard for absolute quantification, this study opted for library-based qualitative profiling. This approach was deemed the most appropriate and efficient for establishing the chemical fingerprint of the extracts and evaluating their suitability for cosmetic formulations. In the context of a circular economy, the primary objective was to determine the functional ingredients of the recycled by-product rather than to achieve comprehensive fine characterization.¹³

Tables 4 and 5 present the chemical compositions of the two selected lemon EOs, *i.e.* EO-A and EO-B, respectively. The

Table 3 Quantitative yields of the supercritical CO₂ extracts from *Citrus limon* peels under different operational conditions

Extract	Yield (g extract per g matrix)
EO-A	2.22%
EO-B	3.70%
EO-C	8.20%
EO-D	10.18%
EO-E	2.26%
EO-F	2.61%

Table 4 Chemical composition of the lemon EO-A. Extraction parameters: pressure of 200 bar; flow rate of 20 kg h⁻¹; and temperature of 60 °C. Extraction yield: 2.22%

Retention time	CAS number	IUPAC name	Chemical class	Abundance %
5.999	594-09-2	Trimethylphosphane	Organophosphorus compound	2.85 ± 0.1
6.293	470-82-6	1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octane	Bicyclic ether	0.39 ± 0.05
6.880	111-87-5	Octan-1-ol	Primary alcohol	0.56 ± 0.07
7.367	78-70-6	3,7-Dimethylocta-1,6-dien-3-ol	Monoterpenoid alcohol	1.93 ± 0.5
8.650	20126-76-5	4-Methyl-1-propan-2-ylcyclohex-3-en-1-ol	Monoterpenoid alcohol	2.74 ± 0.02
8.868	10482-56-1	2-[4-Methylcyclohex-3-en-1-yl]propan-2-ol	Monoterpenoid alcohol	9.43 ± 0.05
9.414	106-25-2	(2Z)-3,7-Dimethylocta-2,6-dien-1-ol	Monoterpenoid alcohol	8.97 ± 0.07
9.590	106-26-3	(2Z)-3,7-Dimethylocta-2,6-dienal	Monoterpenoid aldehyde	4.21 ± 0.03
9.791	106-24-1	(2E)-3,7-Dimethylocta-2,6-dien-1-ol	Monoterpenoid alcohol	9.96 ± 0.03
10.026	5392-40-5	(2E)-3,7-Dimethylocta-2,6-dienal	Monoterpenoid alcohol	6.22 ± 0.05
11.007	3564-98-5	8-Hydroxymenthol	Monoterpenoid aldehyde	1.46 ± 0.02
11.309	141-12-8	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate (Z)	Monoterpenoid diol	1.75 ± 0.1
11.561	16409-44-2	3,7-Dimethylocta-2,6-dienyl acetate	Monoterpenoid ester	0.63 ± 0.05
11.905	121-33-5	4-Hydroxy-3-methoxybenzaldehyde	Monoterpenoid ester	0.78 ± 0.09
14.338	617-05-0	Ethyl 4-hydroxy-3-methoxybenzoate	Phenolic aldehyde	0.52 ± 0.03
15.260	134-96-3	4-Hydroxy-3,5-dimethoxybenzaldehyde	Aromatic ester	0.55 ± 0.08
16.812	3622-84-2	N-Butylbenzenesulfonamide	Phenolic aldehyde	6.06 ± 0.02
18.532	57-10-3	Hexadecanoic acid	Sulfonamide	0.92 ± 0.02
18.851	120-08-1	6,7-Dimethoxychromen-2-one	Coumarin derivative	1.26 ± 0.06
19.002	487-06-9	5,7-Dimethoxychromen-2-one	Coumarin derivative	9.56 ± 0.01
20.352	112-80-1	(Z)-Octadec-9-enoic acid	Fatty acid	3.14 ± 0.01
20.529	57-11-4	Octadecanoic acid	Fatty acid	0.49 ± 0.05
23.364	1000130-81-0	11,13-Dimethyl-12-tetradecen-1-ol acetate	Fatty acid	0.91 ± 0.01
25.679	24724-52-5	4-(2,3-Dihydroxy-3-methylbutoxy)furo[3,2-g]chromen-7-one	Long-chain aliphatic ester	1.72 ± 0.03
26.694	55806-41-2	9-(2-Hydroxy-3-methyl-3-butenyloxy)-4-methoxyfuro[3,2-g]chromen-7-one	Furanocoumarin derivative	0.75 ± 0.05



Table 5 Chemical composition of the lemon EO-B. Extraction parameters: pressure of 300 bar; flow rate of 10 kg h⁻¹; and temperature of 60 °C. Extraction yield: 3.70%

Retention time	CAS number	IUPAC name	Chemical class	Abundance %
5.773	1604-28-0	(3 <i>E</i>)-6-Methylhepta-3,5-dien-2-one	α,β-Unsaturated ketone	0.71 ± 0.03
5.807	86951-58-8	1-(2-Methylenecyclopropyl)cyclopentanol	Cyclopentanol derivative	1.37 ± 0.04
7.442	78-70-6	3,7-Dimethylocta-1,6-dien-3-ol	Monoterpenoid alcohol	2.84 ± 0.03
8.709	562-74-3	4-Methyl-1-propan-2-ylcyclohex-3-en-1-ol	Monoterpenoid alcohol	3.04 ± 0.02
8.936	10482-56-1	2-[4-Methylcyclohex-3-en-1-yl]propan-2-ol	Monoterpenoid alcohol	14.02 ± 0.04
9.456	106-25-2	(2 <i>Z</i>)-3,7-Dimethylocta-2,6-dien-1-ol	Monoterpenoid alcohol	9.48 ± 0.04
9.624	106-26-3	(2 <i>Z</i>)-3,7-Dimethylocta-2,6-dienal	Monoterpenoid aldehyde	1.40 ± 0.01
9.833	106-24-1	(2 <i>E</i>)-3,7-Dimethylocta-2,6-dien-1-ol	Monoterpenoid alcohol	10.14 ± 0.01
10.051	5392-40-5	(2 <i>E</i>)-3,7-Dimethylocta-2,6-dienal	Monoterpenoid aldehyde	1.95 ± 0.3
11.343	1000360-39-7	(2 <i>E</i>)-3,7-Dimethylocta-2,6-dienyl-2-methylbutanoate	Monoterpenoid ester	0.52 ± 0.02
16.880	3622-84-2	<i>N</i> -Butylbenzenesulfonamide	Sulfonamide	3.64 ± 0.1
18.633	57-10-3	Hexadecanoic acid	Fatty acid	1.94 ± 0.05
18.893	120-08-1	6,7-Dimethoxychromen-2-one	Coumarin derivative	0.76 ± 0.02
19.036	487-06-9	5,7-Dimethoxychromen-2-one	Coumarin derivative	3.35 ± 0.02
20.512	60-33-3	(9 <i>Z</i> ,12 <i>Z</i>)-Octadeca-9,12-dienoic acid	Fatty acid (polyunsaturated)	10.75 ± 0.01
23.423	54766-91-5	Bicyclo[10.1.0]tridec-1-ene	Polycyclic hydrocarbon	3.95 ± 0.05
23.523	1000336-54-1	Butyl-9,12-octadecadienoate	Fatty acid ester	0.55 ± 0.03
25.260	16106-03-9	Methyl (7 <i>E</i> ,10 <i>E</i>)-hexadeca-7,10-dienoate	Fatty acid ester	1.16 ± 0.05
38.422	1000336-77-8	<i>n</i> -Propyl-9,12-octadecadienoate	Fatty acid ester	2.97 ± 0.02

rationale for selecting EO-A and EO-B from a chemical point of view was based on their low levels of potentially allergenic constituents, such as limonene, and other potential hazardous chemicals, thereby reducing sensitization risks and enhancing the overall safety of the final formulation. In this context, a higher quantitative extraction yield does not necessarily translate into a qualitative advantage.

Indeed, during limoncello manufacturing, the ethanol maceration of lemon peels leads to the extraction of volatile compounds such as *D*-limonene into the liqueur, leaving the residual peel matrix considerably depleted in these compounds. This depletion is advantageous for cosmetic applications. *D*-Limonene, upon air exposure and subsequent oxidation, produces hydroperoxides that are recognized as potent sensitizers.¹⁴

Despite the depletion of high-volatility terpenes, both lemon EOs retained a complex phytochemical composition, mainly characterized by monoterpenes and oxygenated monoterpenes, with additional contributions from sesquiterpenes and coumarin derivatives.

SFE of exhausted biomass produces non-conventional EO profiles, yielding compositions markedly enriched in oxygenated monoterpenes. This composition differs from typical profiles obtained by conventional methods, as SFE is particularly effective in recovering residual, more polar and matrix-bound compounds that are not easily accessible through traditional techniques.

Moreover, the presence of coumarin suggests that SFE enhanced the solubilization of higher molecular weight and less volatile constituents. Importantly, the relatively high abundance of oxygenated compounds indicates that SC-CO₂ extraction minimized thermal and oxidative transformations, which are typically associated with conventional extraction methods.

Overall, these results highlight that, when applied to exhausted citrus matrices, SFE extraction shifts the volatile

profile from hydrocarbon-dominated compounds to oxygenated and semi-volatile compounds, enabling the recovery of a distinct fraction with potential added value and a composition less affected by artefact formation.

The comparison between EO-A and EO-B highlights a crucial trade-off between total yield and the concentration of active bioactive markers. Indeed, while EO-B resulted in a higher extraction yield (3.70%) compared to EO-A (2.22%), the latter showed a significantly higher relative abundance of key coumarin derivatives, such as citropten (5,7-dimethoxycoumarin) and scoparone (6,7-dimethoxycoumarin). Furthermore, EO-A was significantly more concentrated in oxygenated monoterpenes, particularly monoterpenoid alcohols (~30–35%) and phenolic compounds, whereas EO-B showed a higher percentage of fatty acids and their esters, for instance, linoleic acid reached 10.75%. The higher pressure used for EO-B increased the extraction of bulk lipids, which explains the higher overall yield. Coumarins are associated with antioxidant and dermo-protective action,¹⁵ while monoterpene alcohols and esters are known to contribute not only to olfactory and sensorial properties, but also to antimicrobial, antioxidant, and anti-inflammatory effects.¹⁶

Additionally, coumarin derivatives were significantly higher in EO-A (~10–11%) compared to EO-B (~4%), suggesting the selective extraction of these semi-polar bioactive compounds at lower pressure conditions.

Conversely, increasing the pressure to 300 bar enhanced the extraction of less volatile, higher molecular weight compounds, including fatty acids and polycyclic hydrocarbons.

The comparative analysis of the terpenic profiles reveals a significant divergence between the data reported in the literature and our experimental results. According to a recent review,¹⁰ conventional and green extraction techniques typically preserve a high hydrocarbon fraction, with *D*-limonene dominating the bioactive profile at concentrations ranging



from 46% to 76%, followed by significant percentages of gamma-terpinene and beta-pinene. In our study, these specific monoterpenes are either absent or present only in trace amounts. This discrepancy is primarily due to the unique nature of our starting matrix. Unlike the fresh peels used in conventional studies, our raw material consists of lemon peels that have already undergone exhaustive ethanol infusion for liqueur production. Nevertheless, despite their lower limonene concentration, the potential for these extracts remains significant.

Biological cell toxicity investigation

In addition to qualitative–quantitative chemical characterization, the selection of EO-A and EO-B was further supported by a biocompatibility-driven criterion. Specifically, both extracts were evaluated on human keratinocytes (HaCaT cell line), showing no cytotoxic effects across the tested concentration with cell viability consistently exceeding 80%.

This biological screening allowed the exclusion of potentially irritating or cytotoxic extracts and ensured the selection of samples with favourable safety profiles for topical application. Therefore, the final choice of EO-A and EO-B was based not only on their extraction yield and chemical composition, but also on their demonstrated *in vitro* biocompatibility, strengthening their suitability as cosmetic ingredients.

To evaluate the biocompatibility of the two selected EOs, namely EO-A and EO-B, obtained from ethanol-exhausted *Citrus limon* (L.) peels, targeted *in vitro* bioscreening assays were performed on immortalized human keratinocytes (HaCaT). The extracts were tested at concentrations ranging from 0 to 1000 $\mu\text{g mL}^{-1}$ for 48 h. The resulting data, reported in Fig. 1 as concentration–effect curves of the “cell survival index”, showed no significant changes following *in vitro* treatment compared with untreated cells. No biological effects or interference with cell viability or proliferation were observed under the tested conditions. Overall, these findings suggest favourable safety profiles for both EO-A and EO-B, supporting their biocompatibility in the skin cell model. Extracts are considered biocompatible if a cell survival index of at least 80% relative to untreated control cells was observed, including at the highest tested concentration after 48 h of exposure. This threshold was adopted as an additional selection criterion for EO-A and EO-B.

Antioxidant activity

As previously reported, based on comprehensive MTT cytotoxicity screening conducted on the two selected EOs derived from ethanol-exhausted *Citrus limon* (L.) peels, and considering their distinctive chemical profiles and extraction yields, EO-A and EO-B were selected as representative samples for further biological characterisation and subsequent evaluation in a clinical trial.

Antioxidant substances provide significant protection against various diseases related to oxidative stress, typically induced by free radicals such as reactive nitrogen species (RNS) and reactive oxygen species (ROS).¹⁷ Consequently, the

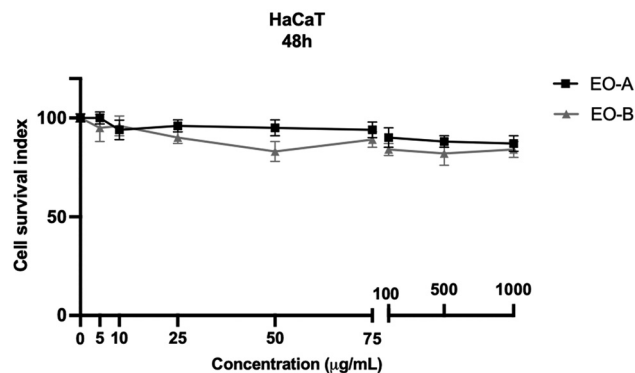


Fig. 1 Preclinical biocompatibility assessment of EO-A and EO-B, the essential oils derived from ethanol-exhausted *Citrus limon* (L.) peels, in HaCaT cells. Concentration–response curves for the cell survival index for HaCaT cells after 48 h of treatment with EO-A and EO-B over a concentration range of 0–1000 $\mu\text{g mL}^{-1}$. Results are expressed as a percentage of the untreated control cells and are reported as the mean \pm SEM of three independent experiments ($n = 15$). Statistical analysis was performed by one-way ANOVA, followed by Tukey's multiple comparison test. No statistically significant differences were observed compared with untreated control cells.

radical scavenging activities of phenolic components in natural molecules, characterized by their electron donor/acceptor behaviour, have been extensively discussed. In the ABTS assay, the antioxidant capacity was quantified as Trolox equivalent antioxidant capacity (TEAC), derived from a linear calibration curve ($R^2 = 0.998$). EO-A exhibited a TEAC value of 135.6 ± 0.01 mmol Trolox equivalents per gram (mmol TE per g), whereas EO-B reached a significantly higher value of 167.3 ± 0.09 mmol TE per g; these values were validated using Trolox as a positive reference standard. Regarding the DPPH test, the free radical scavenging activity was evaluated by determining both the percentage of inhibition and the IC_{50} value (the concentration required to inhibit 50% of the DPPH radical). At the tested concentration, EO-A achieved an inhibition of $59.0\% \pm 0.05\%$, while EO-B showed a stronger effect with $69.0\% \pm 0.08\%$ inhibition. The corresponding IC_{50} values further confirmed the superior potency of EO-B, showing a higher efficiency in neutralizing the DPPH radical compared to EO-A. Ascorbic acid was employed as a positive control, providing a benchmark for the EO performance. These results, as summarized in Fig. 2a and b and reported in Table 6, demonstrate that both samples possess significant radical scavenging capacity, although EO-B consistently displayed a higher antioxidant performance across both complementary assay systems. In the DPPH test, EO-A achieved inhibition of $59.0\% \pm 0.05\%$, while EO-B showed a stronger effect with $69.0\% \pm 0.08\%$ inhibition. These results demonstrate that both samples possess significant radical scavenging capacity, although EO-B consistently displayed a higher performance in both assay systems (Fig. 2a and b).

According to the literature, the antioxidant activity of *Citrus limon* (L.) EOs is generally attributed to their major constitu-



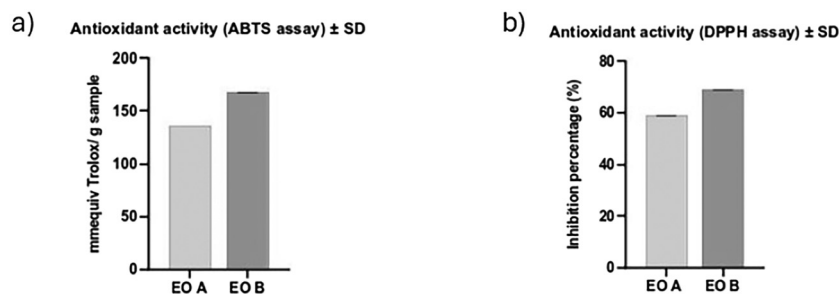


Fig. 2 Antioxidant activity of EO-A and EO-B as measured by (a) ABTS and (b) DPPG assays. Vertical bars represent the standard deviation.

Table 6 Antioxidant capacity and radical scavenging activity of the essential oils (EO-A and EO-B)

Sample	ABTS assay (mmol TE per g)	DPPH inhibition (%)	DPPH IC ₅₀ ($\mu\text{g mL}^{-1}$)
EO-A	135.6 \pm 0.01	59.0 \pm 0.05*	45.2 \pm 0.15*
EO-B	167.3 \pm 0.09*	69.0 \pm 0.08	31.8 \pm 0.11
Reference standard	Trolox	Ascorbic acid	5.4 \pm 0.03*

Results are expressed as mean \pm standard deviation (SD). The antioxidant capacity in the ABTS assay was quantified as Trolox equivalent antioxidant capacity (TEAC). The values represent the concentration required to inhibit 50% of the DPPH radical; lower values indicate higher antioxidant potency. Statistical significance was evaluated using one-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* test for multiple comparisons (*p* value < 0.05). Linear regression analysis was employed to determine the Trolox equivalent antioxidant capacity (TEAC) and to calculate the values for the DPPH radical scavenging assay.

ents, primarily monoterpenes such as limonene, β -pinene, and γ -terpinene.

The ability of β -pinene and limonene to scavenge hydroxyl radicals contributes to the reduction of oxidative reaction rates.¹⁸ D-Limonene has indeed been reported to exert measurable antioxidant effects, although oxygenated monoterpenes typically provide stronger contributions. In particular, the relatively high levels of (*Z*)-citral, α -terpinene, and α -terpineol have been associated with marked scavenging effects in DPPH assays, while γ -terpinene and (*Z*)-citral appear to be more effective in ABTS radical neutralization.¹⁹ Notably, coumarins, also known as benzopyrones, are plant-derived products with several pharmacological properties, including antioxidant and anti-inflammatory activities, among which scoparone is recognized for its potent radical scavenging properties. Its detection provides a mechanistic basis for the enhanced antioxidant activity of EO-B, as evidenced by its higher responses obtained in both the ABTS and DPPH assay systems.²⁰

Antimicrobial properties

The antimicrobial activity of both EO samples, evaluated through the disk diffusion (DDA) and minimum inhibitory concentration (MIC) assays, is quantitatively summarized in

Table 7. The samples were tested against a representative panel of Gram-positive bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus cereus*, and *Listeria monocytogenes*) and Gram-negative strains (*Salmonella typhi* and *Escherichia coli*). The most pronounced inhibition was observed against *S. typhi*, where EO-A and EO-B exhibited mean inhibition diameters of 10.8 mm and 8.7 mm, respectively. Significant activity was also recorded against *E. coli*, particularly for EO-B (10.2 mm), and against *B. cereus*, where both samples produced inhibition zones exceeding 8 mm. Conversely, lower susceptibility was observed for *E. faecalis* and *L. monocytogenes*, with inhibition zones not exceeding the disk diameter (6 mm). The MIC values strongly correlated with the DDA trends; *S. typhi* showed the highest susceptibility with MICs of 125 $\mu\text{g mL}^{-1}$ for EO-A and 100 $\mu\text{g mL}^{-1}$ for EO-B, supporting the previously noted inhibition zones. Similarly, *E. coli* demonstrated moderate sensitivity, especially to EO-B (MIC = 125 $\mu\text{g mL}^{-1}$). Both EOs showed comparable inhibitory effects against *B. cereus* (MIC = 250 $\mu\text{g mL}^{-1}$), while relatively high MIC values (>500 $\mu\text{g mL}^{-1}$) were recorded against *E. faecalis* and *L. monocytogenes*, indicating reduced efficacy. These quantitative data confirm the broader antimicrobial potential of EO-B, particularly against Gram-negative strains. To ensure assay validity, the positive control (ampicillin + clavulanic acid) yielded substantial inhibition zones (30–40 mm) and full growth inhibition at the expected concentrations, while the negative control (sterile water) showed no activity, confirming that the observed antimicrobial effects are strictly attributed to the EO samples.

In agreement with our findings, previous studies have reported that fresh citrus essential oils exhibit antibacterial activity against *B. cereus*, *E. coli* and *S. aureus*.²¹ In the present study, both EO-A and EO-B demonstrated measurable antimicrobial activity, with EO-B generally exhibiting stronger inhibitory effects, particularly against *E. coli* and *S. typhi*. The antimicrobial effects observed for the EOs investigated in this study may be ascribed to their relative abundance of aromatic and phenolic molecules. Although the mechanism underlying the antibacterial action of phenolic compounds is not yet fully elucidated, it has been hypothesised that these molecules can interact with the active sites of key bacterial enzymes, inducing irreversible alterations in membrane permeability and cell wall



Table 7 Antimicrobial activity of EO-A and EO-B. Minimum inhibitory concentration (MIC) values of EO-A and EO-B against selected bacterial strains. MIC is expressed as the lowest concentration (micrograms per milliliter, $\mu\text{g mL}^{-1}$) of the tested natural extract that inhibited visible bacterial growth. Corresponding inhibition zones from the disk diffusion assay (DDA) are reported in millimeters (mm)

	<i>S. aureus</i>	<i>E. faecalis</i>	<i>B. cereus</i>	<i>S. typhi</i>	<i>E. coli</i>	<i>L. monocytogenes</i>
EO-A						
DDA (mm)	6.5 ± 0.04	5.8 ± 0.1*	8.3 ± 0.09**	10.8 ± 0.02**	8.5 ± 0.09**	5.4 ± 0.02*
MIC ($\mu\text{g mL}^{-1}$)	125 ± 0.02	579 ± 0.06*	250 ± 0.04**	125 ± 0.09*	144 ± 0.03**	544 ± 0.07*
EO-B						
DDA (mm)	8.6 ± 0.06	5.7 ± 0.02*	9.5 ± 0.05**	8.7 ± 0.01**	10.2 ± 0.08**	5.2 ± 0.04*
MIC ($\mu\text{g mL}^{-1}$)	100 ± 0.07	576 ± 0.04*	256 ± 0.09**	100 ± 0.01**	125 ± 0.06**	566 ± 0.02*

DDA: diffusion disk; MIC: minimum inhibitory concentration; and antimicrobial assays were carried out by broth microdilution method in nutrient broth. Replicates were from three independent experiments. Two-way ANOVA showed significant effects for bacterial strain and assay type ($p < 0.001$), with a significant interaction effect ($p < 0.01$). *Post-hoc* Tukey's test was used to determine pairwise significance. Asterisks denote the statistical significance compared with the mean antimicrobial activity across all strains and assays: $p < 0.05$ (*); $p < 0.01$ (**); and $p < 0.001$ (***). The significant interaction implies that antimicrobial efficacy depends on the combination of bacterial species and assay type.

Table 8 Effect of 1.0% EO-A and EO-B o/w emulsion on the investigated skin parameters after 1 hour application, average value ± SD

Parameter	EO-A o/w emulsion Average value ± SD	EO-B o/w emulsion Average value ± SD	Placebo emulsion Average value ± SD
Skin hydration	T_0 : 34.41 ± 7.57 T_1 h: 63.54 ± 10.30	T_0 : 34.70 ± 7.72 T_1 h: 61.94 ± 8.46	T_0 : 32.00 ± 9.72 T_1 h: 33.29 ± 10.17
Skin softness	T_0 : 2.54 ± 0.09 T_1 h: 2.60 ± 0.10	T_0 : 2.51 ± 0.08 T_1 h: 2.55 ± 0.08	T_0 : 2.19 ± 0.26 T_1 h: 2.12 ± 0.39
Skin elasticity (R^2)	T_0 : 0.597 ± 0.0258 T_1 h: 0.585 ± 0.0224	T_0 : 0.603 ± 0.0183 T_1 h: 0.621 ± 0.0179	T_0 : 0.587 ± 0.137 T_1 h: 0.567 ± 0.180

integrity, ultimately leading to bacterial death.²² These considerations are consistent with our results, where the largest inhibition zones were recorded against the Gram-negative strains *S. typhi* and *E. coli*, ranging between 8.5 and 10.8 mm. Consistently, MIC values as low as 100–125 $\mu\text{g mL}^{-1}$ were determined for these strains, confirming their higher susceptibility to both EOs. This finding is particularly noteworthy, since Gram-negative bacteria are generally considered less susceptible to EOs due to their protective outer membrane, which is rich in lipopolysaccharides and proteins and limits the penetration of hydrophobic molecules.²³ Nevertheless, literature data indicate that volatile constituents of citrus EOs, such as limonene and γ -terpinene, can disrupt the bacterial membrane, inhibit respiration and ion transport, and thereby increase membrane permeability.²⁴ This mechanism provides a plausible explanation for the measurable inhibitory action observed in this study. Furthermore, in agreement with our results, the antibacterial activity of *C. limon* EOs has also been documented against *L. monocytogenes* and *E. faecalis*. The reported inhibition zones and MIC values indicate that *L. monocytogenes* was one of the most sensitive Gram-positive strains to *C. limon* EO, while activity was also observed against *E. faecalis*. These findings corroborate the moderate, yet significant inhibitory effects obtained in these assays and suggest that the observed antibacterial activity may be attributed to the specific chemical profiles of the investigated samples.

Clinical findings

Before evaluating the short-term effects on skin hydration and mechanical properties, the cutaneous tolerability of both for-

mulations was confirmed through a 48 hour occlusive patch test. No adverse skin reactions or irritation were observed, indicating good tolerability profiles for both emulsions. A pilot short-term clinical evaluation of o/w emulsions containing EO-A or EO-B revealed a similar efficacy profile on the assessed skin parameters.

As reported in Table 8, both formulations induced a pronounced and statistically significant increase in skin hydration after 1 hour, with mean percentage variations of +89.0% for EO-A and +84.0% for EO-B (*vs.* T_0 , $p < 0.001$), confirming a strong short-term moisturizing effect. This finding is further supported by the very large effect sizes observed for both treatments compared to the placebo ($d = 3.11$ and 3.76 , respectively), indicating a clear separation between the treated and untreated conditions.

In addition, assessment of skin softness indicated an overall improvement for both formulations, with small-to-moderate effect sizes observed for both treatments compared to the placebo (EO-A: $d = 0.557$ and EO-B: $d = 0.668$), suggesting enhanced skin softness and improved biomechanical pliability.

With regard to skin elasticity, assessed through the Cutometer® R^2 parameter, EO-A showed a modest positive effect ($d = 0.331$), whereas EO-B exhibited a negligible effect compared to the placebo ($d = 0.058$), despite the statistical significance observed *versus* the baseline (+3.0% *vs.* T_0 , $p < 0.01$). The variability observed, particularly in the placebo group, together with partial overlap of confidence intervals, suggests a certain degree of inter-individual response variability. All the results are shown in Fig. 3a–c.



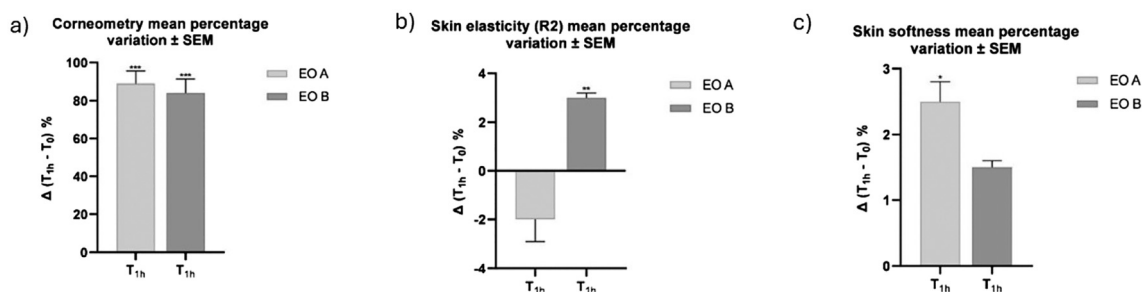


Fig. 3 *In vivo* instrumental assessment of the EO-A and EO-B creams after 1 h of topical application. Data are expressed as mean percentage change \pm SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: not significant, and Student's *t*-test T_{1h} vs. T_0).

Previous studies have demonstrated that the viscoelastic properties of the skin are influenced by its hydration level, as the water content of the stratum corneum represents a key determinant of its flexibility and mechanical properties.²⁵ Further studies are warranted to elucidate the long-term effects and underlying mechanisms associated with these results. The skin parameters are reported in the SI in Table S1.

Conclusions

The successful identification of bioactive compounds in a matrix originally intended as waste, together with the promising experimental outcomes, supports their potential use as multifunctional and eco-sustainable bioactive ingredients for innovative cosmetic formulations aimed at delivering natural, effective solutions to promote skin health.

A key strength of this work lies in the adoption of supercritical CO₂ extraction, which proved to be a pivotal enabling technology. This approach provided a solvent-free, highly selective, and tuneable extraction method, allowing the efficient recovery of thermolabile and non-polar compounds without inducing chemical alterations. It enabled the valorisation of exhausted lemon peels, by-products of limoncello production, yielding essential oils (EO-A and EO-B) with preserved chemical integrity and high functional value.

GC-MS played a fundamental role in the comprehensive chemical characterisation of the extracts. The integration of supercritical CO₂ extraction with GC-MS constitutes a robust and advanced analytical platform, enabling rapid and sensitive profiling of complex mixtures with minimal sample preparation and reduced environmental impact. This combined approach significantly strengthens the reliability, reproducibility, and depth of chemical insight, which are essential for the standardisation and future industrial exploitation of such bioactive ingredients.

EO-A and EO-B contributed significantly to potent anti-oxidant capacity, as demonstrated by consistent radical scavenging activity, as well as antimicrobial properties against Gram-positive and Gram-negative bacterial strains. *In vitro* assays conducted on the HaCaT cell line confirmed the biocompatibility and favourable safety profile of both EOs, exhibiting

minimal cytotoxicity, thereby supporting their suitability for topical application. Furthermore, clinical evaluations revealed significant enhancements in skin hydration, skin softness and elasticity, further underscoring their potential to promote overall skin wellness.

Moreover, they were found to reduce collagenase and elastase enzyme activities, inhibit melanogenesis, and act against *Cutibacterium acnes*, suggesting their possible use as cosmetic additives against skin aging and hyperpigmentation.²⁶

However, further investigations are required to elucidate the molecular mechanisms underlying the biological effects of lemon EOs on skin physiology. Both formulations significantly improved skin hydration, with very large effects compared to the placebo, while softer and more moderate improvements were observed in skin mechanical properties. Overall, EO-A showed a more consistent performance across parameters, whereas EO-B exhibited greater variability, particularly in elasticity. From this perspective, future research should encompass larger and long-term clinical studies to confirm the preliminary results and to further evaluate their potential cosmetic applications. Although the present study focuses on lemon peel waste from limoncello production, the proposed approach may be extended to other agro-industrial residues characterized by similar biochemical compositions, although further investigation is required to assess its applicability under different process and matrix conditions for a large-scale application, requiring dedicated techno-economic and supply chain analyses.

Author contributions

R. D. L.: formal analysis, conceptualization, writing original draft, and supervision; I. N.: validation, formal analysis, and investigation; R. R.: validation, formal analysis, and investigation; D. D. M.: data curation; C. I.: validation and supervision; M. G. F.: formal analysis; and data curation; M. V.: formal analysis; T. P.: data curation; T. D. S.: data curation; S. L.: visualization and conceptualization; and L. G. conceptualization, writing original draft, and supervision. All authors have read and agreed to the published version of the manuscript.



Conflicts of interest

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

Data availability

The datasets generated and/or analyzed during the current study are included within the article.

Supplementary information (SI) is available. See DOI: <https://doi.org/10.1039/d6an00048g>.

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