Acta Pharm. **71** (2021) 657–667
https://doi.org/10.2478/acph-2021-0044

Short communication

Propolis – quality analysis and use in topical formulations

KRISTINA RAMANAUSKIENE[1]
ASTA MARIJA INKENIENE[1]*
KRISTINA PERMINAITE[1]
MODESTAS ZILIUS[1]
DAIVA MAJIENE[2]

[1] *Department of Clinical Pharmacy Lithuanian University of Health Sciences Sukileliu Street 13, LT-50161 Kaunas Lithuania*

[2] *Neuroscience Institute, Lithuanian University of Health Sciences, Eivenių Street 4, Kaunas, Lithuania*

Accepted November 23, 2020
Published online January 26, 2021

The aim of this study was to produce propolis extracts, assess their quality and effect on skin cells and determine the penetration of active ingredients from designed semi-solid topical formulations. The use of higher-concentration ethanol and a larger amount of raw material allows extracting a larger quantity of active ingredients from raw propolis. Ultrasound extraction is an effective method for the production of aqueous extracts of propolis. The results show that depending on concentration, propolis extracts reduce the viability of keratinocytes. The phenolic compounds under observation penetrated the epidermis and dermis from designed formulations. The base of semi-solid formulation influences the efficacy of propolis preparations. The overall quantity of phenolic compounds that penetrated the skin was around 2 % from the ointment and 1.5 % from the cream.

Keywords: phenolic compounds, propolis extracts, skin penetration, cell viability

Nowadays, products containing natural compounds are gaining more interest due to their biological activity, various possible applications and use for the treatment of a wide range of diseases. Propolis, a beneficial product naturally occurring in nature, is a resinous, viscous material of plant and animal origin (1). Propolis has antimicrobial and anti-inflammatory properties, reduces oxidative stress, is suited for the treatment of burns and can even fight cancer cells (2). It should be noted that propolis kills different types of bacteria, viruses and fungi; therefore, it is very important for the treatment of skin diseases. Previous studies found that phenolic acids and flavonoids contained in propolis act as excellent antioxidants and can protect cells against lipid peroxidation (1). It is important to note that many studies of the chemical composition of propolis have been carried out over the past 50 years. During studies, a total of over 300 chemical compounds was found in propolis, and each sample may contain 80–100 of them (1, 2). Due to such rich chemical composition, products containing propolis can be applied in various commercial sectors. Therefore, it is relevant to find the most suitable extraction conditions for the isolation of active compounds. Unprocessed raw propolis cannot be used: it must be cleaned using a solvent that extracts the main ingredients. Generally, the quality of extracts depends on

* Correspondence; e-mail: asta.inkeniene@lsmuni.lt

the proper choice of extraction method. Bioactive compounds may be extracted using traditional extraction methods (3). The main drawback of traditional methods is the long time necessary for extraction. One of the most promising methods of extraction is using ultrasound (4). For green extraction, water is more and more often used as a solvent. In order to ensure both effective and safe use of propolis extracts, non-harmful solvents should be used. Therefore, water and ethanol solutions were chosen as solvents for the production of propolis extracts. Due to such a broad spectrum of action, aqueous and ethanolic extracts of propolis may be used as active components for designing topical semi-solid pharmaceutical formulations to be used on the skin (5). The purpose of such pharmaceutical formulations may be therapeutic, *e.g.* treatment of a disease, or protective, where a disease is not diagnosed. Protective dermatological products should be developed aiming to ensure distribution of the active ingredient in the epidermis and dermis. Potential toxic effects of propolis on skin cells should be assessed in order to design safe semi-solid formulations with propolis extract. The effectiveness and safe use of semi-solid preparations depend on the characteristics of both the active ingredient and the excipients. Targeted selection of excipients must ensure effective penetration of active compounds into the human skin. One of the essential steps in the research of dermatological preparations is determining how an active ingredient, having passed the skin barrier, penetrates and moves into deeper layers of the skin (6). Penetration of active ingredients into the skin is a complex process dependent on many factors; therefore, research of the distribution of the active compounds of propolis in skin layers is required. The aim of this work is to produce propolis extracts, assess their quality and effect on skin cells and determine the penetration of active ingredients from designed semi-solid topical formulations.

EXPERIMENTAL

Materials

Rectified ethanol for food purposes, 96.3 % (JSC "Vilniaus degtine", Lithuania), purified water (LUHS) and propolis raw material (Lithuania) were used in this study. Folin-Ciocalteu's reagent, 2 mol L^{-1}, *p*-coumaric acid, caffeic acid, chlorogenic acid, ferulic acid, vanillic acid and vanillin reference standards as well as acetonitrile were purchased from Sigma-Aldrich (Germany).

Propolis extraction methods

Propolis used for extraction was collected in Lithuania Obelynė (Kamša forest), Kaunas region in late July of 2018. Predominating plants harvested propolis in the region were leafy forest, coniferous wood.

Production of propolis liquid extracts by maceration

The studied extracts containing 2.5, 5 and 10 % propolis were prepared. As solvents, purified water, 40 and 70 % (*V*/*V*) ethanol were used. Crushed crude propolis was soaked in an appropriate amount of solvent and left for maceration 24 hours at 15–25 °C temperature. Manufactured extraction was filtered using a paper filter and stored in a refrigerator at a maximum of 4 °C (3).

Production of propolis liquid extracts by ultrasonic extraction

Aqueous propolis extracts of different concentrations (2.5, 5 and 10 %) were prepared by using the ultrasound extraction method. Crushed crude propolis was placed in a centrifuge tube and filled with an appropriate volume of solvent. The aqueous extracts were placed in an ice bath and an ultrasonic probe was inserted into the tube. Extracts were prepared under the following conditions: 100 % ultrasonic amplitude, 50 % ultrasonic cycle (1 cycle is 1 second). Extraction was carried out for 15, 30, 60 minutes. After extraction, the extracts were centrifuged, filtered through a paper filter and stored in a refrigerator at a maximum of 4 °C (4).

Production of propolis soft extract

The liquid extract of propolis 10 % obtained with 70 % ethanol was evaporated on a water-bath until it became thick consistency. The propolis soft extract was stored in a glass bottle.

Preparation of semisolid samples with propolis extract

Propolis soft extract was introduced to semisolid preparations: ointment and cream for assessing the impact of excipients on the penetration of active propolis compounds. Ointment (T) base contained the beeswax and sunflower oil (ratio 1:10) and cream (K) was prepared to introduce propolis extract into emulsion base (w/o) contained water 30 %, vaseline and stearyl alcohol as an emulsifier. The concentration of propolis soft extract was 3 %.

Determination of total phenolic compounds in propolis extracts

The determination of the total phenol content of propolis extracts. The total amount of phenolic compounds was determined using the Folin-Ciocalteu reagent. All samples were analysed with a spectrophotometer (Agilent 8453, Australia) at 765 nm wavelength. During the reaction, the phenolic compounds are capable of reacting with the Folin-Ciocalteu reagent to form blue complex compounds. Total phenolic compounds were expressed in *p*-coumaric acid equivalent (7).

Qualitative and quantitative analyses of propolis phenolic compounds

Capillary Liquid Chromatography (Agilent Infinity 1260 Capillary LC-DAD, Agilent technologies®, USA) is used for qualitative and quantitative analyses of phenolic compounds in propolis samples. Phenolic compounds were separated by using ACE C18 column (Advanced Chromatography Technologies, Scotland) with such parameters: 150 × 0.5 mm, particle size was 5 μm. The conditions of validated HPLC method: mobile phase was composed of eluent A (0.5 % acetic acid in deionized water) and eluent B (acetonitrile); the linear elution gradient was changed from 1 to 21 % of eluent B and 99 to 79 % of eluent A for 25 min; the flow rate was 20 μL min^{-1}; the column temperature was 25 °C and the injection volume was 0.2 μL. Detection of phenolic compounds was carried out at 290 nm. All the samples prepared for analysis were filtered through nylon membrane syringe filters (pore size 0.22 μm) (8).

Cell lines and cell culture

Immortalized human keratinocyte cell line HaCaT (CLS Cell Lines Service, 300493) was purchased from the Cell Lines Service GmbH (Germany). Cells of convenient concentration were seeded in culture flasks containing DMEM with 10 % fetal bovine serum, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. Cultures were then incubated at 37 °C with 5 % CO_2 and saturated humidity; culture transfer was performed once a week, and the medium was renewed twice a week.

Cell viability and proliferation assessment

Transformed human keratinocytes line HaCaT was selected to evaluate the cytotoxic properties of propolis extracts. HaCaT cell line was selected to represent the main cells in the human epidermis (9). Cell viability was assessed by measuring their ability to metabolize MTT and by nuclear fluorescent staining assay as previously described in the literature (9). After treatment with 10 % propolis ethanolic 70 % extract prepared by maceration and aqueous extract prepared by ultrasound 30 min, the cells were double-stained with Hoechst 33258 (15 μg mL^{-1}) and propidium iodide (PI; 5 μg mL^{-1}) for 15 min, and the viability was assessed under fluorescence microscope OLYMPUS IX71SIF-3 (Olympus Optical Co., Ltd.).

Penetration experiment through the full-thickness undamaged human skin

Abdominal skin of Caucasian women (age range: 25–40 years) was obtained from the Department of Plastic and Reconstructive Surgery (the Hospital of the Lithuanian University of Health Sciences, Lithuania) after cosmetic surgery. It was stored at –20 °C for not longer than 6 months before use. Kaunas Regional Biomedical Research Ethics Committee has approved the use of human skin for transdermal penetration studies. A Bronaugh-type flow-through diffusion cell with full-thickness human skin was used for *ex vivo* skin penetration experiments (n = 3). The efficient diffusion area in the cells was 0.64 cm^2. The diffusion cells were placed on the metallic heating block maintaining 37 °C temperature by a Grant TC120 thermostated circulating water bath (Grant Instruments Ltd., Great Britain). The acceptor medium (0.9 % NaCl solution with 0.005 % NaN_3) was circulated underneath the skin samples maintaining 0.6 mL min^{-1} of circulation rate by Masterflex L/S peristaltic pump with multichannel pump head (Cole-Parmer Instrument Co., USA). The infinite dose of the propolis ointment or cream was applied on the outer human skin-side surface, and the diffusion cells were covered with aluminum foil. After 24 hours, semisolid formulations were removed from the human skin surface. The epidermis was separated from the dermis by applying the dry heat separation method. The experiment was conducted according to the methods proposed by Zilius *et al*. (10).

Statistical analysis

All tests were performed three times. The mean values and standard deviations of the results were calculated using IBM SPSS statistics 27 and Microsoft Office Excell 2016 programs. The significance of differences was evaluated using Student's t-test. The differences were statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Two extraction methods were employed in order to obtain the biologically active components of propolis. Water and ethanol solutions were used as solvents for the production of extracts. The results are summarized in Fig. 1.

The amount of phenolic compounds is directly dependent on the quantity of propolis used in the production of extracts. The results showed a trend of a larger total quantity of phenolic compounds with increasing ethanol concentration. Research findings showed that the largest quantity of active compounds was extracted using 70 % ethanol as a solvent, and the smallest total quantity of phenolic compounds was found in propolis aqueous extract. In the case of ultrasound extraction, the yield of phenolic compounds increases with time. Based on the quantities of phenolic compounds in produced extracts, it can be stated that the optimum extraction time using ultrasound is 30 min. It can be stated that the mechanical effect of ultrasound accelerates the extraction process and larger quantities of active compounds are extracted in comparison with the production of the aqueous extract by maceration. According to research data, ultrasound extraction is a fast, simple, effective and economical method (11).

In the next step, the analysis of the active compounds was performed using the HPLC analysis method. The aim of this study was to compare the content of phenolic acids and vanillin in propolis extracts obtained using different solvents. Liquid extracts samples containing 10 % propolis prepared by maceration and ultrasonic extraction (duration 30 min) were analysed. The five extracts with the highest content of phenolic compounds by the spectrophotometric method were selected for the study. HPLC analysis of active compounds of liquid propolis extracts showed that *p*-coumaric acid was prevalent in all extracts. Total *p*-coumaric acid content was about 35 % of all phenolic compounds found in these extracts (Fig. 2).

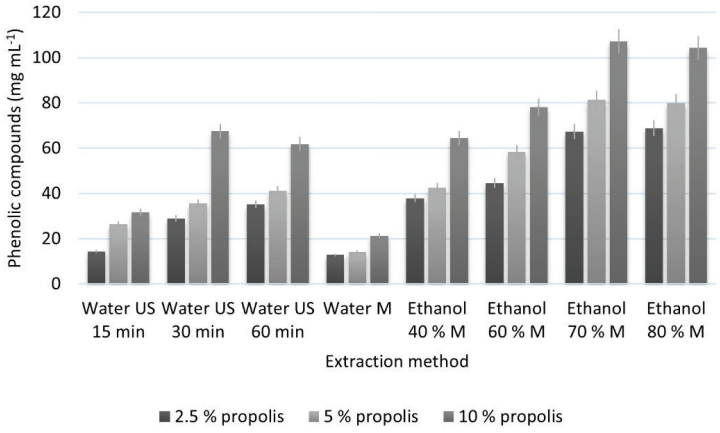

Fig. 1. Influence of ultrasound and maceration extraction methods on the extraction of phenolic compounds from aqueous and ethanolic propolis extracts of different concentrations (2.5, 5 and 10 %). Values are for mean ± SD for $n = 3$. US – ultrasonic extraction, time 15, 30, 60 min; M – maceration, extraction time 24 h.

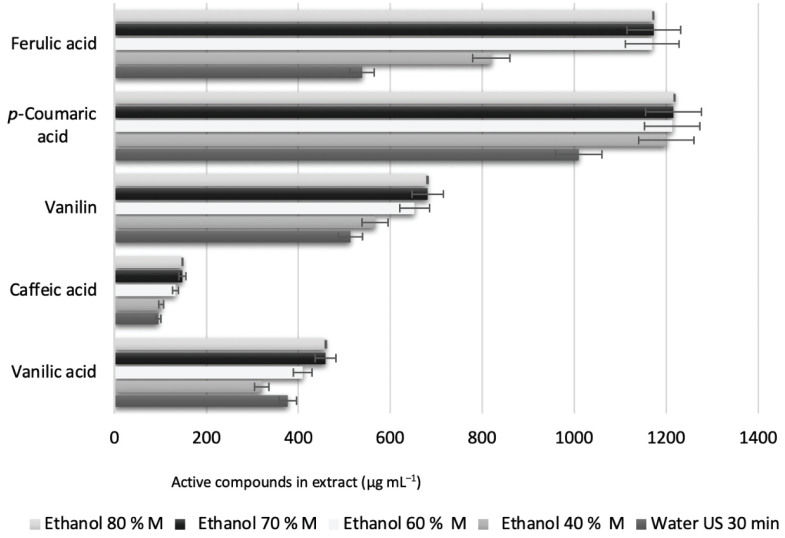

Fig. 2. Active compounds in propolis extracts. Values are for mean ± SD for $n = 3$. US – ultrasonic extraction, time 30 min; M – maceration, extraction time 24 h.

Such findings that propolis mostly contains *p*-coumaric acid coincide with the results of other researches (8). The second by quantity was ferulic acid (about 25 %) and the third component among all tested compounds was vanillin (about 20 %). The number of active compounds in the aqueous extract was smaller in comparison to ethanolic extracts ($p < 0.05$). The difference between the total amount of phenolic compounds in extracts produced using 70 and 80 % ethanol was not statistically significant ($p > 0.05$) (10). In order to avoid the presence of ethanol in the extracts due to the possible dermatological side effects and at the same time ensure a higher content of active compounds than in the aqueous extracts, a soft propolis extract was prepared. The same predominant compounds were detected in the soft extract (ferulic acid 9.79, *p*-coumaric acid 12.45, vanillin 5.43, caffeic acid 1.41 and vanillic acid 3.05 mg mL^{-1}) as in the liquid propolis extracts (Fig. 2). This extract contained a statistically significantly higher amount of active compounds compared to other propolis extracts.

In vitro research methods using cell cultures were chosen to assess the potential effect of propolis extracts on human skin. A stably transformed adherent human keratinocyte cell culture HaCaT was chosen as the epidermal model (9). The research aimed to assess the potential toxicity of propolis for HaCaT cells. Propolis 10 % extract produced using 70 % ethanol by maceration was used for the research, because this extract contained the largest quantity of active compounds. Propolis 10 % aqueous extract sonicated for 30 min was selected in view of the fact that the use of water as a solvent for green extraction is becoming increasingly common. Research results are presented in Figs. 3. and 4. The results are expressed as relative cell viability compared with control cells, which were not exposed to propolis extracts. The effect of propolis aqueous extracts (Fig. 3) on cell viability was compared with control cells.

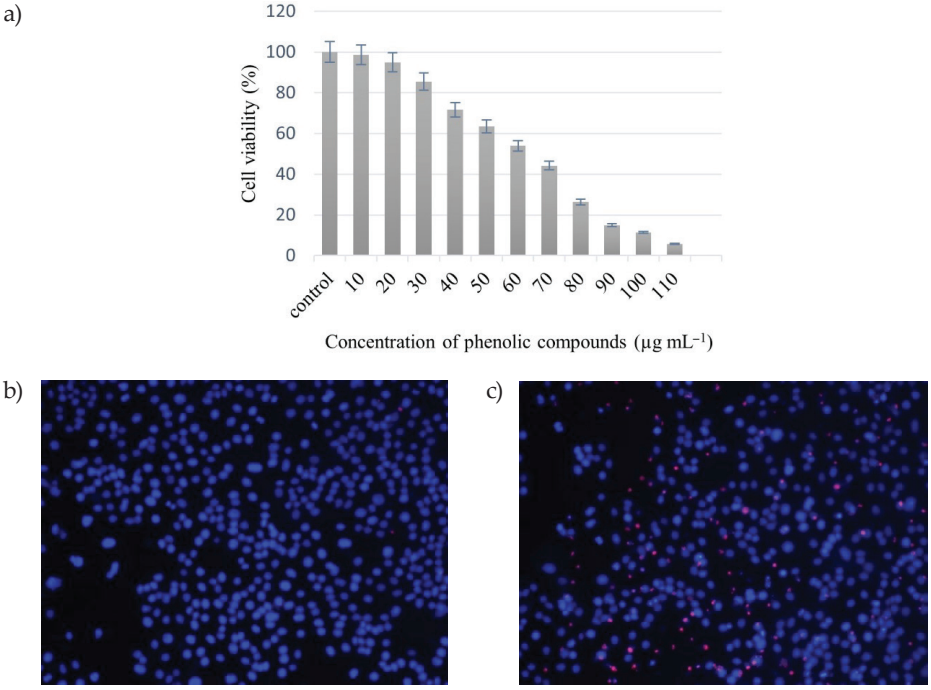

Fig. 3. a) The effect of propolis 10 % aqueous extracts (Water US 30 min) on cell viability; b) assessment of cell morphology; control; c) assessment of cell morphology, mean ± SD, $n = 3$.

The results obtained showed that after exposure of keratinocyte cells (HaCaT) to aqueous extract with up to 10 μg mL^{-1} of total phenolic compounds, cell viability remained almost unchanged. After exposure to extracts with 10–20 μg mL^{-1} concentration, the difference in cell viability from control cells was not statistically significant ($p > 0.05$). After exposure to extracts with 30 μg mL^{-1} concentration and higher, the difference of cell viability compared to control cells was statistically significant ($p < 0.05$). Cell viability decreased from 85.5 to a minimum of 5.7 %. The effect of propolis extracts obtained using 70 % ethanol (Fig. 4) on cell viability was compared to control cells.

When exposed to extracts containing up to 2 μg mL^{-1} active compounds, cell viability remained unchanged. After exposure of HaCaT to 70 % ethanolic extracts with a concentration of 3.0 μg mL^{-1} or higher, cell viability decreased from 85.3 to the minimum of 0.5 % compared to control cells. Research results showed a statistically significant ($p < 0.05$) difference between viability of control cells and viability of cells exposed to 70 % ethanolic extract of propolis with a concentration of 3.0 μg mL^{-1} or higher. During the assessment of propolis aqueous and ethanolic extracts on cell viability, it was found that with the increase of total concentration of phenolic compounds in samples, cell viability decreased. Cell viability tests showed that if propolis aqueous extract is used, a higher concentration of active compounds is necessary to achieve a statistically significant decrease in HaCaT cell viability compared to propolis ethanolic extracts. It can be assumed that this may have

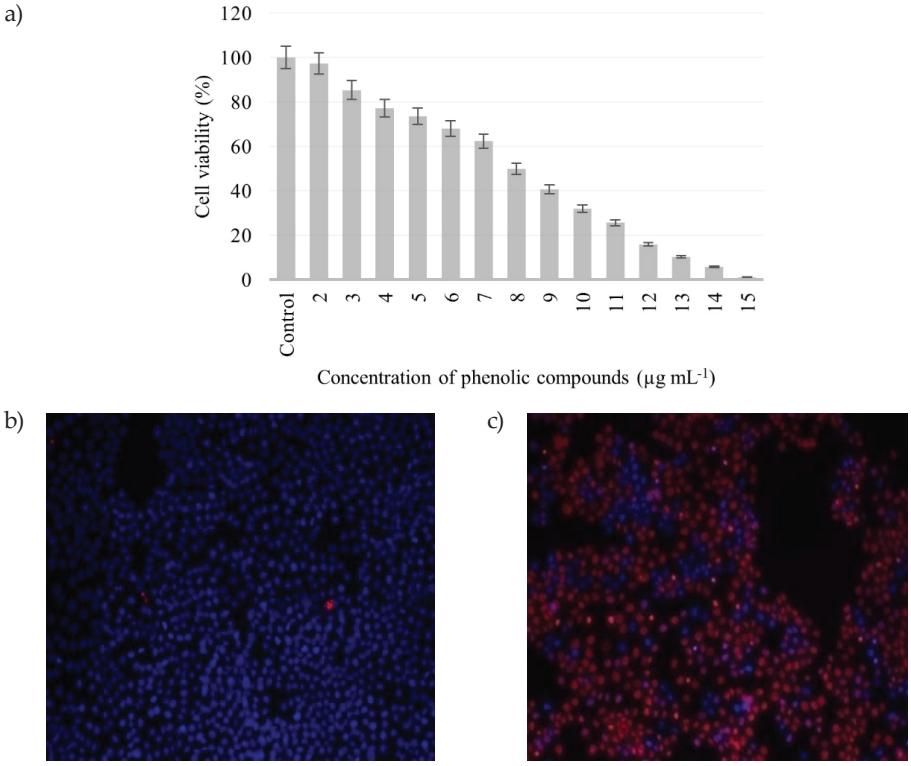

Fig. 4. a) The effect of propolis 10 % extracts obtained using 70 % ethanol on cell viability; b) assessment of cell morphology; control; c) assessment of cell morphology, mean ± SD, $n = 3$.

been caused by other compounds that had been extracted but not identified by HPLC and could have affected the viability of cells. In order to determine the effect of propolis on HaCaT cell viability, cell morphology was assessed, it was found that the main way of cell death caused by aqueous (Fig. 3b) and ethanolic extracts (Fig. 4b) of propolis was necrosis. Research results showed that the viability of cells exposed to aqueous extract of propolis is higher compared with cells exposed to propolis ethanolic extract.

In order to achieve topical effects of propolis preparations, phenolic compounds should penetrate the epidermis and into the dermis. This is particularly important if topical skin products containing propolis are intended to have anti-inflammatory effects.

In order to assess the impact of excipients on the penetration of active compounds, an ointment with soft propolis extract and cream, with propolis introduced into emulsion base (w/o) were produced. In all formulations, propolis soft extract concentration was 3 %. This extract was selected because of the highest content of active compounds. The phenolic compounds penetrated the epidermis and dermis from all formulations. Having carried out an *ex vivo* test, the best penetration of all phenolic compounds was found in ointment (Fig. 5).

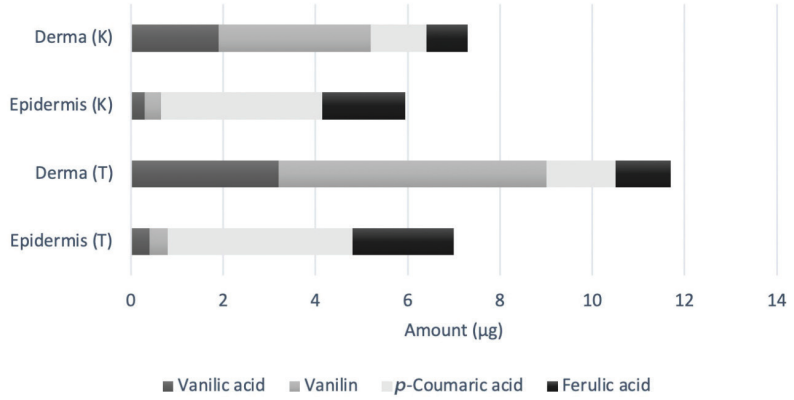

Fig. 5. Penetration of the phenolic compounds into the skin from semisolid formulations, mean ± SD, $n = 3$.

The quantity of ferulic and *p*-coumaric acids found in the epidermis was significantly greater ($p < 0.05$) than in the dermis. The quantities of vanillin and vanillic acid found in the dermis were significantly greater ($p < 0.05$) than found in the epidermis. In total, 0.77 ± 0.12 % of all phenolic compounds contained in propolis were found in epidermis, 1.25 ± 0.12 % were found in the dermis, and the total quantity of compounds absorbed by the skin from ointment was 2.05 ± 0.2 %. Caffeic acid was found neither in the epidermis nor the dermis. The number of active compounds absorbed from ointment was greater compared with the emulsion system. This could have been due to fatty acids contained in sunflower oil and beeswax, which are described in the literature as effective enhancers of penetration (12). The results of penetration of active compounds from the emulsion system into the skin showed that according to penetrated quantities, tested phenolic components ranked in the same order as with the hydrophobic system, but the concentration of all compounds was smaller in both the epidermis and the dermis compared to ointment. Petroleum jelly contained in the emulsion system does not have penetration-enhancing properties, which could have resulted in lower penetration of active compounds into the skin. Based on the penetration results obtained, an assumption can be made that the choice of base components influences the penetration of biologically active compounds of propolis.

Scientific literature provides data about the assessment of the quantitative composition of Lithuanian propolis determining the quantities of phenolic acids and vanillin (8). The fact that the content of ferulic acid in propolis is higher than that of vanillin is described in the studies of other researchers as well (13). During the research, the quantity of vanillic acid was significantly higher than caffeic acid. However, the studies of other researchers showed that the quantity of caffeic acid in propolis extract was higher than vanillic acid (14). Such differences may be due to different propolis collection sites and different types of plants used for making propolis (15). Ethanol and water are suitable solvents for the extraction of phenolic compounds from plant raw materials. Research results confirmed that excipients can impact the effectiveness of preparations (16). According to Barry theoretical guidelines for active ingredients used for the formulation of dermatological products, phenolic acids and vanillin identified in propolis extracts have positive properties:

relatively small molecular weight (< 600 Da), logarithmic value log *P* (between 1 and 3) of the partition coefficient between octanol and water, and good solubility in organic solvents (17). Excipient carrier substances are essential in the formulation of dermatological products because they influence the penetration and distribution of the active ingredient in the skin (16). Skin penetration results are strongly influenced by the top, corneous layer of the epidermis, consisting of keratinocytes whose main component is watertight α-keratin fibers (18). It is essential that the base contains substances capable of dissolving the active ingredients, hydrating the corneous layer of the epidermis or breaking down the lipid formations of this layer, thus improving the penetration of active ingredients. It was found, that due to a lower log *P* value of vanillic acid and vanillin molecules as described in another study (10), they tend to penetrate into the deeper layers of the skin and accumulate in the hydrophilic dermis. Ferulic and *p*-coumaric acids with a higher log *P* value are more lipophilic; therefore, they tend to stay in the lipophilic epidermis and their penetration into the deeper layers of the skin is limited. Penetration results obtained during the experiment can be linked with the information published in the scientific literature that lipophilic bases form a layer on the skin protecting against water loss. This increases the hydration of the corneous layer, which possibly also makes the penetration of active ingredients more effective (19).

CONCLUSIONS

Ultrasound extraction is an effective method for the production of aqueous extracts of propolis. The results show that depending on concentration, propolis extracts reduce the viability of keratinocytes; therefore, a safe concentration of active ingredients must be chosen when designing preparations to be used on the skin. *Ex vivo* research of the penetration of the active ingredients into human skin allowed us to compare the penetration of soft propolis extract compounds into skin layers from different semi-solid formulations. Active ingredients of propolis penetrate into the deeper layers of the skin and may be used in designing topical formulations as active ingredients.

Acknowledgement. – The authors acknowledge the financing BITE-KK-19-1-00676-PR001 "Applied research programmes for beekeeping and apiculture products" of the Lithuanian Beekeeping Sector Support Programme 2017–2019 supported by the National Paying Agency under the Ministry of Agriculture.

The study was approved by Kaunas Regional Biomedical Research Ethics Committee, Lithuania, (2008-11-05, No. BE-2-53, renewed 2016-07-14), with written consent of participation.

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