Acta Poloniae Pharmaceutica – Drug Research, Vol. 74 No. 4 pp. 1063–1070, 2017 ISSN 0001-6837
Polish Pharmaceutical Society

THE INFLUENCE OF TERPENES ON HUMAN *STRATUM CORNEUM* BY FLUORESCENCE MICROSCOPY, ATOMIC FORCE MICROSCOPY AND SCANNING ELECTRON MICROSCOPY

KRZYSZTOF CAL[1*], JUSTYNA STEFANOWSKA[1], BILJANA GOVEDARICA[2], ODON PLANINSEK[2], STANE SRCIC[2], KAROLINA BAZELA[3] and RENATA DEBOWSKA[3]

[1]Department of Pharmaceutical Technology, Medical University of Gdansk, Hallera 107, 80-416 Gdańsk, Poland
[2]Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Ljubljana, Askerceva 7, SI-1000 Ljubljana, Slovenia
[3]Dr Irena Eris Centre for Science and Research, Puławska 107A, 02-595 Warszawa, Poland

Abstract: Numerous preparations containing single terpenes or complexes of essential oils are applied onto the skin in their pure form or in the form of ointments, gels, oily solutions, etc. The aim of this study was to visualize changes in the *stratum corneum* by terpinen-4-ol, racemic camphor, eucalyptol, racemic menthol and levomenthol incorporated in various concentrations into lipophilic, ethanolic or hydrophilic vehicle. Additionally, an irritation *in vitro* test was performed to determine the influence of individual formulations and pure vehicles on viability of skin cells.

Keywords: terpenes, *stratum corneum*, AFM, SEM, fluorescence microscopy, irritation test

Terpenes are components of natural essential oils. These are organic compounds built of carbon, hydrogen and oxygen in an isoprene arrangement (C_5H_8). There are numerous classes, groups and subgroups of terpenes that can be distinguished and their division depends on the number of carbon atoms. They are broadly distributed in nature and have been used in medicine and cosmetics for a long time. Numerous preparations containing single terpenes or complexes of essential oils are applied on the skin in their pure form or in the form of ointments, gels, oily solutions, where they have anaesthetic, antiseptic or irritating action. In the pharmaceutical technology, selected terpenes are added in small amounts to preparations intended for topical applications to increase the permeation of the active substance into and through the skin; these are penetration enhancers (1, 2).

This study was aimed at examining changes by means of various techniques occurring in the structure of the *stratum corneum* of the human skin under the influence of terpenes such as terpinen-4-ol, racemic camphor (Ph. Eur.), eucalyptol (Ph. Eur.), racemic menthol (Ph. Eur.) and levomenthol (Ph. Eur.) incorporated in various concentrations into typical dermatological vehicles:

- grapeseed oil (lipophilic vehicle),
- ethanol 40^o (ethanolic vehicle),
- hydrogel (hydrophilic/ethanolic vehicle).

Additionally, an irritation *in vitro* test was performed to determine the influence of individual formulations and pure vehicles on viability of skin cells.

Terpenes were selected on the basis of frequency and commonness of their occurrence in medicinal products where they occur as active ingredients in greater amounts or fulfil the role of penetration enhancers.

EXPERIMENTAL

Terpenes formulations

All chemicals were of Ph. Eur. or cosmetic grade. Terpenes formulations were made by dissolving terpenes in ethanol (40^o), grapeseed oil or carbomer gel (Table 1). In the last case, some amounts

* Corresponding author: e-mail: kcal@wp.pl

of ethanol 96° were used to dissolve the terpene before incorporation into the vehicle. No ethanol solutions of 5% menthol and 5% levomenthol and no hydrogel formulations of levomenthol were prepared either due to the poor solubility of terpene and their re-crystallization. Finished preparations were stored at 8°C and protected from light.

***Ex vivo* observations by fluorescent microscopy**

The studies were approved by the local Ethics Committee. Human skin was collected from cadavers in the area of the chest and abdomen. It was then cleaned from any remains of fat and subcutaneous tissue and divided into smaller fragments. The tissue was wrapped in aluminium foil and frozen at -40°C. Before the start of tests, the skin was thawed at room temperature. Its surface was cleaned by 2-times sticking and removing fragments of adhesive tape.

To separate the epidermis with the *stratum corneum* from the dermis, a thawed and cleaned fragment of skin was immersed in a beaker with purified water at 65°C for 45 s. After separating the layers and drying the epidermis surface delicately using tissue paper, the tissue was placed on a glass microscope slide. A small amount (approx. 0.5-1.0 µL) of ethanol or oily solution, or a thin layer of hydrogel, was applied on the surface of isolated *stratum corneum*. Skin with applied substrate not containing terpenes was the control. The sample on a microscope slide was placed on a Petri dish in which tissue saturated with 0.9% sodium chloride solution had been placed to ensure a constant moisture level. The closed Petri dish was placed in an incubator and incubated at 37°C for 30 min. After this period of time, the dish with the sample was taken out and the excess of the preparation was delicately removed from the surface and dried using tissue paper. A fluorescent stain solution (sodium fluorescein – NaFl, rhodamine B hexyl ester – RBHE or sulforhodamine B – SRB) was applied where the preparation had been. The microscope plate was replaced in the Petri dish and incubated again at 37°C for 15 min, protecting it from light. Next, the sample surface was dried delicately using tissue paper and observed under a fluorescent microscope.

The samples were observed under an epi-fluorescent microscope (Nikon eclipse e-50i) with a mercury lamp, using appropriate filters and wavelengths for stains used in the test and under objectives of 10×, 40× and 100×. The main color of the obtained images depended on the kind of the applied stain and an appropriate filter in the fluorescent microscope. The green color was observed for NaFl, orange for derivatives of rhodamine, i.e. SRB and RBHE.

Given the equipment of the microscope in an automatic drive of the *z* axis, a series of photographs were taken at various depths of the sample, changing the position of the table by 0.5–1.0 µm on average. Following that, a subsequent series of photographs were put together to create 3D image. The image was edited and recorded using Nis Elements AR 3.2. software. Also, 3D images and graphs were constructed so as to present the intensity of fluorescence recorded for individual tests.

***In vitro* observations by atomic force microscopy (AFM) and scanning electron microscopy (SEM)**

After a 30-minute application of formulations containing terpenes or control on forearm or abdominal region of adults, the remains of the preparation were removed using tissue paper. Next, the tape striping procedure was conducted, that is, fragments of adhesive tapes were applied on the skin surface and pressed at a constant pressure of 1 kg (10 N) for 10 s (3, 4). In this way, corneocytes were collected from further, deeper layers of the skin and directly observed under an AFM or SEM microscope. The microscopic observation involved accurate scanning of subsequent corneocyte samples. The images obtained reflected the surface area, interaction forces and the topography of the tested sample.

Table 1. Summary of terpenes formulations used for research.

Terpene	Concentrations in ethanol 40° (w/w)	Concentrations in oil (w/w)	Concentrations in hydrogel (w/w)
Terpinen-4-ol	0.5%, 1%, 5%	0.5%, 1%, 5%	0.5%, 1%, 5%
Camphor	0.5%, 1%, 5%	0.5%, 1%, 5%	0.5%, 1%, 5%
Menthol	0.5%, 1%	0.5%, 1%, 5%	0.5%, 1%, 5%
Levomenthol	0.5%, 1%	0.5%, 1%, 5%	-
Eucalyptol	0.5%, 1%, 5%	0.5%, 1%, 5%	0.5%, 1%, 5%

Conditions of AFM and SEM observations were presented earlier (5).

Irritation test

The visualization analyses were supplemented by the determination of the degree of skin irritation by tested preparations, expressed by the percentage of survivability of the epidermis model cells EpiDerm®-200. The test was performed according to the procedure "ECVAM: *In vitro* skin irritation test: Human skin model".

The tested formulations were applied on the insert surface for 60 min. At the same time, a positive application of 5% SDS solution (i.e., sodium lauryl sulfate in water) and a negative (application of purified water) control sample has been done. After this time, inserts were washed a few times to remove the tested preparation, and placed in a fresh substrate. After 24 h of incubation, the medium was subjected to analysis for interleukin IL-1α content, and the tissue was incubated for another 18 h to perform an MTT test for cytotoxicity of the tested substance. The survivability of cells was defined for each insert as the percentage of the average survivability as compared to the negative control. The tested sample is defined as irritating if cell survivability is lower than 50%.

RESULTS AND DISCUSSION

***Fluorescent microscopy* (*ex vivo* studies))**

Images of *stratum corneum* and single corneocytes selected from among several hundred pictures and the intensity of fluorescence before and after the application of tested preparations are presented in Figure 1.

In the tests, grapeseed oil, ethanol 40° or hydrogel were used as vehicles. First, vehicles were applied on appropriately prepared skin which was stained with prepared fluorophores after incubation. The aim of this was to visualize the influence of vehicles themselves on *stratum corneum*.

It was found that ethanol 40° destroys the *stratum corneum* structure. This observation confirmed previous reports on the destructive effect of ethanol on *stratum corneum* cells, involving lipid extraction from intercellular spaces. At the same time, it was observed that the application of pure grapeseed oil and hydrogel did not have a significant influence on the *stratum corneum* structure.

In numerous tests, it was proven that the factor determining the intensity of the action of xenobiotics on the skin is, amongst other things, the type of the vehicle used (6, 7). As shown in the tests, the vehicle itself can have a destructive influence on the *stratum corneum*, while a combination of the vehicle with the xenobiotic interfering with the *stratum corneum* structure may cause even greater damage to the *stratum corneum*. The obtained in this study photographs revealed various degrees of destruction to the skin barrier, i.e., the *stratum corneum*, under the influence of terpenes depending on the substrate in which it was incorporated.

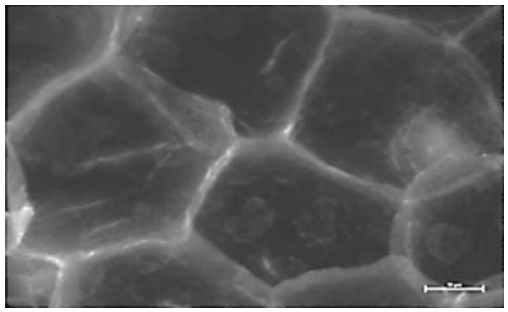
First, oil and ethanol solutions, as well as hydrogels with terpinen-4-oil at concentrations of 0.5%, 1% and 5%, were applied. The photographs taken indicate the destructive influence of this terpene on *stratum corneum* cells. It can be claimed that the degree of disturbing the ordered *stratum corneum* structure is the greatest when this compound was applied in ethanol while the application of oily solution and hydrogel at the same concentration did not induce such drastic changes.

Another terpene applied on the skin was (+)-camphor. Preparations were fixed with the content of 0.5, 1 and 5% of terpene in the same three vehicles. Various types of staining of *stratum corneum* cells were observed after prior application of 1% camphor preparations in various vehicles. *Stratum corneum* stained with RBHE, which is a lipophilic stain, therefore, if the *stratum corneum* is undamaged it has affinity to intercellular lipids, as a result it was stained in a uniform manner. It is probably caused by the destructive action of camphor in combination with ethanol on the corneocyte shell.

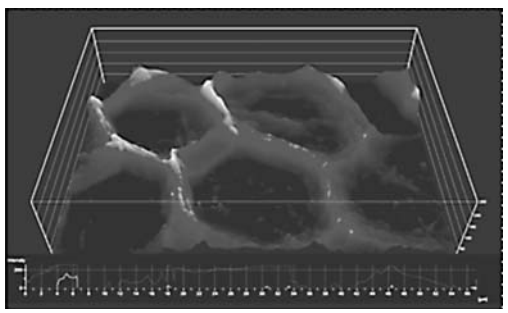
In the case of staining the skin with NaFl, previously subjected to the action of racemic menthol solutions at the same concentration (0.5%) but in various vehicles, various fluorescence intensities were observed. It can be assumed that this is also caused by the influence of the vehicle on the *stratum corneum* structure. The least intense fluorescence was seen for the oil vehicle. This indicates the weakest influence of this vehicle on the menthol action intensity on the *stratum corneum* structure. Hydrogel has an indirect effect on the menthol action intensity.

Levomenthol was applied onto the skin in oil solution at concentrations of 0.5, 1, 5% and in the ethanol solution at concentrations of 0.5 and 1%. Due to the low solubility it was not possible to prepare ethanol solution at a concentration of 5%. While analysing photographs taken after the application of 1% ethanol solution of levomenthol, stronger staining of corneocytes with SRB than in the case of skin subjected to the action of oil solution of this terpene was found. More intense fluorescence could have been caused by additional destruc-

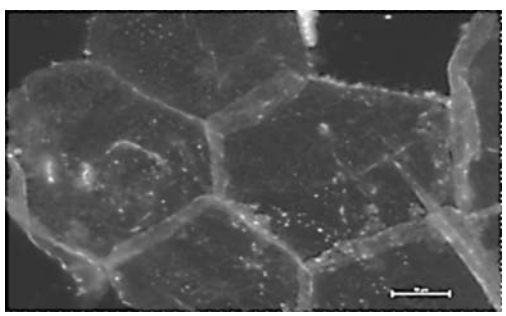
Control samples



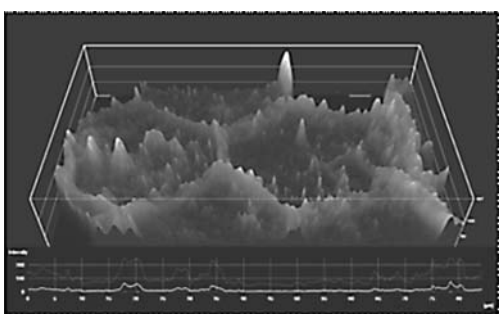
RBHE-stained spaces between corneocytes after the application of ethanol 40° (scale 10 μm).



Fluorescence intensity. RBHE-stained sample after ethanol 40° application.

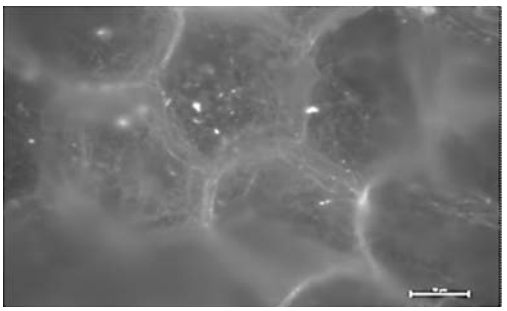


RBHE-stained spaces between corneocytes after the application of oil (scale 10 μm).

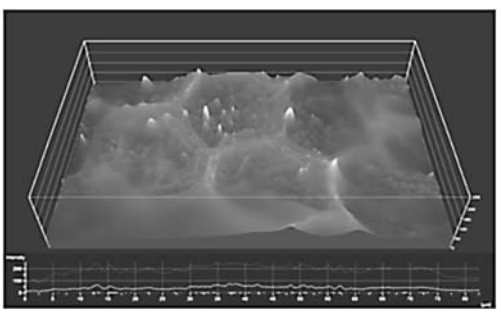


Fluorescence intensity. RBHE sample after the application of oil.

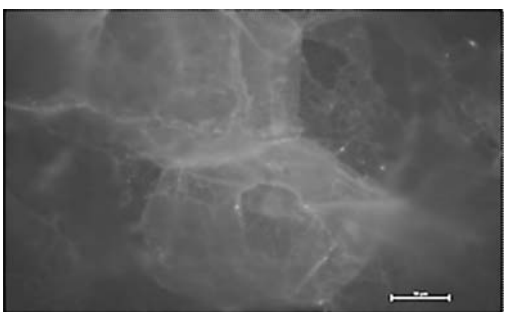
Tested samples



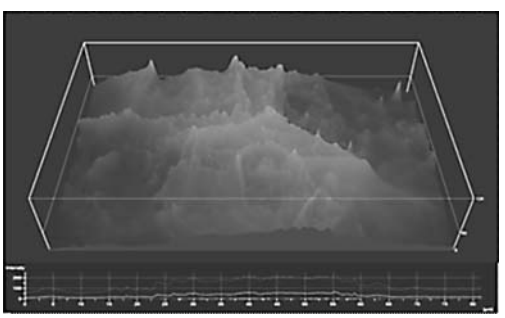
RBHE-stained corneocytes and intercellular spaces after the application of 5% (+)-camphor in ethanol (scale 10 μm).



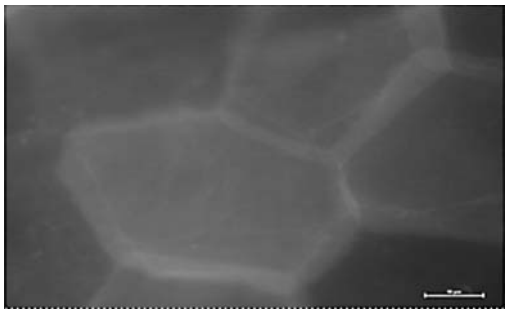
Fluorescence intensity. RBHE-stained sample after application of 5% (+)- camphor in ethanol.



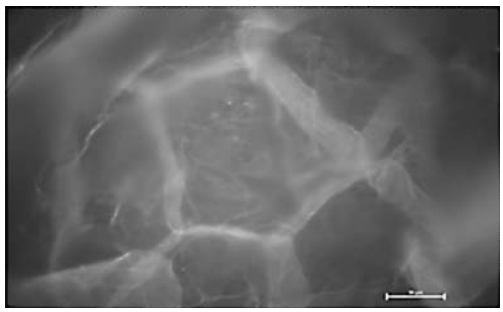
RBHE-stained corneocytes and intercellular spaces after the application of 5% levomenthol oily solution (scale 10 μm).



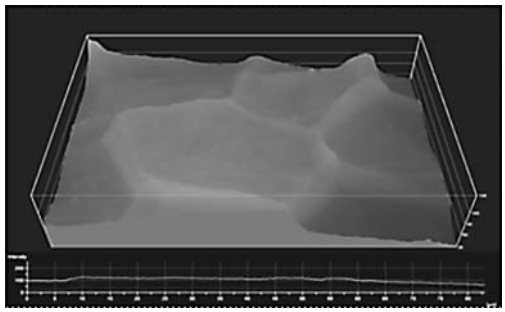
Fluorescence intensity. RBHE-stained sample after the application of 5% levomenthol oily solution.



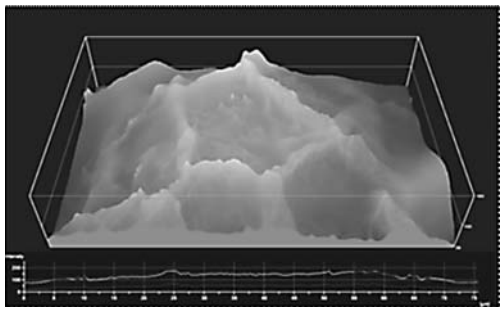
NaFl-stained corneocytes after the application of hydrogel (scale 10 µm).



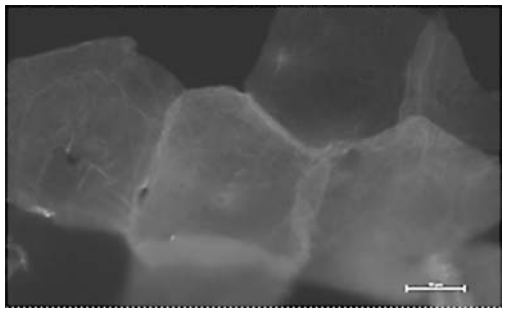
NaFl-stained corneocytes after the application of 5% eucalyptol hydrogel (scale 10 µm).



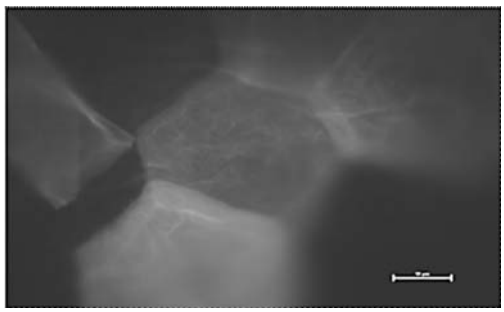
Fluorescence intensity. NaFl-stained sample after hydrogel application.



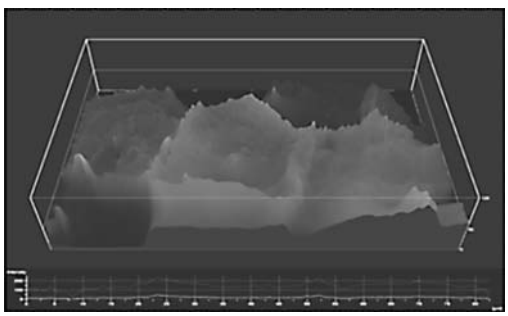
Fluorescence intensity. NaFl-stained sample after application of 5% eucalyptol hydrogel.



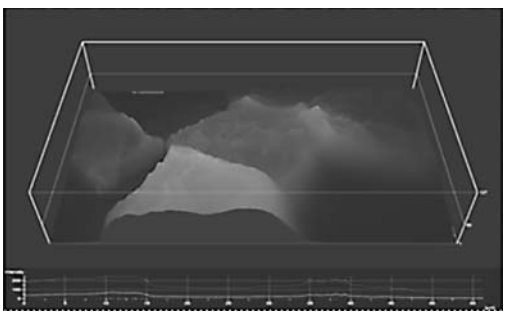
SRB-stained corneocytes after the application of oil (scale 10 µm).



SRB-stained corneocytes after the application of 5% tepinen-4-ol oily solution (scale 10 µm).



Fluorescence intensity. SRB-stained sample after oil application



Fluorescence intensity. SRB-stained sample after the application of 5% terpinen-4-ol oily solution.

Figure 1. Fluorescent images of the *stratum corneum*

tive influence of ethanol on the *stratum corneum* as described in the above cases.

Another tested terpene was eucalyptol. While comparing effects of this terpene in various vehicles, again, the basic difference between the photographs taken was the intensity of fluorescence. Allegedly, this was caused by greater absorption of the stain into corneocytes. Based on the photographs taken, it can be concluded that the ethanol solution contributes the most to the destruction of the *stratum corneum* struc-

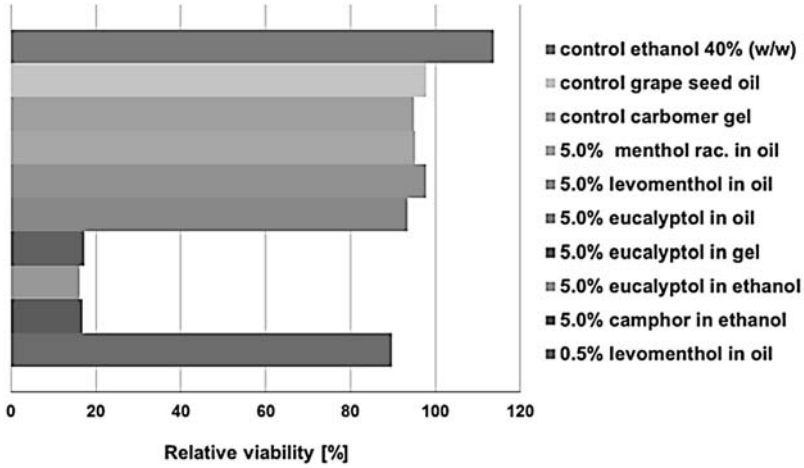
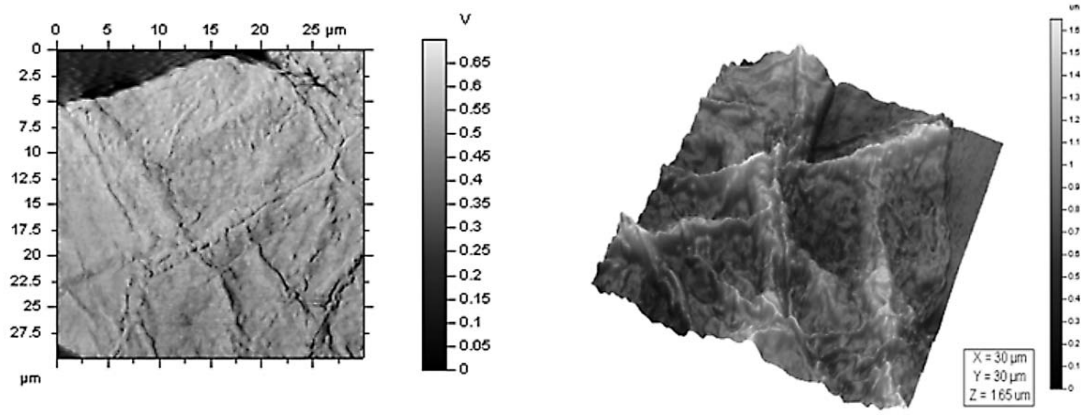


Figure 2. Results of irritation test



2D topography 3D topography

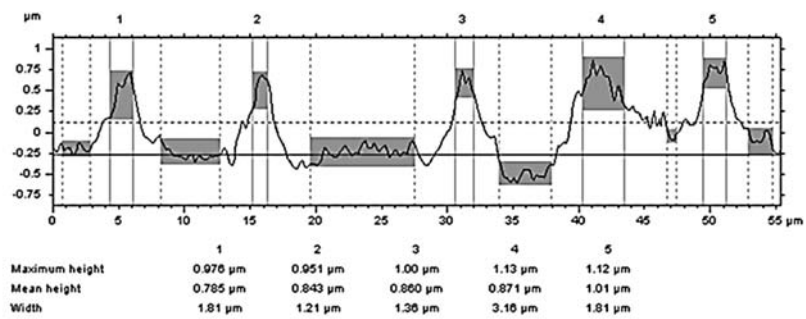


Figure 3. Typical AFM imaging of the *stratum corneum* and data set

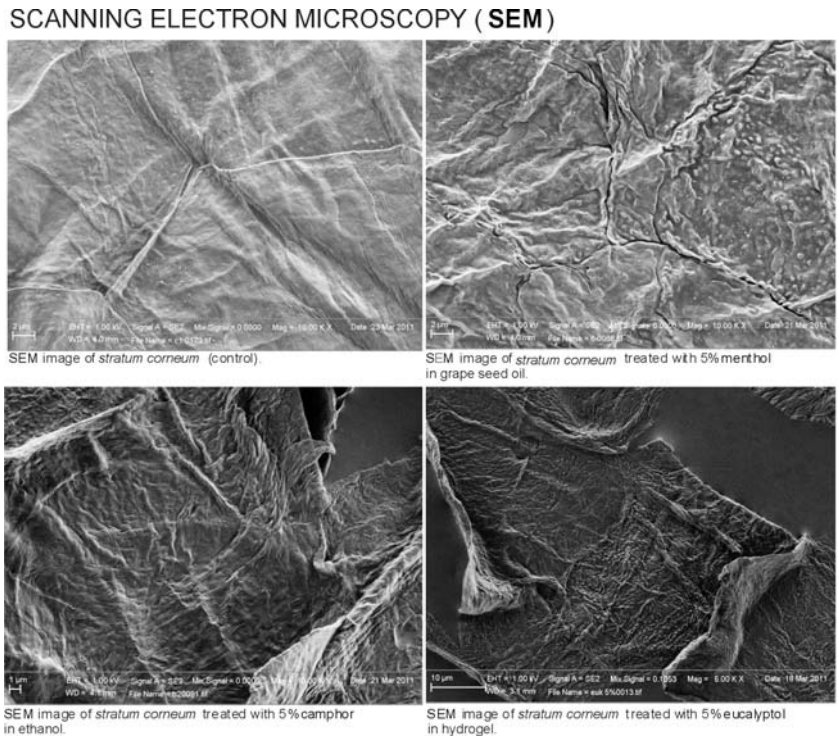


Figure 4. Typical SEM images of the *stratum corneum*

ture causing greater penetration of the stain into corneocytes. Oil solution, on the other hand, has the weakest effect on the *stratum corneum* structure.

While comparing changes in the *stratum corneum* structure induced by terpene preparations at various concentrations, applied in the same vehicle, it can be unambiguously concluded that the terpene concentration has a positive influence on the induced negative effect on *stratum corneum*.

Irritation test (in vitro tests)

Tests were performed using the epidermis model EpiDerm. Preparations with the strongest destructive effect on the *stratum corneum ex vivo* and pure vehicles were used in the test. The results of the test, expressed in the form of percentage of survivability of the cells, are presented in Figure 2. 5% ethanol solutions of (+)-camphor and eucalyptol and hydrogel with 5% of eucalyptol can be classified as irritating (cell survivability < 50%).

Atomic force microscopy (in vivo tests)

AFM imaging was performed by collecting the *stratum corneum* after the application of terpene-containing and control preparations. The obtained sample images of corneocytes and the sets of data are presented in Figure 3. For all preparations tested, no significant and repeatable changes were observed in the topography and in the roughness of cells, and friction forces between the "blade" of the microscope and the cell structure.

Scanning electron microscopy (in vivo tests)

SEM imaging was performed by collecting the *stratum corneum* after the application of terpene-containing and control preparations. Sample images are presented in Figure 4. Only slight changes in *stratum corneum*/corneocyte morphology were observed, which were manifested, for example, by edge curling.

CONCLUSIONS

- Terpenes in ethanol solutions have stronger destructive properties on the *stratum corneum ex vivo* than terpenes dissolved in oil.
- Among tested compounds, (+)-camphor and eucalyptol exhibit the strongest action destroying the *stratum corneum* structure *ex vivo*, in particular, when ethanol is the vehicle.
- 5% ethanol solutions of eucalyptol and camphor and hydrogel with 5% eucalyptol had the

strongest irritating effect on *stratum corneum* cells in the irritation test *in vitro*.

- In the *in vivo* tests (AFM, SEM), no negative effect of terpenes on the *stratum corenum* and its cells was confirmed.
- For terpenes, the *stratum corneum* layer *ex vivo* and its *in vitro* equivalents may not be analyzed as models equivalent to *in vivo* tests.

REFERENCES

1. Williams A.C., Barry B.W.: Pharm. Res. 8, 17 (1991).
2. Williams A.C., Barry B.W.: Adv. Drug Deliv. Rev. 56, 603 (2004).
3. Moser K., Kriwet K., Naik A., Kalia Y.N., Guy R.H.: Eur. J. Pharm. Biopharm. 52, 103 (2001).
4. Surber C., Schwarb F.P., Smith E.W.: J. Toxicol. Cut. Ocular Toxicol. 20, 461 (2001).
5. Wosicka-Frackowiak H., Cal K., Stefanowska J., Glowka E., Nowacka M., Struck-Lewicka W., Govedarica B. et al.: Int. J. Pharm. 495, 807 (2015).
6. Cal K.: Planta Med. 72, 311 (2006).
7. Cal K.: Arch. Dermatol. Res. 297, 311 (2006).

Received: 22. 08. 2016