Evaluation of sesquiterpenes as permeation enhancers for a model macromolecule across human skin *in vitro*

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ABSTRACT

The objective of this study was to assess the *in vitro* transdermal permeation of a model macromolecular compound namely; fluorescein isothiocyanate - dextran 4 kDa (FD-4) across full thickness human skin by means of chemical enhancers. For this purpose, two sesquiterpenes derived from farnesol were synthesized and further evaluated. Transport studies were conducted using vertical Franz diffusion cells followed by tape stripping in an attempt to examine the disposition of FD-4 on and within the skin. The transport of FD-4 across skin was significantly enhanced in the presence of farnesol and two of its derivatives: A 3.02, 2.67 and 3.74-fold increase was observed compared to a control formulation free of sesquiterpenes. Light microscopy studies showed detachment of the superficial layers and in some cases loosening and swelling of cell layers. Differential scanning calorimetry (DSC) studies of stratum corneum (SC) sheets treated with the sesquiteprenes indicated that enhanced permeation of FD-4 was mainly achieved by disruption and fluidization of the SC lipid bilayers whereas infrared spectroscopy (AT-FTIR) data indicated significant decreases in peak intensity. The results showed that the co-administration of selected sesquiterpenes with FD-4 increased its transport across the skin and might be advantageous for transdermal delivery of macromolecules.

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1. Introduction

Transdermal drug delivery is an alternative route for systemic delivery of active compounds with many advantages compared to the conventional oral route of administration such as avoidance of poor drug absorption or enzymatic degradation in the gastrointestinal tract and/or the first pass effect as well as better patient compliance [1]. Stratum corneum (SC) the outmost layer of the skin imposes the main barrier to drug permeation. A strategy to circumvent the properties of this barrier is the use of physical and chemical enhancers [2–4].

Chemical permeation enhancers (sulfoxides, pyrrolidones, fatty acids, alcohols and glycols, surfactants, urea and its derivatives, essential oils, terpenes and their derivatives, phospholipids) have been used extensively to promote the transport of actives across human skin [2]. Among them active compounds derived from essential oils (terpenes) have been widely used as permeation enhancers for both hydrophilic and hydrophobic drugs [5]. Terpenes can interact with intracellular lipids in the SC in order to increase the diffusion of the drugs [6].

In the current study, we assessed the ability of two farnesol derivatives (sesquiterpenes) as chemical enhancers for the transdermal delivery of a model macromolecular compound namely; fluorescein isothiocyanate - dextran 4 kDa (FD-4) *in vitro*. Farnesol belongs to acyclic sesquiterpenes and it exists in cosmetic formulations mainly for its anti-bacterial activity. It has low toxicity and skin irritation and it issued in cosmetics to a concentration up to 5% [7]. Although there are several studies using terpenes as permeation enhancers for transdermal delivery reports with sesquiterpenes are scarce [8]. To the best of our knowledge this is the first study enrolling chemically synthesized sesquiterpenes as permeation enhancers for skin delivery.

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2. Materials and methods

2.1. Materials

Fluorescein isothiocyanate - dextran with Mw 4 kDa (FD-4) and dimethyl isosorbide ether (DMI), were purchased from Sigma Aldrich, Germany. Trypsin from bovine pancreas, trypsin inhibitor from Glycine max (soybean), farnesol and formaldehyde solution was also purchased from Sigma Aldrich, Germany. Phosphate buffered saline (PBS) pH 7.4, was prepared by dissolving NaCl (8.0 g), KCl (0.20 g), Na_2HPO_4 (1.44 g), and KH_2PO_4 (0.24 g) in 1 L of distilled water. All chemicals and solvents were of analytical grade and all components of buffer solutions were purchased from Merck (Germany). Adhesive tape (Scotch® Magic™ Tape 810, 3M France) was used for tape stripping method.

2.2. Synthesis of farnesol's derivatives

The followed synthetic procedure to access farnesol's derivatives is shown in Fig. 1. The commercially available farnesol (1) -as a mixture of isomers-, was protected through an acetylation reaction using Ac_2O. The effect of NBS on the acetylated product 2 resulted in the formation of bromohydrin 3, which was then transformed into epoxide 4 (1st derivative) upon reaction with NaH, in 90% yield. Then, oxidative cleavage of the epoxide 4 was carried out by the use of periodic acid (HIO_4), delivering aldehyde 5. The final step involved the Wittig reaction between aldehyde 5 and the ylide 6 and subsequent hydrolysis of the formed methyl ester to produce the corresponding carboxylic acid 7 (2nd derivative), in 42% yield [9,10].

2.3. Tissue preparation and transport studies

Abdominal human skin was obtained from a local hospital, following cosmetic surgery or reconstructive abdominal and was used within 24 h after surgery. The specimens were surgically treated, using surgical scalpel to remove connective and subcutaneous fatty tissue. Care was taken to prevent any contamination of the skin surface with subcutaneous lipids.

Full thickness skin was immediately placed between the donor compartment and receptor compartment with the stratum corneum (SC) side facing the donor compartment in vertical Franz cells (Permegear®, USA) with a diffusion area of 4.9 cm^2 and a compartment volume of 15 mL. Following an equilibration period of 30 min the acceptor compartment was filled with the PBS pH 7.4 and the donor compartment with the following formulations: 1 mL, 200 μM of FD-4 plain solution in (i) PBS pH 7.4, (ii) 1% w/v of farnesol (PBS: DMI 1:1 v/v) (iii) 1% w/v of 1st derivative (PBS: DMI 1:1 v/v) and (iv) 1% w/v of 2nd derivative (PBS: DMI 1:1 v/v). Dimethyl isosorbide was chosen due to its relatively small effect on the rate of drug absorption through human skin [8]. The diffusion studies were carried out under constant stirring at 32 °C. The donor compartment was covered with a tape to establish occlusive conditions. At regular time intervals, samples of 700 μL were taken from the acceptor compartment and were replaced with the same amount of fresh pre-warmed buffer solution. The fractions of the acceptor phase were collected over a period of 24 h and were further analysed by fluorescence spectroscopy. In all cases the results were reported as means of six different repetitions.

2.4. Tape-stripping

Upon the completion of transport studies the surface of the skin was wiped with filter paper and placed in a cork plate with pins. For drug determination in epidermis, twenty adhesive strips were cut to a length equal to the diameter of the active area of diffusion cells. The SC was then subsequently removed by tape stripping. The tape strips were slightly pressed onto the skin with the aid of a roller for 5 times back and forth. Every time the direction of the strips was alternated. The tape strips were then rapidly removed and were immersed in vials containing 2 mL PBS according to the following sequence: vial 1 (strip 1), vial 2 (strips 2–3), vial 3 (strips 4–5), vial 4 (strips 6–8), vial 5 (strips 9–12), vial 6 (strips 13–16), and vial 7 (strips 17–20). The samples were sonicated for 1 h at ultrasonic bath following centrifugation for 10 min at 10,000 rpm. Subsequently the vials containing 2 mL PBS and the drug was extracted using an ultrasonic bath for 1 h [11] and further analysed by fluorescence spectroscopy.
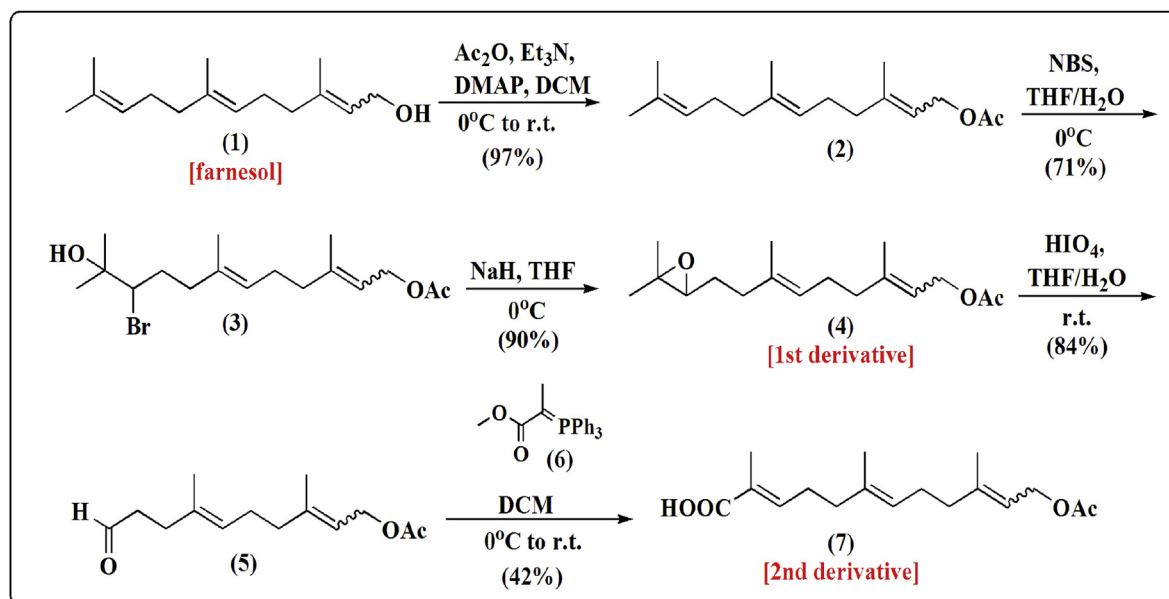

Fig. 1. The synthetic route of farnesol's derivatives.

2.5. Sample analysis

Drug content was quantified using a fluorescence spectroscopy [RF 5301 (Shimadzu)]. The samples immediately after collection were centrifuged for 15 min at a speed of 12,000 rpm and then the supernatant analysed. Fluorescence intensity of the samples was measured with excitation (EX) and emission (EM) wavelengths of 492 and 518 nm respectively. A good linearity ($r^2 = 0.999$) was established in the range of 0.006−0.125 μM. Additionally tape-strips were spiked with known concentrations of FD-4 and subsequently extracted and further analysed in an attempt to determine the extraction efficiency. In all cases it was found above 92%.

2.6. Analysis of permeation data

The steady state flux (J_{ss}) across the skin membranes were calculated using the following equation,

$$J_{ss} = Q/At \; (\mu g/cm^2 \; hr) \tag{1}$$

where Q is the cumulative amount of drug penetrating the skin (μg), A is the diffusional area (cm^2) and t is the total time of the experiment (s).

The permeability coefficient (P) was then calculated according to the following equation,

$$P = J_{ss}/C_d \; (cm/hr) \tag{2}$$

where C_d is the drug concentration in the donor compartment [12].

In order to normalize the permeability data in the presence of the enhancer compared to control, enhancement ratios (ER) were calculated according to equation (3),

$$ER = P_{enh}/P_{control} \tag{3}$$

where P_{enh} is the permeability coefficient corresponding to the enhancer treated skin (terpenes), and $P_{control}$ is the permeability coefficient respectively for the control (FD-4 solution).

2.7. Degradation studies of FD-4 in the skin

Any degradation of FD-4 occurred during the transport studies was assessed by sampling fractions from the donor and the acceptor phases and further analysed by size exclusion chromatography (SEC). After the transport study, 250 μL of donor solution containing FD-4 were collected from the donor compartment and diluted 10 times to a final volume of 2.5 mL in PBS. In a similar manner 600 μL was collected from the acceptor compartment and diluted to a final volume of 2.5 mL in PBS. Finally, samples containing stock solution of FD-4 was analysed by SEC.

The size exclusion chromatography (SEC) setup comprised of: a SpectraSystem SCM 1000 degasser (Thermo Separation Products, San Jose, CA); a SpectraSystem P 2000 chromatographic pump (Thermo Separation Products, San Jose, CA); a column assembly set-up comprising of a 2 mm frit (Idex, Oak Harbor,WA); one GPC/SEC PL-Aquagel-OH 50 × 7.5 mm guard column (8 mm) (Varian Inc, Palo Alto, CA); one PSS Suprema Ultrahigh (10 μm) Suprema column (Polymer Standards Service GmbH, Mainz, Germany); two tandem connected GPC/SEC PL-Aquagel-OH 300 (8 μm) columns (Varian Inc, Palo Alto, CA), all encased in a Model 605 column oven (Scientific Systems Incorporated, State College, PA) set at 30 °C. The detector was a Fasma 520 fluorescence detactor (Rigas Labs, Thessaloniki, Greece) operating at an excitation wavelength of 492 nm and an emission wavelength of 518 nm. The results were also verified by a tandem detector setup comprising of a BI-DNDC

differential refractometer (refractive index, RI) detector set at 30 °C (Brookhaven Instruments Corporation, Brookhaven, Holtsville, NY) and a BI-MwA multi-angle laser light scattering (static light scattering, SLS) detector (Brookhaven Instruments Corporation, Brookhaven, Holtsville, NY) (results not shown). All results were acquired and treated using a dedicated software package (ParSEC, Brookhaven Instruments Corporation, Brookhaven, Holtsville, NY). The analytes were injected using 200 μL loop into the eluent, which was ultrapure water with a flow rate of 0.8 mL min^{-1}.

2.8. Histological evaluation

The effect of the permeation enhancers to skin membrane was assessed by light microscopy. Using 4 μm-thick sections of each of the formalin-fixed and paraffin-embedded samples stained by conventional hematoxylin-eosin. Specimens were analysed in an Olympus CX31 optical microscope and microphotographs were taken via the OLYMPUS analysis getIT software. All skin membranes were from the same donor.

2.9. Differential scanning calorimetry (DSC) and infrared-red spectroscopy (IR) of SC sheets treated with sesquiterpenes

Biophysical changes of SC in presence of sesquiterpenes were monitored by means of differential scanning calorimetry (DSC) and fourier transformed infrared spectroscopy (FTIR). SC sheets were obtained by incubating epidermal membranes obtained by heat separation in a solution of 0.1% (w/v) trypsin in PBS, overnight at 4 °C and 1 h at 37 °C. SC was peeled off from the epidermis. Because of the remaining activity of trypsin, the SC was treated with 0.1% (w/v) trypsin inhibitor solution in PBS. Subsequently SC was washed with distilled water twice, blotted dry and stored at room temperature in a silica gel containing desiccator in N_2 in order to avoid any oxidation of the lipids [13]. Samples of dry SC were hydrated for 2 days to 20−40% hydration over a saturated solution of potassium sulphate (R.H. 97%). The chosen hydration range is representative of the normal in vivo level of SC hydration throughout much of the tissue [14] since no major changes in the properties of the membrane are expected at these conditions [15,16]. Hydrated samples were immersed in a solution of 1% w/v of terpenes in DMI, for 24 h at 32 °C, to mimic the diffusion experiments. Furthermore, samples were blotted dry with tissue paper prior to analysis [17].

For DSC experiments, DSC 204 F1 Phoenix (NETZSCH) was used. Approximately 12 mg of SC sheets were hermetically sealed into stainless-steel capsules to prevent water vaporisation from the tissue. Samples were heated from 0 to 120 °C, with heating rate of 10 °C/min. Molecular investigation was also conducted with IR. Analysis of the samples was carried using a spectrophotometer ATR-FTIR (Prestige-21, SHIMADZU). Spectra of tissues were obtained in the wavelength range of 650−4000 cm^{-1}, with a spectral resolution of 4 cm^{-1} and 64 scans per minute. In order to collect ATR-FTIR spectra, the tissue samples were placed directly on the ATR crystal with the uppermost layer facing down on the surface of the crystal. Intact (untreated) stratum corneum was used as the control. DSC and IR measurements were conducted with stratum corneum sheets from the same donor.
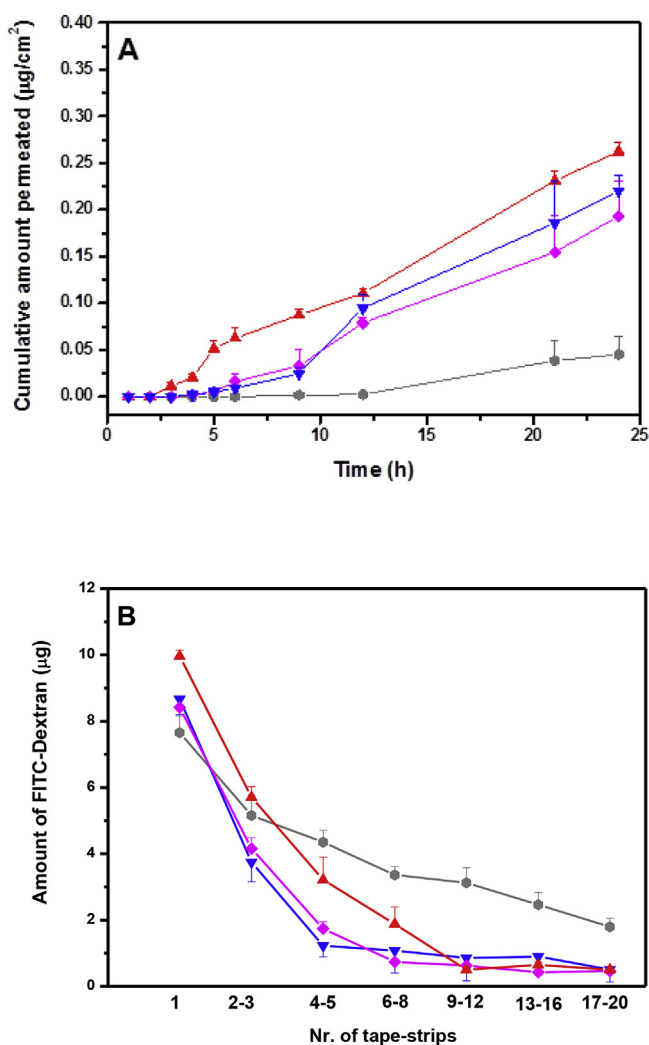
2.10. Statistical analysis

The results are presented as mean ± standard deviation of 6 experiments. Statistical differences were tested using unpaired Student t-test (p < 0.05).

3. Results and discussion

3.1. Transport studies

Permeation profiles of plain FD-4 and its permeation enhancement congeners, expressed as the cumulative amount permeated *versus* time, are shown in Fig. 2A. The steady state flux (J_{ss}) across the human skin, the permeability coefficient (P) and the enhancement ratios are illustrated at Table 1. The permeability coefficient of plain FD-4 across full thickness skin was $P = 0.43 \pm 0.11 \times 10^{-5}$ cm/h which is in close agreement with previous studies [18,19]. The presence of farnesol increased 3.02 fold (*t*-test, p < 0.05) the transport of FD-4 across skin membrane compared to the control resulting in a permeability coefficient of $P = 1.30 \pm 0.11 \times 10^{-5}$ cm/h. Although the presence of 1st derivative resulted in 2.67 fold increase of FD-4 across the skin compared to control (plain FD-4 in PBS) the permeability coefficient $P = 1.15 \pm 0.21 \times 10^{-5}$ cm/h was lower compared to that of farnesol (*t*-test, p > 0.05). In the presence of the 2nd derivative the transport of FD-4 increased 3.74 fold compared to control exhibiting a permeability coefficient

Table 1
Calculated steady state flux values (J_{ss}), permeability coefficients (P) and enhancement ratio for RH across human skin in the presence of permeation enhancers.

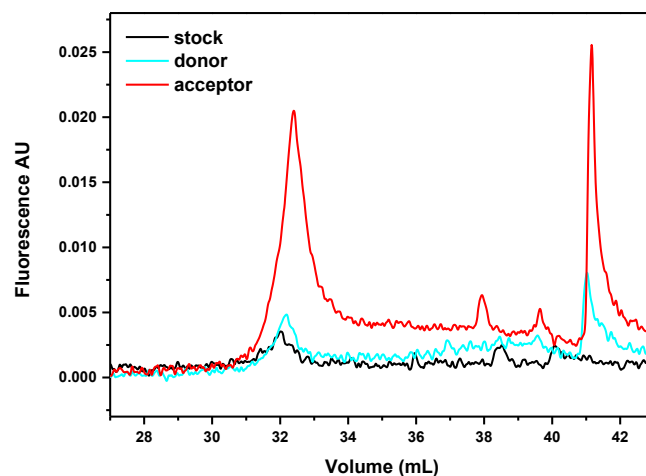|	J_s ($\mu g/cm^2h^{-1}$)	P *10^{-5} (cm/h)	ER
FD-4 (passive)	0.0034 ± 0.0009	0.43 ± 0.11	-
FD-4 and 1st derivative	0.0092 ± 0.0015	1.15 ± 0.21	2.67
FD-4 and 2nd derivative	0.0129 ± 0.0009	1.61 ± 0.08	3.74
FD-4 and farnesol	0.0104 ± 0.0008	1.30 ± 0.11	3.02

*Values are reported as mean ± standard deviation (n = 6).

$P = 1.61 \pm 0.08 \times 10^{-5}$ cm/h, which was significantly higher compared to that of farnesol and 1st derivative (*t*-test, p < 0.05). The rank order effect of the permeation enhancers for the transport of FD-4 across human skin *in vitro* was the following: $P_{2nd\ derivative} > P_{farnesol} > P_{1st\ derivative} > P_{FD-4\ solution}$. The presence of the derivatives significantly reduces the diffusional lag-times compared to the control. Terpenes due to their polar group have the ability to insert into the lipid lamellae and disrupt them [8]. In a similar manner in the current study the highest transport rate (among terpenes) of FD-4 across skin was obtained for the more polar 2nd derivative whereas the lowest transport rate of FD-4 was recorded for less polar 1st derivative accordingly.

The disposition of FD-4 on and within the skin was further assessed using the tape stripping method. In Fig. 2B is presented the amount of FD-4 as a function of tape-strips. The curves for farnesol and its derivatives show similar profile: the amount of FD-4 decreases fast with increasing numbers of tape-strips. Notably the 1st derivative reaches a plateau at 4—5 strips, farnesol 6—8 strips and 2nd derivative 9—12 strips. However in the case of plain FD-4 the amount of model compound found in tissue is higher compared to its farnesol congeners.

3.2. Stability of FD-4 in the skin

In Fig. 3 are presented chromatograms for donor, acceptor and stock samples. In all chromatograms four fluorescent peaks are observed under the used conditions, of which one taken at 32 mL corresponds to a substantial constituent of the sample under study, corresponding to the elution times of small biopolymers. The two peaks that follow correspond to small-molecular weight entities, while the last peak should be attributed to very small constituents (akin to an RI system peak).

Fig. 2. (**A**) Cumulative transport of FD-4 across human skin (▲): in presence of 1% (w/v) 2nd derivative (◆), in presence of 1% (w/v) 1st derivative (▼), in presence of 1% (w/v) farnesol, (●) FD-4 solution, (n = 6) (**B**) Amount of transport of FD-4 proportional to the number of fractions of the tape-strips (▲): in presence of 1% (w/v) 2nd derivative (◆), in presence of 1% (w/v) 1st derivative (▼), in presence of 1% (w/v) farnesol (●), FD-4 solution (n = 3).

Fig. 3. Size exclusion chromatography of donor and acceptor fluids from a transport experiment and stock solution of FITC-Dextran (FD-4).

3.3. Light microscopy studies

Light microscopy was performed to assess any histological changes resulting from the presence of the permeation enhancers. In the histological analysis tissue specimens were treated with plain FD-4, and 1% (w/v) of the sesquiterpenes. As control was considered the non-treated skin.

As is shown in Fig. 4B-D, the presence of farnesol and its derivatives induced changes to the skin membrane compared to the control Fig. 4A. The presence of farnesol induced limited detachment of the superficial layers (Fig. 4B) of the skin membrane whereas in the case of 1st derivative (Fig. 4C) and the 2nd derivative (Fig. 4D) almost complete detachment of the SC was observed. Notably the presence of farnesol and the 2nd derivative (Fig. 4B and D) transformed the highly compact cells of the SC into a looser network resulting in swelling of SC cell layers of human epidermis. This is in accordance with the transport studies were the highest fluxes of FD-4 were observed in the presence of farnesol and the 2nd derivative.

3.4. DSC studies

In Fig. 5A are presented the thermograms of SC treated with different permeation enhancers. DSC of the non-treated SC sheet exhibited the following endothermic peaks: $T_{m2} = 75.2\ °C$ (ascribed to lipids) and $T_{m3} = 87.6\ °C$ (lipid proteins) accordingly [20] [21]. In the presence of farnesol these peaks were shifted to lower values namely; $T_{m2} = 73.4\ °C$ and $T_{m3} = 84.4\ °C$ whereas these changes were more pronounced when 2nd derivative was present ($T_{m2} = 72.8\ °C$ and $T_{m3} = 84.8\ °C$). Small shifts were observed to these peaks when SC was treated with the 1st derivative ($T_{m2} = 74.9\ °C$ and $T_{m3} = 86.7\ °C$ accordingly). Such shift toward

lower temperatures, are related with the disruption of the intercellular lipid bilayer and the detachment of proteins and lipids [20]. These results are in accordance with the permeation studies where the higher fluxes recorded for farnesol and the 2nd derivative and can be attributed to lipid disruption and posting of proteins and lipids from SC [22].

3.5. ATR-FTIR studies

At Fig. 5B, are demonstrated the spectra of plain and terpene-treated SC. In plain SC the peak at $3282\ cm^{-1}$ represents the functional group C-H, but could also represents the group N-H (amide I) which belong to lipids, proteins and water. The peaks at $2922\ cm^{-1}$ and $2849\ cm^{-1}$ illustrate the CH_2 groups of lipids (ceramides, phospholipids), which give information on the internal composition of the lipid bilayer. Ceramides, cholesterol, fatty acids and cholesterol esters are laminates of lipids that impart barrier properties to the stratum corneum. The peak at $1740\ cm^{-1}$ represents the group C = O of ester lipids. While the peaks at $1639\ cm^{-1}$ and $1536\ cm^{-1}$ respectively represent the group C = O of amide I and the group C-N amide II of epidermal keratin [20,23,24].

When SC sheets were treated with farnesol and the 2nd derivative the intensity of all assigned peaks were significantly reduced and were almost extinct which might be attributed to lipid detachment [20,24]. However in the case of 1st derivative these changes were not so pronounced in accordance with the DSC studies.

4. Conclusion

Chemically synthesized sesquiterpenes were utilised as permeation enhancers for skin delivery of macromolecules. Results
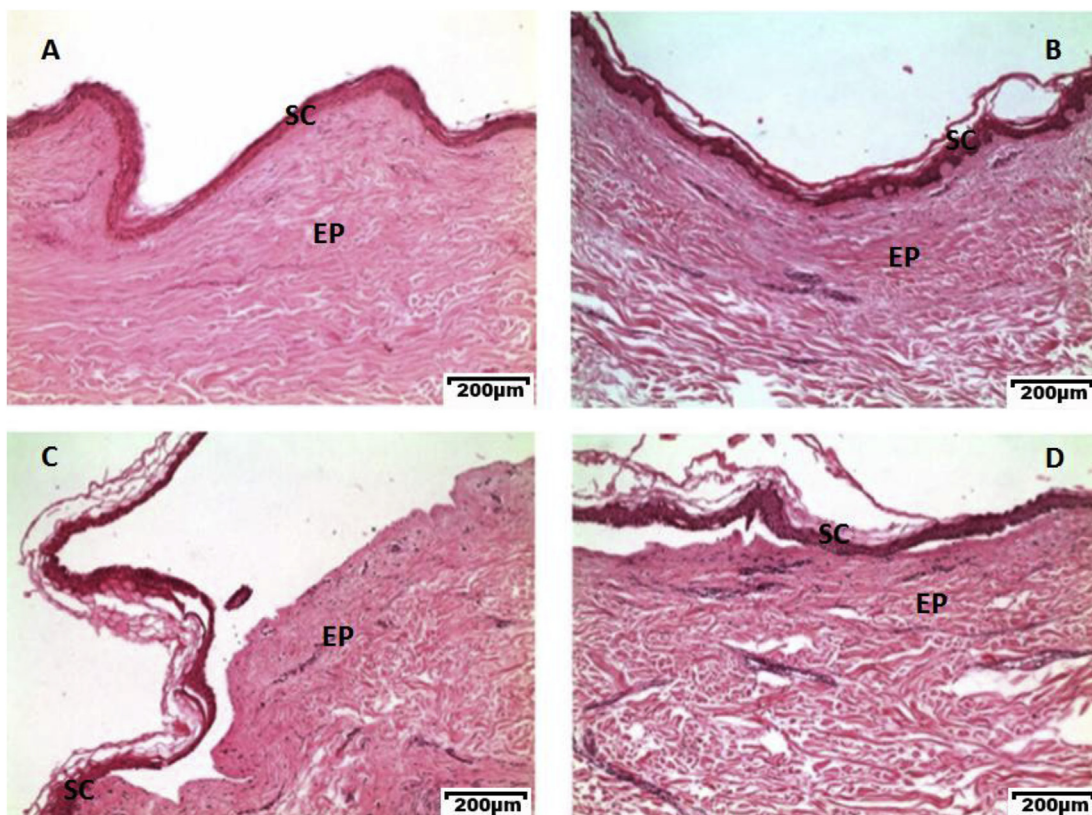

Fig. 4. Light micrographs of human skin with **A.** FD-4 solution, **B.** farnesol, **C.** 1st derivative of farnesol, **D.** 2nd derivative of farnesol. Bar represents 200 μm.
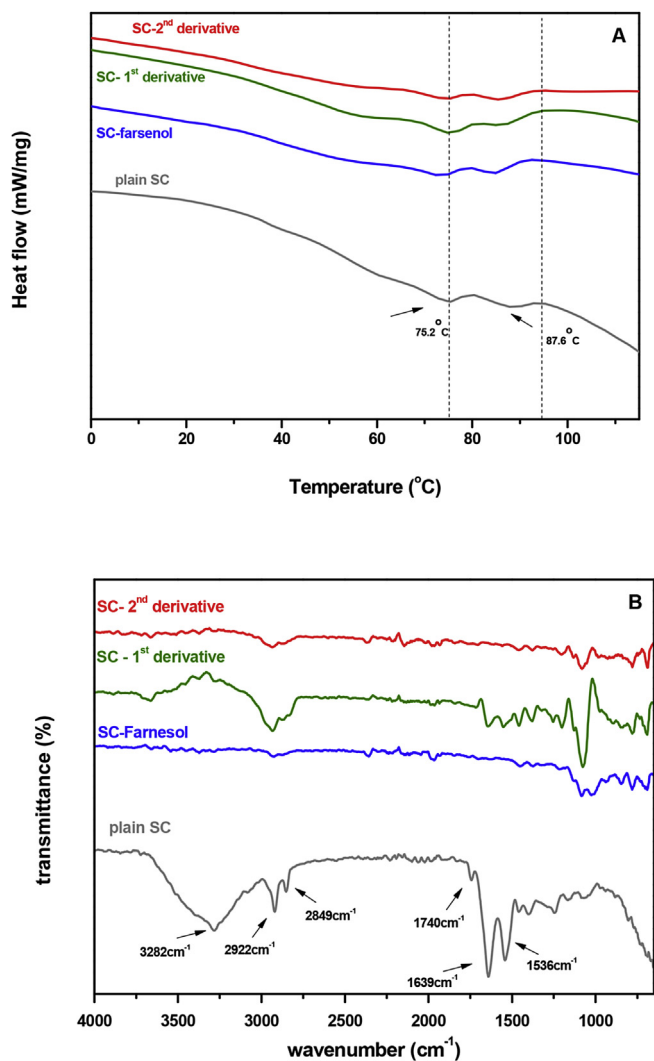
Fig. 5. (**A**) DSC traces of plain hydrated SC and stratum corneum treated with terpenes. Endothermic transitions appear as peaks. (**B**) ATR-IR spectra of untreated, 20–40% hydrated SC and SC treated with sesquiterpenes.

have shown that the transport rate of the macromolecular compound across full thickness skin *in vitro* was increased when co-applied with these derivatives. Due to their low toxicity sesquiterpenes hold promise as permeation enhancers. However more studies are needed enrolling more farnesol derivatives with different properties (e.g. lipophilicity).

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