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A new blend of *Litsea cubeba*, *Pinus mugo*, and *Cymbopogon winterianus* essential oil active as antityrosinase ingredient in topical formulations

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ABBREVIATIONS

DMSO	dimethyl sulfoxide
EO(s)	Essential oil(s)
HS	HeadSpace
HS-SPME	HeadSpace Solid Phase MicroExtraction
LOD	Limit of Detection
LOQ	Limit of Quantitation
MHE	Multiple Headspace Extraction
NOEL	No Observed Effect Level
O/W	oil/water
SCCNFP	Scientific Committee on Cosmetic Products and Non-Food Products
SCCS	Scientific Committee on Consumer Safety
SDS	sodium dodecyl sulphate

Abstract

Tyrosinase is a target enzyme to be inhibited to reduce excessive melanin production and prevent typical age-related skin disorders. Essential oils are complex mixtures of volatile compounds, belonging mainly to monoterpenoids and sesquiterpenoids, which have been relatively little studied as tyrosinase inhibitors. Among the monoterpenoids, citral (a mixture of neral and geranial) is a fragrance compound in several essential oils, that has shown interesting tyrosinase inhibitory activity. Although citral is listed as an allergen among the 26 fragrances in Annex III of the Cosmetics Directive 2003/15/ EC, it can be safely used for the formulation of topical products in amounts that are not expected to cause skin sensitization, as shown by the various commercially-available products.

The aim of this work is to evaluate two different formulations (oil/water emulsion, oily solution) containing a new combination of essential oils (*Litsea cubeba*, *Pinus mugo*, *Cymbopogon winterianus*) applied to the skin both in non-occlusive and partially occlusive mode. The blend is designed to reduce the concentration of citral to avoid potential skin reactions while taking advantage of the inhibitory activity of citral. Specifically, the amount of citral and other bioactive compounds (myrcene, citronellal) delivered through the skin was studied as a function of formulation and mode of application.

The results show that an oil/water emulsion is preferable because it releases the bioactive compounds rapidly and minimises their evaporative loss. In addition, semi-occluded conditions are required to prevent evaporation, resulting in higher availability of the bioactive compounds in viable skin.

Keywords: essential oils, dermal permeation, *Litsea cubeba*, Lauraceae, *Pinus mugo*, Pinaceae, *Cymbopogon winterianus*, Poaceae

Introduction

Tyrosinase, a key enzyme in the biosynthesis of melanin pigments, is a common target that should be downregulated to reduce excessive melanin production and prevent typical age-related skin disorders [1]. For this reason, research on tyrosinase inhibitors as skin-lightening agents is of particular importance for the cosmetic field, not only in the form of single molecules but also as mixtures of bioactive compounds. In particular, it is becoming increasingly important to search for inhibitors that can cross the skin barrier to interact with the enzyme when administered locally. Essential oils (EOs) are complex mixtures of potentially bioactive compounds, mainly belonging to the group of monoterpenoids and sesquiterpenoids. Few terpenoids have been studied as tyrosinase inhibitors, including citral (i.e., a mixture of neral and geranial) and β-myrcene, which have been shown to block the enzymatic activity of fungal tyrosinase [2,3].

Citral is characteristic of several EOs, including lemon balm, citronella, verbena, and litsea [4]. In 2021, Capetti et al. [2] reported the possible synergistic and/or additive effect of the minor components of the above-mentioned EOs on the activity of citral by using a bioassay-guided fractionation approach. The study showed the advantages of using mixtures of compounds/EOs instead of single molecules and highlighted the possible additive effect of β-myrcene and citronellal on the inhibitory activity of citral [2].

Citral is listed as an allergen among the 26 fragrances added to Annex III of the Cosmetics Directive by the 7th Amendment (2003/15/ EC), based on the opinion of the Scientific Committee on Cosmetic Products and Non-Food Products-SCCNFP (SCCNFP/0017/98) and it has been identified as a suspected responsible of allergic contact dermatitis by the European Commission's advisory committee with a rather high No Observed Effect Level (NOEL) of 1400 μg/cm^2 [5]. Although citral is known to be a contact allergen by consumers, it can be used in topical formulations in amounts that do not cause skin sensitization, as demonstrated by several commercially available products formulated with citral-containing EOs.

The study aims to investigate the amount of citral and other bioactive compounds (i.e. myrcene, citronellal) from the selected EOs delivered through the skin depending on the type of formulation and the application mode. Two different formulations (O/W emulsion and oil solution) containing a justified and reasoned blend of three EOs - *Litsea cubeba* (Lour.) Pers. (Lauraceae), *Pinus mugo* Turra (Pinaceae), *Cymbopogon winterianus* Jowitt ex Bor (Poaceae) - applied to the skin both in non-occlusive and partially occlusive mode were evaluated. A solvent-free headspace sampling based on solid phase microextraction (HS-SPME) in combination with a Fast GC-MS analysis was used to monitor the target bioactive volatiles.

Results and Discussion

L. cubeba EO showed promising results in inhibiting tyrosinase *in vitro* [2]. At a concentration of 166.7 µg/mL (i.e., the EO concentration in the final *in vitro* test mixture), the observed percent inhibitory activity was 59 ± 6. *L. cubeba* EO contains three bioactive constituents, namely neral and geranial (i.e., *cis*- and *trans*-3,7-dimethyl-2-6-octadienal) and β-myrcene. Neral and geranial (30.8% and 39.4%, respectively) are the most abundant components in the EO and are responsible for most of its bioactivity. β-Myrcene is present only in trace amounts (0.5%), but also contributes to the EO tyrosinase inhibitory potential, with its inhibitory activity being about 10 times higher than that of neral and geranial [2].

In the present study, a new blend containing 45% *L. cubeba* EO, 45% *P. mugo* EO and 10% *C. winterianus* EO was formulated and then tested for its tyrosinase inhibitory activity. *P. mugo* EO was selected because it contains a relatively high percentage of β-myrcene (i.e., 12%), which explains its interesting inhibitory activity (percent inhibitory activity 40 ± 3 at a concentration of 166.7 µg/mL). *C. winterianus* EO was also added to the blend due to its high content of citronellal (42.8%), which has been shown to enhance neral and geranial activity [2]. When this blend was tested at the same concentration as the individual EOs (i.e., 166.7 µg/mL), the results showed similar enzyme inhibitory activity (i.e., 48 ± 4) compared to the individually tested EOs of *L. cubeba* and *P. mugo*. In terms of

potential use as an active ingredient in dermatological formulations, the use of the specially designed blend instead of the individual EOs has a number of advantages, including the ability to halve the concentration of neral and geranial from *L. cubeba* EO while maintaining the same inhibitory activity, reducing the risk of developing contact dermatitis due to citral (see above) [5] and better fragrance.

The chemical composition of the tested EO blend is given in **Table 1** along with the normalised relative percent abundance of each constituent. The absolute quantitative amounts of β-myrcene, citronellal, neral and geranial in the EO blend were also measured using an external standard calibration and are listed in **Table 2** along with their physicochemical properties. **Table S1** reports the diagnostic ions (m/z) used for SIM-MS quantification, the calibration range, and the linear regression parameters (i.e., slope, intercept, correlation value, and regression standard error).

In the current study, *in vitro* release tests were performed with two dermatological formulations, namely an oily solution (i.e., almond oil-based) and an O/W emulsion, to determine the best carrier that would allow the release of bioactive compounds from the formulation to the skin while minimising evaporative loss (of the selected markers in the environment). In fact, the volatility of monoterpenes is a major challenge for the use of the EO blend as an active ingredient in topical or transdermal formulations, as the active components are significantly lost through evaporation before being absorbed through the skin. The proportion lost to evaporation depends on several factors, including the interaction of the active compounds with the carriers [6,7]. Each formulation was applied under non occluded mode and its amount was chosen to mimic expected conditions of use (average amount of formulation applied 12 ± 1.6 mg). **Figure 1** shows the *in vitro* kinetic release profiles of the studied markers from the formulations determined using the Higuchian model [8,9] (i.e., cumulative amount per unit surface area (µg/cm^2) plotted against the square root of time expressed as hour). **Table 3** and **Table 4** report the mass balance of the studied markers, i.e., the distribution of the different investigated markers in all compartments of the system at the end of the *in vitro* release study for the O/W emulsion and the oily solution, respectively (i.e., the amount released in the receptor phase, the residual amount detected on the membrane, and the percentage

recovered in the system, measured by the ratio between the initial amount of each marker introduced into the system and the amount recovered at the end of the experiments). The amount of released markers selected above was determined using a solvent-free approach based on HS-SPME sampling, optimised and validated in a previous work [10], followed by GC-MS analysis. Quantitation of each marker was performed by HS-SPME-GC-MS with in-matrix external standard calibration built analysing the pure standards at different concentrations. The same approach was also used to quantify the amount of permeated marker compounds.

Of the investigated markers, β-myrcene and citronellal recorded the highest evaporative loss, regardless of formulation. In the case of the oily solution, their evaporative loss was 99.9 ± 0.01% and 98.9 ± 0.4%, respectively. Both the residual amount recovered from the formulation at the end of the release test and the amount detected in the receptor phase were negligible (i.e., below LOQ or undetectable) regardless of the sampling time. In the case of the O/W emulsion, the evaporative losses of β-myrcene and citronellal were slightly lower, and at the end of the test, 1.2 ± 0.24% and 2.1 ± 0.12%, respectively, of the total applied doses were found in the receiving phase. Regardless of formulation, the evaporative losses of neral and geranial were less significant, as expected from their relatively low vapour pressures. The release rate of the selected markers was higher in the O/W emulsion than in the oily solution, as shown by the steepest slopes of the linear portions of their kinetic release profiles in Figure 1. The total amount of neral and geranial released from the formulation in the receiving phase was on average three times higher in the O/W emulsion than in the oily solution.

Tyrosinase is present in melanocytes, which are dendritic cells located in the basal cell layer of the epidermis (i.e., *the stratum germinativum*). *In vitro* permeation studies were performed to determine the actual amount of bioactive compounds (i.e., β-myrcene, citronellal, neral, and geranial) that can reach their biological target (i.e., tyrosinase) and exert their inhibitory effects. To find out whether this amount could be sufficient for an effective inhibitory effect, two types of data were used: 1) literature data on the dermal absorption profile of kojic acid obtained under similar *in vitro*

conditions [11]; 2) the results of our previous work [2], in which β-myrcene and citral showed 10- and 100-fold lower inhibitory activity than kojic acid (IC_{50} values: 13.3 ± 3.1 μg/mL, 121.8 ± 13.7 μg/mL, 1.0 ± 0.4 μg/mL, respectively), suggesting that their bioavailable amount should be at least 10- and 100-fold greater, respectively, than that observed for kojic acid to expect a significant and comparable inhibitory effect.

According to a study reported in the opinion of the European Scientific Committee on Consumer Safety on kojic acid [11], the average amount of kojic acid that penetrated the skin over the entire 24-hour exposure period was 0.142 ± 0.265 μg/cm². *In vitro* percutaneous absorption of kojic acid was determined on human dermatomed skin by applying a leave-on skin care formulation containing 1% kojic acid at a finite dose. A similar experimental setup was used in the current study: *in vitro* permeation studies were performed by applying a finite amount of the formulation under both non-occluded and semi-occluded conditions. Under semi-occluded conditions, the entire skin surface was covered with aluminium foil, leaving no headspace between the skin sample and the cover to minimise evaporative loss of the bioactive volatile compounds. The system was defined as semi-occluded because it was not hermetically sealed and evaporative losses could still occur, although to a lesser extent than in the non-occluded condition. The formulation chosen for the *in vitro* permeation test was the O/W emulsion, which, according to the *in vitro* release tests, minimised the evaporative loss of the selected markers compared to the oily solution.

The amount of the investigated markers that was considered potentially available for interaction with tyrosinase was that detected in the receiving phase (i.e., dermally absorbed) and that accumulated in the viable epidermis and dermis. Therefore, the stratum corneum was removed from the skin at the end of the exposure period before the compounds were quantified in the viable skin. **Table 5** shows the amount/cm^2 recovered in the skin for each bioactive marker studied and the total amount absorbed dermally (i.e., the total amount of markers studied that reached the receiving phase). Under non-occluded conditions, percutaneous absorption of all markers studied was negligible. In the receiving phase, the compounds were not detected or were detected only in trace amounts (i.e., below

LOQ), regardless of the time of sampling. A very small portion of the applied dose was recovered from viable skin, but this was considered too small to exert an inhibitory effect on tyrosinase. To exclude the possibility that the low dermal absorption was due to the barrier function of the *stratum corneum*, which, however, is very unlikely given the physicochemical properties of the markers studied, the amount of the investigated marker was also examined in the removed *stratum corneum*. The *stratum corneum* was free of the studied markers, with the sole exception of β-myrcene, the amount of which did not exceed 0.006% of the applied dose, suggesting that the low dermal absorption was due to a significant evaporative loss.

Under semi-occluded conditions, the results were more promising. **Figure 2** shows the *in vitro* permeation kinetics of the monitored markers, while **Table 5** gives the absolute amount of each compound that remains in viable skin and is absorbed dermally (i.e., the percentage amount recovered in the receiving phase). For citronellal, neral, and geranial, 6.2%, 44.5%, and 47.2% of the applied dose was detected in the receiving phase, while 2.3, 0.67, and 0.72% remained in viable skin, respectively. The hydrocarbon β-myrcene was barely absorbed by the skin after 27 hours of exposure, as only 0.43% of the applied dose was recovered in the receiving phase, while retention in viable skin increased to 2.1% of the applied dose. The total bioavailability (i.e., the amount of compound recovered in the receiving phase and detected in the viable skin layers) of the investigated markers was significantly higher than that reported for kojic acid (i.e., 0.14 ± 0.26 μg/cm²) and was 0.4 ± 0.11 μg/cm², 19.1 ± 4.4, and 25.5 ± 5.6 μg/cm² for β-myrcene, neral, and geranial, respectively. These results indicate that a significant inhibition of tyrosinase may occur after application of the studied formulation under semi-occluded conditions, provided that the *in vitro* inhibitory activities observed for the selected markers mirror the *in vivo* conditions for human tyrosinase.

The cell toxicity of the EOs and the two different formulations (oil/water emulsion, oily solution) containing the blend of three EOs was evaluated by performing MTT assay on HaCaT cells and compared to gemcitabine (selected as positive control at a concentration of 30μM). No cytotoxic effects were observed in any of samples investigated at a concentration of 1 μg/mL (Figure 3).

Materials and Methods

Origin and chemical characterization of the employed essential oils

The essential oils of *Litsea cubeba*, *Pinus mugo*, and *Cymbopogon winterianus* were obtained by steam distillation and supplied by Witt Italia. A blend of 45% *L. cubeba* EO, 45 % *P. mugo* EO and 10 % *C. winterianus* EO was prepared by mixing an appropriate amount of each essential oil. The chemical composition of the essential oils used to prepare the blend, as well as that of the mixture itself, were determined by GC-MS, analysing 1 µL of a 5 mg/mL solution in cyclohexane of each sample and of the final mixture. The absolute amount of citronellal, geranial (i.e., *trans*-citral), β-myrcene, and neral (i.e., *cis*-citral) in the EO blend was determined using the multilevel external standard calibration method. Pure standards of citronellal (purity > 99%), geranial (purity > 99%), β-myrcene (purity > 99%), neral (purity > 99%) and the solvents cyclohexane and acetone were provided by Merck. A stock solution containing all the above markers at a concentration of 10 mg/mL was prepared in cyclohexane. Appropriate calibration levels in the range of 5.0 – 0.01 mg/mL were prepared from the stock solution and analysed by GC-MS.

Analyses were carried out on a Gerstel MPS-2 multipurpose sampler installed on a Shimadzu GC-FID-MS system consisting of a Shimadzu GC 2010 system, equipped with FID, in parallel with a Shimadzu QP2010-PLUS GC–MS mass spectrometer. A MEGA-5 95% methyl-polysiloxane 5% phenyl column was used. Column characteristics were as follows 15 m × 0.18 mm dc, 0.18 µm df. Analyses were carried out in triplicate under the following conditions. Temperatures: injector: 250°C, transfer line: 270°C, ion source: 200°C; carrier gas: He; flow control mode: constant linear velocity; flow rate: 0.72 mL min^{-1}; injection mode: split; split ratio: 1:20. The MS was operated in electron ionisation (EI) mode at 70 eV, scan rate: 666 u/s, mass range: 35–350 m/z. Temperature programme: 50°C (30 s) //7.2°C/min// 250°C (2 min) (obtained by translating the method commonly used for conventional columns - 30 m × 0.25 mm dc, 0.25 µm df - by the Agilent method translator software

[12]). Identification was made by comparisons of linear retention indices and mass spectra either with those of authentic standards, or with data stored in commercial [13] and in-house libraries.

Data were processed using the ChemStation version E.02.02.1431 data processing system (Agilent Technologies).

Tyrosinase inhibitory activity of the essential oils and corresponding blend

The tyrosinase inhibitory activities of the EOs and the resulting mixture were investigated *in vitro* using a colorimetric enzyme assay based on a method optimized in our previous work [2]. Mushroom tyrosinase from *Agaricus bisporus* and kojic acid as positive control inhibitor were selected for this study and provided by Merck. Spectrophotometric measurements were performed using a Thermo spectronic Genesys 6. The solutions of the investigated essential oils and mixture were prepared in dimethyl sulfoxide (DMSO) at a concentration of 50 mg/mL. Mushroom tyrosinase solution 200 U/mL (27.9 µg/mL) was prepared in sodium phosphate buffer (pH 6.8) and aliquots of 9 mL were stored at -18 °C and thawed just before the assay. Tyrosine solution 0.1 mg/mL was prepared in sodium phosphate buffer (pH 6.8) and renewed daily. The components of the reaction mixture were added to the vial in the following order: 1 mL of mushroom tyrosinase solution 200 U/mL; 1 mL of sodium phosphate buffer solution; 10 µL of EO blend/kojic acid solution, and, finally, 1 mL of tyrosine solution 0.1 mg/mL. The final DMSO content in the reaction mixture was 0.3 %, while the EO blend concentration in the final reaction mixture for the *in vitro* assay was 166.7 µg/mL. The assays were performed in sealed 4 mL vials to avoid the loss of EO components to the environment and to minimize their release into the headspace above the reaction mixture. The reaction mixture was incubated in a thermostatic water bath at 25 °C for 6 minutes. Then, the absorbance was measured at 475 nm, since this wavelength allows the identification of dopachrome. The absorbance corresponding to 100% of tyrosinase activity was measured by replacing the essential oil/pure standard compound/kojic acid solution with 10 µL of pure DMSO. Blank solutions were prepared as follows: 2 mL sodium phosphate buffer solution, 10 µL EO/individual compound/kojic acid and 1

mL tyrosine solution 0.1 mg/mL. The percentage of tyrosinase inhibition was measured according to the following equation:

$$\% \, Inhibition = \frac{\Delta A \, (Control) - \Delta A \, (Sample)}{\Delta A \, (Control)} \times 100$$

where

ΔA (Control) or (Sample)= A_{475} (Control) or (Sample)- A_{475} (Control Blank) or (Sample Blank)

Vehicle formulation

The EOs mixture was incorporated into two different dermatological formulations, an O/W emulsion and an oily solution (almond oil) in both cases at a concentration (w/w) of 5%. The O/W emulsion consisted of (w/w %): deionized water (84.6%), glycerin (4.6%), PEG 400 (0.6%), disodium EDTA (0.1%), carbomer 341 (0.4%), mineral oil (3.6%), cetyl alcohol (0.2%), triethylamine (0.3%), dimethicone (0.5%), methylparaben (0.2%), EOs blend (5%). All the components of the formulation were purchased from Merck.

***In vitro* release studies**

A static vertical Franz cell diffusion system with a receiving chamber of 6 mL volume and a surface area of 2.54 cm^2 was used. For the *in vitro* release studies, dialysis Dialysis tubing made of Membra cell cellulose (Spectrapore, cut-off = 14000 Da) was used to separate the donor and receiving phase. The receiving phase consisted of a 50 mM phosphate buffer solution (pH 5.5) containing 0.1% sodium dodecyl sulphate (SDS) as a solubilizer to achieve sinking conditions. The vehicle was dispensed using a transfer pipette. The exact amount of vehicle dispensed was determined by weighing the transfer pipette tip loaded with the vehicle and after dispensing onto the membrane. The average amount of vehicle applied was 12 ± 1.6 mg. The *in vitro* release test temperature was set at 32 ± 0.5°C to reflect the usual skin temperature and lasted 24 hours; at specific sampling intervals (i.e. 0.5 h, 1 h, 2 h, 4 h, 8 h, 24 h) 6 mL of the receiving phase were withdrawn, replaced with fresh new phosphate

buffer solution, and analysed to determine the concentration of each marker. At the end of the assay the cell was disassembled and the membrane was properly analysed to measure the residual amount of the selected markers in the vehicle. The *in vitro* release study was repeated five times.

In vitro permeation studies

Pig ear skin was used for the *in vitro* permeation tests. The pig ears were purchased from a local slaughterhouse a few hours post mortem. Ethical approval from the Animal Welfare Commission was not required because the animals were not slaughtered for the study. The ears were first washed with water, then all visible hairs were cut off with scissors, and finally a 1-mm-thick layer was cut from the dorsal side of the ear with a dermatome. The samples were stored in aluminium foil at -20 °C. The integrity of the barrier was measured before each experiment. The same static Franz diffusion cell system and uptake phase were used, differing only in pH (i.e., 7.4 in the case of the *in vitro* permeation studies) from those described for the *in vitro* release studies. The skin sample was placed between the donor and receiving chamber with the *stratum corneum* facing the donor chamber. The receiving chamber was set at 37.0 °C ± 0.5 °C, ensuring that the skin surface temperature was set at 32.0 °C ± 1 °C. The vehicle containing the formulation was applied in a finite dose regimen to mimic the expected conditions of application. The average of vehicle amount applied was 12 ± 3.2 mg to remain under finite dose conditions (i.e. below 10 mg/cm^2 [14]) considering the surface area of the static Franz cell used (i.e. 2.54 cm^2). The vehicle was applied with the same procedure as that described for the *in vitro* release test. The skin sample was exposed to the vehicle for 27 hours. At specific sampling intervals (i.e. 1h, 2h, 4h, 8h, 24h and 27h) 6 mL of the receptor phase were withdrawn, replaced with fresh phosphate buffer and analysed to determine the concentration of each marker. The *in vitro* permeation tests were performed under both semi-occluded and non-occluded conditions. Under semi occluded conditions, the skin surface area was covered by a foil disk. At the end of the exposure time, the skin sample was removed from the system; the residual vehicle was gently mechanically removed using absorbent paper. To isolate the *stratum corneum* from the viable

epidermis and dermis, adhesive tape (Scotch Book Tape, 3M) was pressed onto the skin sample and then firmly torn off. The procedure was repeated 20 times, each time with a new piece of adhesive tape. The tape samples as well as the residual epidermis and dermis were analysed to determine both the localization and the concentration of the investigated marker compounds within the skin, as required by the aims of the study. The *in vitro* permeation study was repeated five times.

Downstream analytical platform for *in vitro* release and permeation studies

At the end of the permeation and release tests, the amounts of citronellal, geranial, β-myrcene and neral were determined in all the system compartments: in the receiving phase (both release and permeation tests), in the *stratum corneum*, in the viable epidermis + dermis (*in vitro* permeation studies), membrane (*in vitro* release studies). Quantitative analysis was performed using an analytical protocol based on HS-SPME sampling online combined to fast GC-MS analysis [15]. A schematic diagram of the HS-SPME device and detailed information on its operation are reported in Supporting Information (Figure 1S). GC-MS instrument and analysis conditions were identical to those mentioned above.

HS-SPME conditions were the following*:* Divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 μm (2 cm length) fibres were employed for the experiments. The fibres were purchased from Merck and conditioned according to the manufacturer's instructions. Three fibres were tested by analyzing a set of standard solutions of the target compounds in the receiving phase at different concentrations. ANOVA was carried out to confirm the homogeneity of fiber performance and to discard those with different sampling behaviour. The consistency of fiber performance was checked every 50 analyses using an in-fiber external standardization approach and a standard mixture of hydrocarbons (C_9-C_{25}) in cyclohexane (1 μL of a 0.1 mg/mL solution) [16,17]. Sampling conditions: sampling temperature 35°C, sampling time 30 min, vial volume 20 mL.

Receiving phase: For each sampling time, 1.8 mL of the collected receptor fluid were spiked with 3.0 μL of 1.0 mg/mL tridecane (C_{13}) solution in acetone and finally sampled by HS-SPME for 30 minutes

at 35 °C. After the sampling, the fiber was withdrawn and the SPME device moved to the GC-MS system for analysis. GC desorption lasted 10 minutes to minimise carry-over. Appropriate calibration levels in the range 5.0 – 0.01 mg/mL were prepared in 50 mM phosphate buffer solution (pH 5.5 and 7.4) containing 0.1% sodium dodecyl sulphate (SDS) and 1.8 mL of each calibration level was sampled in triplicates by HS-SPME online combined to GC-MS analysis following the procedure described above. GC-MS condition were identical to those described above.

Stratum corneum and viable epidermis + dermis (in vitro permeation studies), membrane (in vitro release studies). The investigated markers were sampled from the listed matrices by HS-SPME and subsequently analysed by GC-MS analysis and quantified by Multiple Headspace Extraction (MHE-SPME) method. The HS-SPME sampling parameters were the same as those employed for the receiving phase. For the quantitative evaluation of MHE experiments, sample vapour standards (10 to 0.01 µg) were prepared in the vial using the full evaporation technique, sampled by MHE-SPME and subsequently analysed by GC-MS analysis.

Cells and culture conditions

HaCaT is a spontaneously transformed aneuploidy immortal keratinocyte cell line from adult human skin, widely used in scientific research. Cells were purchased from the American Type Culture Collection (ATCC) and were cultured in DMEM high glucose media, supplemented with 10% Fetal Bovine Serum, 100 U/mL penicillin, 100 µg/mL streptomycin in a 5% CO2, 37 °C incubator.

Cell viability assay

Cell viability was assessed using the MTT assay. HaCaT (1500 cells/well) were plated in 100 µl of medium on a 96-well plate. The next day, cells were treated for 72h with the investigated EOs/EOs blend/formulation at a 1/500 dilution. Untreated cells (contr) and cells treated with DMSO (DMSO) at the same dilution served as controls. Gemcitabine (30µM) was selected as positive control (gem). After treatments, the viability was assessed adding 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-

2,5-diphenyltetrazolium bromide) (Merck) for 2h, and absorbance was recorded at 530 nm in a microplate ELISA reader. Data are expressed as percentage of viable treated cells relative to untreated cells (n=3).

Statistical analysis

Data were expressed as mean ± standard mean error (SEM). Results were analysed using a one-way ANOVA and post-ANOVA Tukey's test (Prism Graphpad 5.0, Graphpad Software). We considered statistically significant values of $p \leq 0.05$.

Supporting Information

Table 1S: The diagnostic ions (m/z) used for SIM-MS quantification, calibration range, and linear regression parameters (i.e., slope, intercept, correlation value, and regression standard error); Figure 1S: Schematic representation of an HS-SPME device and relative operation.

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Conflict of Interest Disclosure

The authors declare no conflict of interest.

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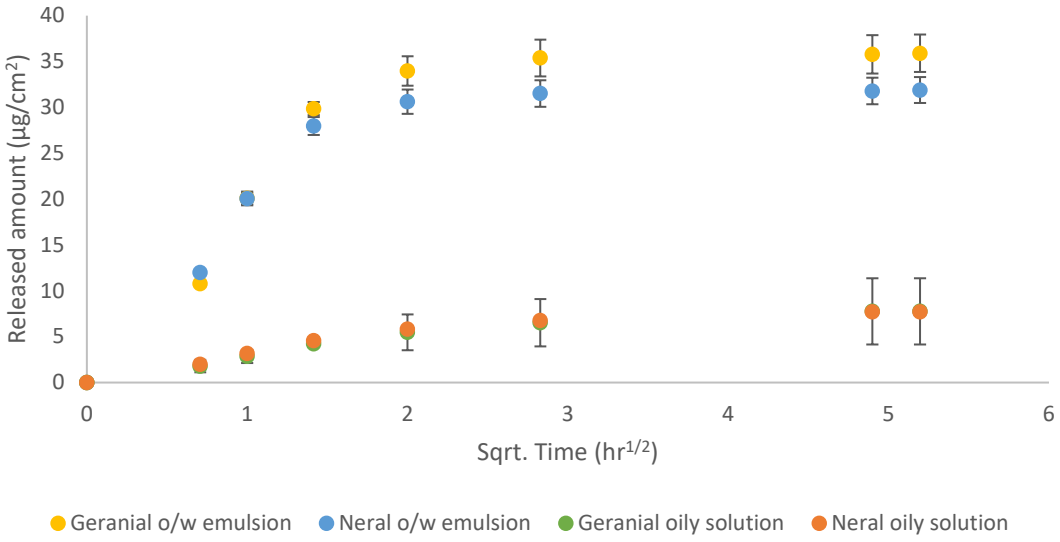
Legends to figures

Figure 1. *In vitro* kinetic release profiles of the investigated markers determined using the Higuchian model (i.e., cumulative amount per unit surface area (μg/cm^2) plotted against the square root of time, expressed as hour). Number of replicates (n): five; error bars represent the standard deviation values.

Figure 2. *In vitro* permeation kinetics of the monitored markers using the Higuchian model (i.e., cumulative amount per unit surface area (μg/cm^2) plotted against the square root of time, expressed as hour). Number of replicates (n): five; error bars represent the standard deviation values.

Figure 3. Effect of EOs on viability of HaCaT cells evaluated after a 72 h incubation with 1 μg/mL of each sample. Legend. Contr: untreated cells, gem: gemcitabine, C: *Cymbopogon winterianus* EO, L: *Litsea cubeba* EO, P: *Pinus mugo* EO, O.M.: oily solution, O/W: oil in water emulsion, MIX: blend of EOs, DMSO: dimethylsulphoxide.

Figure 1


Released amount (µg/cm²)
Sqrt. Time (hr^1/2)
Geranial o/w emulsion
Neral o/w emulsion
Geranial oily solution
Neral oily solution

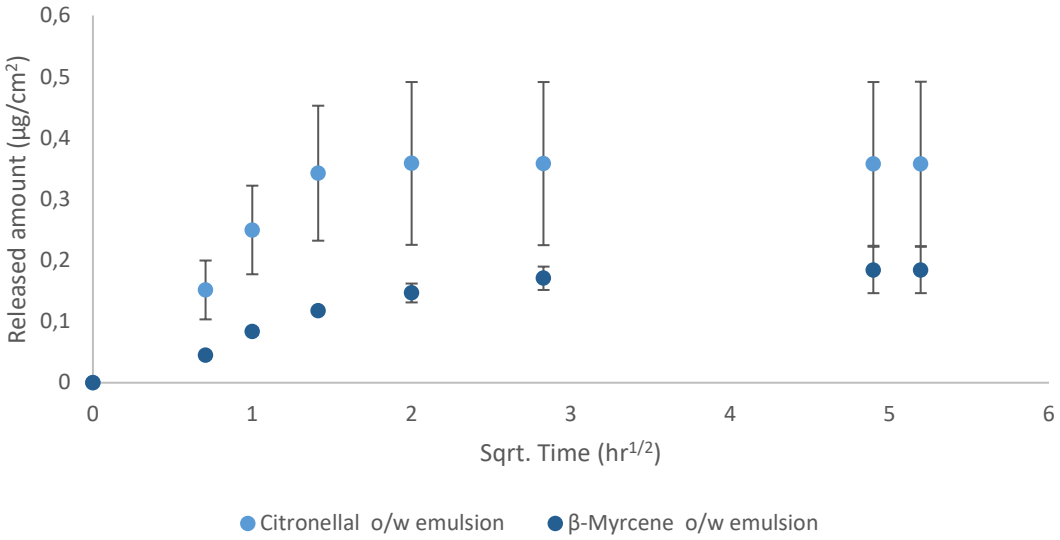

Released amount (µg/cm²)
Sqrt. Time (hr^1/2)
Citronellal o/w emulsion
β-Myrcene o/w emulsion

Figure 2

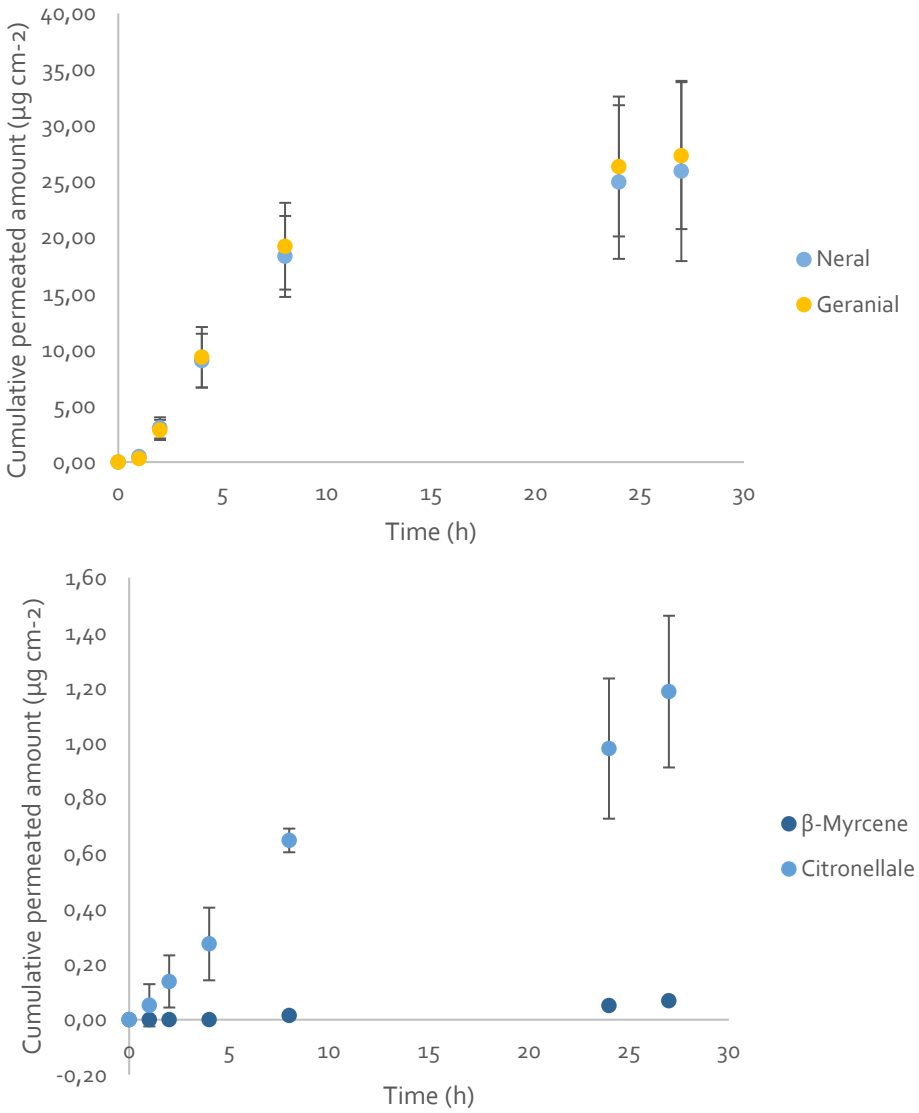

Cumulative permeated amount (µg cm-2)
40,00
35,00
30,00
25,00
20,00
15,00
10,00
5,00
0,00
0
5
10
15
20
25
30
Time (h)
Neral
Geranial
Cumulative permeated amount (µg cm-2)
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
-0,20
0
5
10
15
20
25
30
Time (h)
β-Myrcene
Citronellale

Figure 3

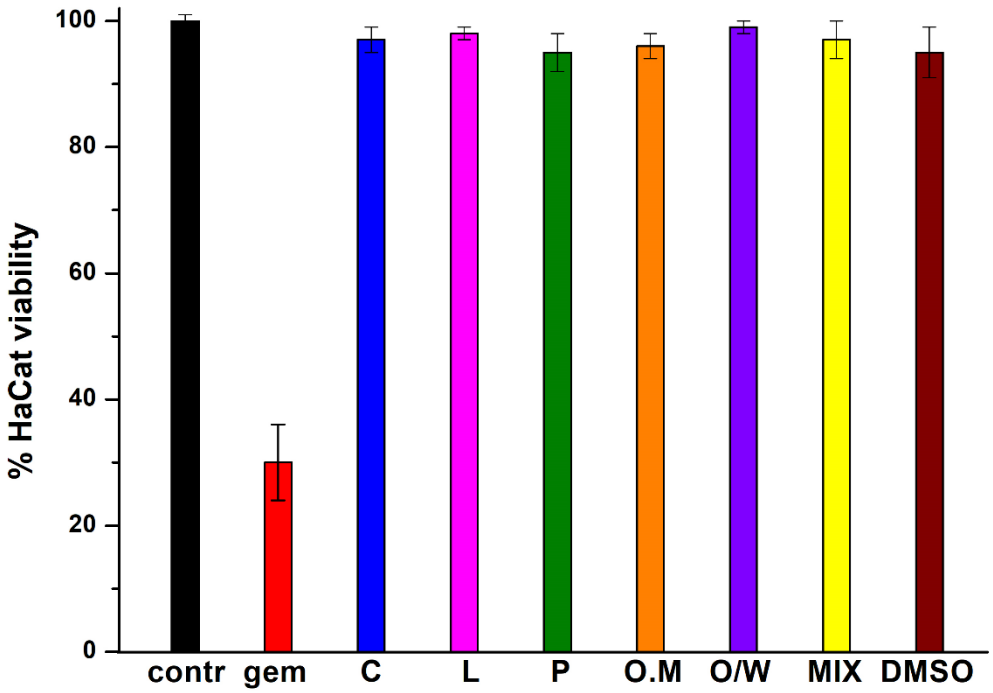


Table 1. Normalized relative percentage abundance of costituents of EO blend investigated.

Compound	I^t_s **Lit**	I^t_s **Exp**	**Abundances (%)**
α-Thujene	931	930	0.1
α-Pinene	939	937	11.3
Camphene	953	950	0.5
Sabinene + β-Pinene	976-980	977	6.8
β-Myrcene	991	990	5.5
α-Phellandrene	1005	1003	0.3
δ-3-Carene	1011	1010	7.4
α-Terpinene	1018	1015	0.2
p-Cimene	1026	1023	1.1
β-Phellandrene	1031	1030	3.5
Limonene	1031	1029	9.7
cis-β-Ocimene	1040	1038	0.1
trans-β-Ocimene	1050	1049	0.1
γ-Terpinene	1062	1059	0.1
α-Terpinolene	1088	1086	0.6
Linalool	1098	1096	0.8
Citronellal	1153	1151	4.8
4-Terpineol	1177	1177	0.2
α-Terpineol	1189	1188	0.4
Neral	1240	1238	16.9
trans-Geraniol	1255	1254	2.9
Geranial	1270	1268	19.2
Bornyl acetate	1285	1284	1.6
Citronellyl acetate	1354	1353	0.3
Geranyl acetate	1383	1382	0.5
β-Elemene	1391	1388	0.2
trans-β-Caryophyllene	1418	1416	3.0
α-Humulene	1454	1455	0.2
Germacrene D	1480	1478	0.4
δ-Cadinene	1524	1523	0.3
Caryophyllene oxide	1583	1581	0.3

Table 2. Absolute amount (expressed as % w/w) of the selected bioactive compounds in the EO blend.

Compound	Molecular Weight	$LogK_{ow}$	Boiling Point (°C) 760mmHg	Vapour pressure (mmHg) 25°C	Water solubility (mg/L) 25 °C	% w/w
β-Myrcene	136	4.17	156	2.40E+00	6.9	6.8 ± 0.8
Citronellal	154	3.83	205	2.54E-01	38.9	7.5 ± 0.5
Neral	152	3.45	217	9.13E-02	84.7	19.9 ± 0.6
Geranial	152	3.45	217	9.13E-02	84.7	21.7 ± 0.8

Table 3. Distribution of the investigated markers in all the compartments of the system at the end of the in vitro release study for O/W emulsion.

Compound	Initial amount (µg) in the applied formulation	Released amount (µg) (i.e. receiving phase)	Residual amount on the membrane (µg)	% Amount detected in the system
β-Myrcene	41.1	0.48 ± 0.10	0.013 ± 0.004	1.2 ± 0.25
Citronellal	45.0	0.93 ± 0.35	0.690 ± 0.128	32.7 ± 4.5
Neral	107.8	82.6 ± 3.8	0.063 ± 0.002	76.7 ± 3.5
Geranial	130.3	93.2 ± 5.1	0.099 ± 0.006	71.6 ± 3.9

Table 4. Distribution of the investigated markers in all the compartments of the system at the end of the in vitro release study for oily solution.

Compound	Initial amount (µg) in the applied formulation	Released amount (µg) (i.e. receiving phase)	Residual amount on the membrane (µg)	% Amount detected in the system
β-Myrcene	41.1	n.d	0.037 ± 0.003	0.090 ± 0.007
Citronellal	45.0	n.d	0.49 ± 0.12	1.1 ± 2.4
Neral	107.8	24.8 ± 6.2	1.1 ± 0.091	24.0 ± 5.8
Geranial	130.3	25.3 ± 7.3	1.2 ± 0.030	20.3 ± 5.6

Table 5. Amount (µg/cm^2) recovered in the skin and total amount absorbed dermally for each investigated bioactive compounds

		Non-occluded condition		**Semi-occluded** condition	
Compound	**µg applied to the skin**	**Epidermis + Dermis (µg/cm^2)**	**Absorbed dermally (µg/cm^2)**	**Epidermis + Dermis (µg/cm^2)**	**Absorbed dermally (µg/cm^2)**
β-Myrcene	41.1	0.029 ± 0.008	n.d.	0.33 ± 0.11	0.068 ± 0.003
Citronellal	45.0	0.024 ± 0.012	n.d.	0.40 ± 0.14	1.1 ± 0.20
Neral	107.8	0.022 ± 0.009	n.d.	0.28 ± 0.073	18.9 ± 4.3
Geranial	130.3	0.028 ± 0.011	n.d.	0.36 ± 0.094	24.2 ± 5.5