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Upcycling Lemon Peel Waste into Sustainable Bioactive Ingredients. Chemical Characterization and Efficacy Assessment

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Abstract

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— residues generated as a by-product of *Limoncello* production, a typical liquor of Southern Italy—were investigated as a potential source of bioactive compounds using supercritical CO_2 extraction. The resulting extracts were chemically characterized by gas chromatography coupled with mass spectrometry, revealed complex phytochemical profiles mainly composed of monoterpenes and oxygenated monoterpenes. Two representative extracts (EO-A and EO-B) were selected based on extraction yield, chemical profile, and safety considerations. The evaluation on human keratinocytes (HaCaT cell line) demonstrated 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, melanogenesis suppression, 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 CO_2 extraction as green, sustainable, and highly efficient technology, coupled with a well-established and widely recognized

analytical technique, converting a waste as lemon peel, into new assets, as multifunctional, eco-sustainable cosmetic ingredients.

Introduction

The "upcycling" paradigm gains increasing attention, to reuse a 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 the environmental advantages, this approach is now being successfully to industrial sectors, particularly, where demand for natural and sustainable ingredients keeps growing.

The food processing industry contributes significantly to the production of organic waste usually discharged in 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 we find citrus by-products - particularly lemon peels - which are abundant, low-cost, and rich in valuable phytocompounds [2].

Citrus plants (*genus Citrus,* family Rutaceae), also known as agrumes, are one of the world's major fruit crops with global availability and popularity contributing to human diet. 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 these, lemon (*Citrus limon*) residues are particularly attractive due to their abundance and phytocompounds 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 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 at developing an important resource for the circular economy, creating a virtuous cycle of by-product reuse, proposing the valorisation of wastes derived from "*Limoncello"* production, using supercritical fluid extraction (SFE). EOs obtained from the extraction were characterized for chemical composition and evaluated for their activity *in vitro* and *in vivo.*

In recent years, green extraction techniques 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 technologies for potential cosmetic applications. This work can represent 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, the integration of chemical characterization with both *in vitro and in vivo* bioactivity assessment reinforces 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 °C, until the extraction procedures were carried out. Frozen samples (18 kg of fresh lemon peel matrix) underwent freeze-drying using a Buchi Lyovapor L-300 system and were subsequently grounded with a food grinder. EOs from *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 seven liters 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 aim of optimizing both the yield and the quality of the extracts, investigating the impact of individual operating parameters on yield, performing the extraction at pressures of 200–300 bar, temperatures of 50–60 °C, and CO_2 flow rates of 10–20 $kg \cdot h^{-1}$. The operating conditions for each variant are reported below:

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

SFE conditions			
Extract	**Pressure (bar)**	**Temperature (°C)**	**Flow (Kg/h)**
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

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

The yield of extraction was calculated as follows:

$$yield = \frac{matrix\ quantity\ (g)}{extract\ quantity\ (g)} \times 100$$

where *matrix quantity* refers to grams of matrix weighted analytically and submitted to extraction; *extract quantity* is the weight of the final collected eluate.

Gas Chromatography-Mass Spectrometry analysis (GC-MS)

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-5ms capillary column (5% phenyl–95% methylpolysiloxane; 30 m × 0.25 mm i.d.; 0.25 µm film thickness). Helium served as the carrier gas, flowing at 1.0 mL 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 a 7683 Automatic Liquid Sampler (Agilent). The oven temperature program started at 50 °C, and increased to 300 °C, at a rate of 10 °C, min^{-1}. Identification of EOs constituents relied on matching both retention times and mass spectra with entries from the National Institute of Standards and Technology (NIST) Library, NIST 2020 MS Database. Each sample was injected in triplicate, and results are reported as the mean relative percentage composition.

Study Design

The study was structured as a multi-phase experimental protocol encompassing the supercritical CO_2 extraction of EOs from exhausted lemon peels, preclinical investigations on human skin cell models (HaCaT) 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 well diffusion (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 (T0) and after 1 hour (T1h) 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, Sigma-Aldrich), and streptomycin (100 µg/mL). Cells were maintained at 37°C in a humidified atmosphere with 5% CO2 and seeded at a density of 2–4 × 104 cells/cm^2 and allowed to grow to approximately 80–90% confluence.

Assessment of EO-A and EO-B biocompatibility

The biocompatibility of two selected essential oils,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/well and allowed to grow for 24 h. Subsequently, cells were treated with EO-A and EO-B using a concentration range from 0→1000 µg/mL 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 by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay procedure, 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 by 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 count of 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 appraised using two complementary methods: the DPPH and ABTS assays.

DPPH Radical Scavenging Assay

For the free radical scavenging activity, 30 µg of EO-A and EO-B were incubated in 0.4 mL of 0.1 M Tris–HCl (pH 7.5 ± 0.1) and 0.5 mL of 0.3 mM DPPH in the dark at room temperature (20 ± 2°C) for 20 min. The absorbance (Asample) was measured at 517 nm using a PerkinElmer Lambda 25 UV/Vis Spectrometer and compared to a reference (Acontrol) consisting of 80% methanol, 0.1 M Tris–HCl,

and 0.5 mL of 0.3 mM DPPH, which served to monitor the maximum radical stability.

The scavenging percentage was calculated using the formula:

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

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 assay's sensitivity and provide a benchmark for high antioxidant potency.

ABTS Radical Cation Decolorization Assay

Parallelly, 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 ABTS·+ 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) were dissolved in 2 mL of DMSO solution (5%), and 100 µL of this solution were added to 1.9 mL of the ABTS·+ working solution.

After 2 min of reaction in the dark, the absorbance was measured at 734 nm using a VitroScan Multiwell 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 (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 were incubated in 0.4 mL of 0.1 M Tris–HCl (pH 7.5 ± 0.1) and 0.5 mL of 0.3 mM DPPH in the dark at room temperature (20±2°C) for 20 min. The absorbance (Asample) was measured at 517 nm using a PerkinElmer Lambda 25 UV/Vis Spectrometer (PerkinElmer Inc., Waltham, MA, USA) and compared to a reference (Acontrol) consisting of 80% methanol, 0.1 M Tris–HCl, and 0.5 mL of 0.3 mM DPPH. The scavenging percentage was calculated as % DPPH scavenging (1−Asample /Acontrol)×100, and results were further expressed as IC50 values (the concentration required to inhibit 50% of the DPPH radical) derived from a dose-response curve, using Ascorbic acid as a positive control. Parallelly, 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 ABTS·+ 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 were dissolved in 2 mL of DMSO solution (5%), and 100 µL of this solution were added to 1.9 mL of the ABTS·+ working solution. After 2 min of reaction in the dark, the absorbance was measured at 734 nm using a VitroScan Multiwell 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 (R2=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 by disk diffusion assay (DDA) and broth microdilution method (MIC). 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 that 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 suspensions was placed onto the surface of Mueller–Hinton agar. Disks (6.0 mm diameter) were impregnated with 25 µL of a solution 1.2 mg/mL of each sample placed on the agar Petri dish and incubated at 37 °C, for 24 h. Sterile distilled water (25 µL), and ampicillin and clavulanic acid (30 µg) were used control negative and positive reference, 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 by the broth microdilution method in a Mueller–Hinton broth medium by using sterile 96-well polypropylene microtiter plates. The microbial inoculum size used was 1 ×

10^6 CFU mL^{-1} (NCCLS, 1993). Two-fold serial dilutions of different samples were carried out to obtain concentrations ranging from 10 to 1000 µg/mL. Then, the bacterial cells were inoculated from an overnight culture at a final concentration of about 5×10^5 CFU mL^{-1} per well and incubated with different samples overnight at 37 °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 °C, - were estimated by measuring the spectrophotometric absorbance at 570 nm using VitroScan Multiwell 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 ± 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 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, consisting of deionized water (71.78% w/w) and disodium EDTA (0.10% w/w), was heated under magnetic stirring (200 ± 25 rpm) from 60 °C to 70 °C until complete dissolution.

In parallel, the oil phase (Phase B), composed of Glyceryl Stearate SE (6.00% w/w), Cetylstearyl 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.

Table 2. Composition of EOs-containing o/w emulsion

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	Cetylstearyl 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	Citrus limon (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

Clinical Trial

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

the entire facial area for 1 hour. All measurements were performed at baseline (T_0) and after 1 hour (T1h) by the same trained operator, following a 30-minute acclimatization period under controlled environmental conditions (20 ± 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 the Corneometer® CM 825, skin softness with the Indentometer® IDM 800, and skin elasticity with the Cutometer® MPA 580. The latter was evaluated using the R2 parameter (gross elasticity), calculated as Ua/Uf, which is considered a robust indicator of the skin's ability to recover its original shape after deformation and reflects the condition of collagen, elastin, and extracellular matrix components [9] .

Statistical Analysis in clinical trial

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 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 SAS® software, version 9.4 (SAS Institute Inc., Cary, NC, USA).

Results and Discussion

SC-CO_2 and GC-MS analysis

The extraction of EOs from lemon by-products is a critical process where the method chosen directly impacts the oil's chemical integrity, aroma, and bioactivity. 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 the 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 like essential

oils while preserving their structural integrity. Its non-toxic, inert, and recyclable characteristics further contributes to the environmentally friendly profile of the process.

The extraction process is influenced by several characteristics of matrix such as the particle size, shape, surface area, porosity, moisture, because the diffusion of CO_2 into the matrix is related to its features and for this reason a pretreatment is usually recommended[12]. Indeed, before being extracted, the lemon peels were undertaken freeze-drying and then grinded. Moreover, the different combinations of operational parameters, particularly pressure, influence the solubility of CO_2 and, consequently, the affinity between CO_2 and the bioactive compounds in the matrix. This directly impacts the compositional profile of the obtained extracts, as the extraction efficiency and selectivity of compounds depend on these conditions.

The quantitative yields of the extracts were influenced by the operational conditions:

Table 3. Quantitative yields of the supercritical CO_2 extracts from *Citrus limon* peels under different operational conditions.

Extract	Yield (g extract/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%

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 (EO-D) outperformed the 20 kg/h flow (Extract C), yielding 10.18% versus 8.20%. These findings suggest that a 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 via 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 to establish the chemical fingerprint of the extracts to evaluate their suitability for cosmetic formulations. In the context of a circular economy, the primary objective was to determine the functional ingredients of the recycled byproduct rather than a comprehensive fine characterization[13].

Table 4 and Table 5 report the chemical composition of the two selected lemon EOs, *i.e*. EO-A and EO-B. The rationale for the choice of the EO- A and EO-B by a chemical point of view, was based on their low levels of potentially allergenic constituents — such as limonene) — and/or other potential hazardous chemicals, thereby reducing sensitization risks and enhancing the overall safety of the possible 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 a composition markedly enriched in oxygenated monoterpenes. This composition differs from typical profiles obtained by conventional methods with SFE 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 the SFE enhanced the solubilization of higher molecular weight and less volatile constituents. Importantly, the relatively high abundance of oxygenated compounds indicates that SC-CO_2 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 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 highlight 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, the EO-A is significantly more concentrated

in oxygenated monoterpenes particularly monoterpenoid alcohols (≈30–35%), and phenolic compounds, while 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 have been 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 a selective extraction of these semi-polar bioactive compounds at lower pressure conditions.

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

Table 4. Chemical composition of lemon EO-A. Extraction parameters: pressure 200 bar; flow 20 kg h^{-1}; temperature 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 EO- B. Extraction parameters: pressure 300 bar; flow 10 kg h^{-1}; temperature 60°C. Extraction yield: 3.70%

Retention time	**CAS Number**	**IUPAC Name**	**Chemical Class**	**Abundance %**
5.773	1604-28-0	(3E)-6-Methylhepta-3,5-dien-2-one	α,β-Unsaturated ketone	0.71 ± 0.03
5.807	86951-58-8	Cyclopentanol,1-(methylenecyclopropyl)-	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	(2Z)-3,7-Dimethylocta-2,6-dien-1-ol	Monoterpenoid alcohol	9.48 ± 0.04
9.624	106-26-3	(2Z)-3,7-Dimethylocta-2,6-dienal	Monoterpenoid aldehyde	1.40 ± 0.01
9.833	106-24-1	(2E)-3,7-Dimethylocta-2,6-dien-1-ol	Monoterpenoid alcohol	10.14 ± 0.01
10.051	5392-40-5	(2E)-3,7-Dimethylocta-2,6-dienal	Monoterpenoid aldehyde	1.95 ± 0.3
11.343	1000360-39-7	(2E)-3,7-Dimethylocta-2,6-dienyl-2-methylbutanoate	Monoterpenoid ester	0.52 ± 0.02
16.880	3622-84-2	N-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	(9Z,12Z)-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 (7E,10E)-hexadeca-7,10-dienoate	Fatty acid ester	1.16 ± 0.05
38.422	1000336-77-8	n-Propyl 9,12-octadecadienoate	Fatty acid ester	2.97 ± 0.02

The comparative analysis of the terpenic profiles reveals a significant divergence between the data reported in 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 an exhaustive ethanol infusion for liquor production. Despite the 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 a favourable safety profile for topical application. Therefore, the final choice of EO-A and EO-B was based not only on extraction yield and chemical composition, but also on their

demonstrated in vitro biocompatibility, strengthening their suitability as cosmetic ingredients.

To evaluate the biocompatibility of two selected EOs, namely EO-A and EO-B, obtained from ethanol-exhausted *Citrus limon* (L.) peels, targeted in vitro bioscreen assays were performed on immortalized human keratinocytes (HaCaT). The extracts were tested at concentrations ranging from 0 to 1000 µg/mL for 48 h. The resulting data, reported in Figure 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 a favourable safety profile for both EO-A and EO-B, supporting their biocompatibility in a skin cell model. Extracts were 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.

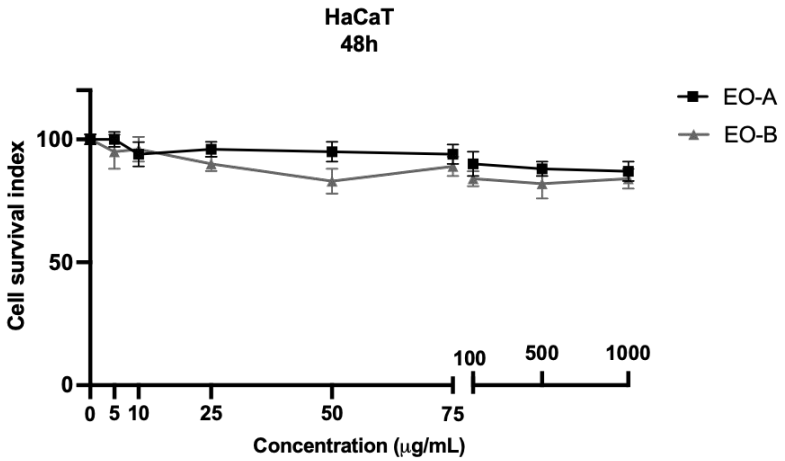

Figure 1. Preclinical biocompatibility assessment of EO-A and EO-B, essential oils derived from ethanol-exhausted *Citrus limon* (L.) peels, in HaCaT cells. Concentration-response curves by the "Cell survival index" for HaCaT cells after 48 h of treatment with EO-A and EO-B over a concentration range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

Antioxidant activity

As previously reported, based on comprehensive MTT cytotoxicity screening conducted on 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 the 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 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

(R2=0.998). EO-A exhibited a TEAC value of 135.6±0.01 mmol Trolox equivalents per gram (mmol TE/g), whereas EO-B reached a significantly higher value of 167.3±0.09 mmol TE/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 IC50 values (the concentration required to inhibit 50% of the DPPH radical). At the tested concentration, EO-A achieved an inhibition of 59.0%±0.05, while EO-B showed a stronger effect with 69.0%±0.08 inhibition. The corresponding IC50 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 EOs' performance. These results, summarized in Figure 2 a-b, and reported in Table 6 demonstrate that both samples possess a significant radical scavenging capacity,

Sample	ABTS Assay (mmol TE/g)	DPPH Inhibition (%)	DPPH IC50 (µg/mL)
EO A	135.6 ± 0.01	59.0 ±0.05*	45.2 ±0.15*
EO B	167.3 ±0.09*	69.0± 0.08	31.8± 0.11
Reference Standard	Trolox	Ascorbic Acid	5.4± 0.03*

Table 6. Antioxidant capacity and radical scavenging activity of Essential Oils (EO A and EO B). Results are expressed as Mean±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. Linear regression analysis was employed to determine the Trolox Equivalent Antioxidant Capacity (TEAC) and to calculate the values for the DPPH radical scavenging assay.

although EO-B consistently displayed a higher antioxidant performance across both complementary assay systems. In the DPPH test, EO-A achieved an inhibition of 59.0 % ± 0.05, while EO-B showed a stronger effect with 69.0 % ± 0.08 inhibition. These results demonstrate that both samples possess a significant radical scavenging capacity, although EO B consistently displayed a higher performance in both assay systems (Figure 2 a-b).

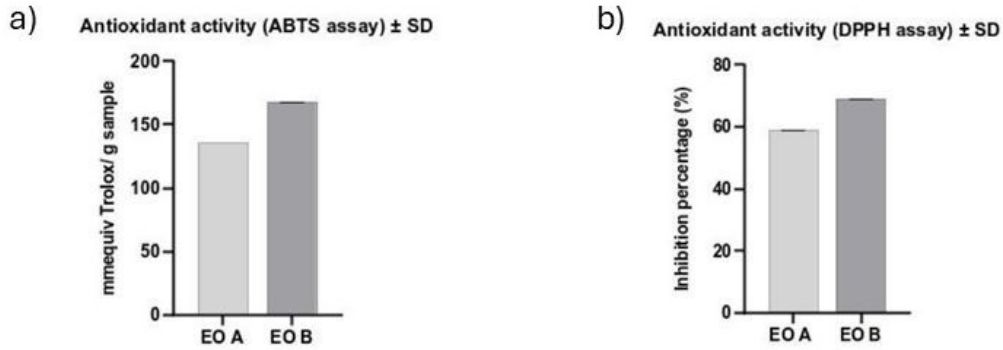

Figure 2. Antioxidant activity of EO-A and B, a) as measured by ABTS and b) DPPG assay. Vertical bars represent the standard deviation.

According to the literature, the antioxidant activity of *Citrus limon* (L.) EOs is generally attributed to its major constituents, primarily monoterpenes such as limonene, β-pinene, and γ-terpinene

According to 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, coumarin also known as benzopyrones, are plant-derived products with several pharmacological properties, including antioxidant and anti-inflammatory activities, by 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 the higher responses obtained in both ABTS and DPPH assay system [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*, *Listeria monocytogenes*) and Gram-negative strains (*Salmonella typhi*, *Escherichia coli*). The most pronounced inhibition was observed against *S. typhi*, which exhibited mean inhibition diameters of 10.8 mm for EO-A and 8.7 mm for EO-B. 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 µg/mL for EO-A and 100 µg/mL for EO-B, supporting the previously noted inhibition zones. Similarly, *E. coli* demonstrated moderate sensitivity, especially to EO-B (MIC = 125 µg/mL). Both EOs showed comparable inhibitory effects against *B. cereus* (MIC = 250 µg/mL), while relatively high MIC values (>500 µg/mL) 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 expected concentrations, while the negative control (sterile water) showed no activity, confirming that the observed antimicrobial effects are strictly attributable to the EO samples themselves.

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 (in micrograms per milliliter, µg/mL) of the tested natural extract that inhibited visible bacterial growth. Corresponding inhibition zones from the disk diffusion assay (DDA) are reported in millimeters (mm).

EO A	**S. aureus**	**E. faecalis**	**B. cereus**	**S. typhi**	**E. coli**	**L. monocytogenes**
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 (µg/mL)	125±0.02	579±0.06*	250±0.04**	125±0.09*	144±0.03**	544±0.07*

EO B	**S. aureus**	**E. faecalis**	**B. cereus**	**S. typhi**	**E. coli**	**L. monocytogenes**
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 (µg/mL)	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; antimicrobial assays were carried out by broth microdilution method in Nutrient Broth. Replicates were from three independent experiments. A 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 statistical significance compared to the mean antimicrobial activity across all strains and assays: $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***). The significant interaction implies that antimicrobial efficacy depends on the combination of bacterial species and assay type.

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 in 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 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 µg/mL 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 the protective outer membrane, rich in lipopolysaccharides and proteins, which 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.* reported inhibition zones and MIC values indicating that *L. monocytogenes* was among 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 profile of the investigated samples.

Clinical findings

Clinical findings

Before evaluating the short-term effects on skin hydration and mechanical properties, the cutaneous tolerability of both formulations was confirmed through a 48-hour occlusive patch test. No adverse skin reactions or irritation were observed, indicating a good tolerability profile 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 T0, $p<0.001$), confirming a strong short-term moisturizing effect. This finding is further supported by the very large effect sizes observed for both treatments compared to placebo (d = 3.11 and 3.76, respectively), indicating a clear separation between 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 placebo (EO-A: d = 0.557; EO-B: d = 0.668), suggesting enhanced skin softness and improved biomechanical pliability.

With regard to skin elasticity, assessed through the Cutometer® R2 parameter, EO-A showed a modest positive effect (d=0.331), whereas EO-B exhibited a negligible effect compared to placebo (d=0.058), despite the statistical

significance observed versus baseline (+3.0% vs T0, $p<0.01$). 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 results are shown in Figure 3 a–b–c.

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. Skin parameters are reported in Supplementary material as Table S1.

Table 8. Effect of 1.0 % EO-A and EO-B o/w emulsion on the investigated skin parameter 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	T0: 34.41 ± 7.57 T1h: 63.54 ± 10.30	T0: 34.70 ± 7.72 T1h: 61.94 ± 8.46	T0: 32.00 ± 9.72 T1h: 33.29 ± 10.17
Skin Softness	T0: 2.54 ± 0.09 T1h: 2.60 ± 0.10	T0: 2.51 ± 0.08 T1h: 2.55 ± 0.08	T0: 2.19 ± 0.26 T1h: 2.12 ± 0.39
Skin Elasticity (R2)	T0: 0.597 ± 0.0258 T1h: 0.585 ± 0.0224	T0: 0.603 ± 0.0183 T1h: 0.621 ± 0.0179	T0: 0.587 ± 0.137 T1h: 0.567 ± 0.180

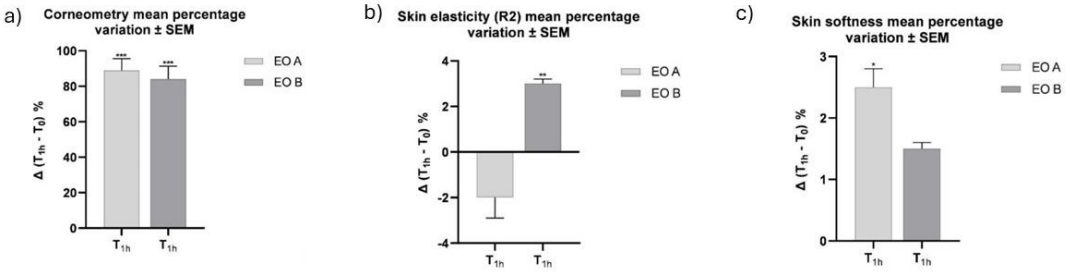

Figure 3 . *in vivo* instrumental assessment of EO-A and EO-B creams after 1h topical application. Data are expressed as mean percentage change ± SEM; (*p<0.05, **p<0.01, ***p<0.001, ns:not significant, t-Student test T_{1h} *vs* T_0)

Conclusions

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

A key strength of this work lies in the adoption of supercritical CO_2 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 plays a fundamental role in the comprehensive chemical characterisation of the extracts. The integration of supercritical CO_2 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 the potent antioxidant capacity, demonstrated by consistent radical scavenging activity, as well as antimicrobial properties against gram-positive and gram-negative bacterial strains. *In vitro* assays conducted on HaCaT cell lines 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, it was found active in reducing collagenase and elastase enzyme activities, inhibiting melanogenesis, and fighting *Cutibacterium acnes,* suggesting a possible use as a cosmetic additive also against skin aging, 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 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. In 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. While 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.

Statements and Declarations

Competing Interests:

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.

Author contribution

R.D.L. formal analysis, conceptualization, writing original draft, supervision; I.N. validation, formal analysis, investigation; R.R validation, formal analysis, investigation; D.D.M data curation; C.I validation, supervision; M.G. F. formal analysis; data curation; M.V. formal analysis, T.P. data curation, T.D.S. data curation, S.L. visualization, conceptualization; L.G. conceptualization, writing original draft, supervision.

All authors have read and agreed to the published version of the manuscript.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

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Declaration

Data Availability Statement

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