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Final scientific report

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Project title**:** ***Delivery to the dermocosmetic market of a modern topical formulation with betulinic acid encapsulated in proniosomes***

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Introduction

The present project was aimed to obtain a novel pharmaceutical formulation based on betulinic acid (BA) encapsulated in proniosomes for topical application in different cutaneous pathologies as adjuvant treatment. The selection of BA as active compound for the topical formulation was based on the data found in the literature, but also on our scientific background on this subject. BA is a natural compound, a pentacyclic triterpene, found in the bark of birch tree, that exhibits potent anticancer activity in different types of cancer including skin cancers (both *in vitro* and *in vivo*) and a low/absent toxicity in healthy cells. The main handicap of this compound is represented by its low solubility in aqueous media, limiting its use *in vivo*. To overcome this issue, this project proposes the formulation of BA as proniosomes, formulation that improves both its solubility and bioavailability.

To accomplish the main objective of the project, the project was organized in seven different objectives that comprise two activities each, except for objective 6.

Scientific and technical report

O.1. Synthesis and physicochemical characterization of proniosomes

A.1.1. Synthesis of the proniosomes – as nanoplatform for natural compounds with hydrophobic character

A.1.2. Physicochemical characterization: surface morphology, size distribution, zeta potential, and stability during formulation process and storage

O.2. **Synthesis and physicochemical characterization of proniosomes loaded with betulinic acid**

A.2.1. Synthesis and physicochemical characterization: surface morphology, size distribution, zeta potential, and stability during formulation process and storage

A.2.2. Assessment of the BA encapsulation efficiency of proniosomes and in vitro release profile

Applied methodology

The methods applied for the preparation and physicochemical characterization are standardized and internationally validated. The coacervation phase-separation method was performed for the preparation of the BA-loaded and blank proniosomes formulated as aqueous

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suspension. The method was conducted according to the protocols described in the literature and adapted to our laboratory conditions. The percentage of BA in the proniosomal suspension was 2.7%. The physicochemical characterization of the proniosomes was performed by the means of SEM, DLS and FT-IR techniques. The entrapment efficacy was analyzed spectrophotometrically.

Results

- the BA-loaded and blank proniosomes have the sizes environ 200 nm
- the suspensions of proniosomes (both blank and BA-loaded) are homogeneous having a polydispersity index < 5
- the suspensions are stable, their zeta potential being -21.90±3.64 mV for blank and -25.62±3.46 mV for BA-loaded, respectively
- after 3 months of storage at 2-8 °C, the suspensions were homogenous, without flocs, and no changes in color or smell were detected
- the entrapment efficacy of BA in the proniosomes was environ 60%
- ongoing studies are performed to improve the entrapment efficacy of BA.

O.3. *In vitro* toxicological profile of hollow and BA-loaded proniosomes

A.3.1. Assessment of selected test compounds impact on healthy human skin cells: keratinocytes, melanocytes, and fibroblasts.

A.3.2. Assessment of selected test compounds impact on 3D skin model: EpiDerm™ (MatTek In Vitro Science Laboratories)

Applied methodology

The toxicological profile of the proniosomal suspensions (blank and BA) and of BA-DMSO solution was assessed on healthy cell lines (keratinocytes – HEKa, fibroblasts – HDFa and 1BR3, and melanocytes – HEMa - Figure 1) and on 3D human reconstructed skin tissues (EPI-200 - Figure 2; EPI-200-SIT and EPI-200-PHO) purchased from MatTek Corporation.

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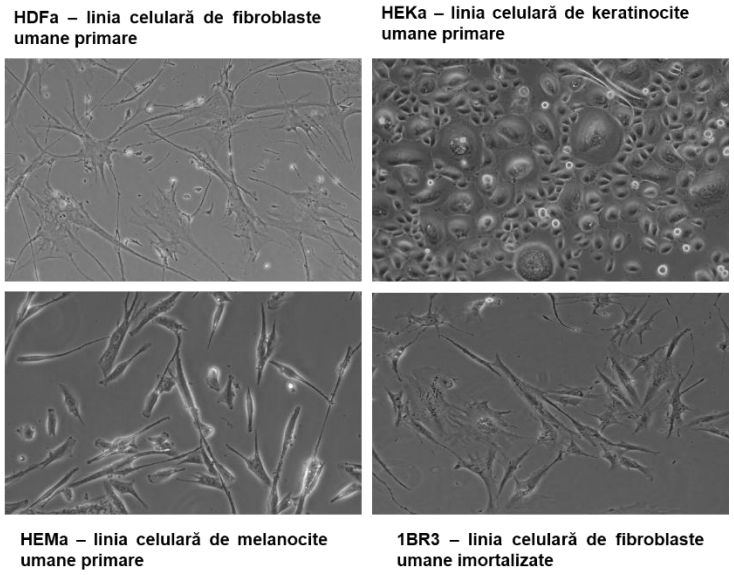

Figure 1. The healthy cell lines used for the in vitro toxicological profile of BA- loaded proniosomes

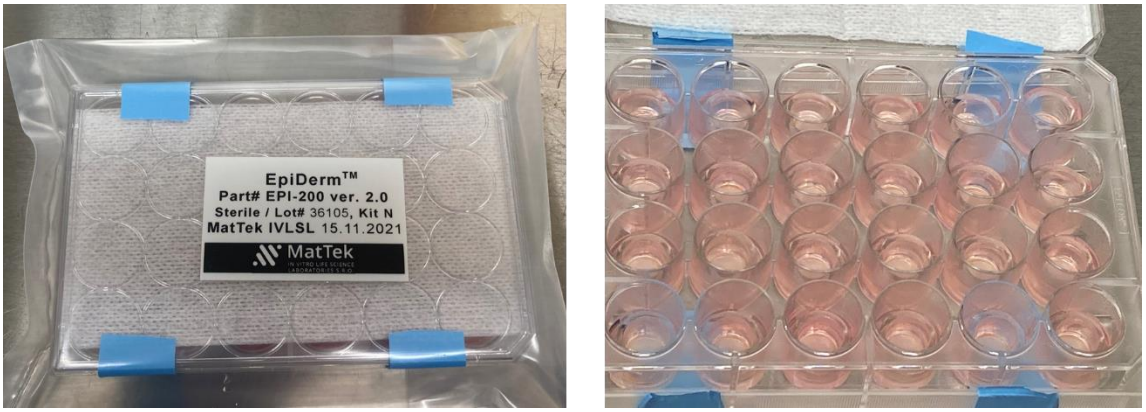

Figure 2. 3D human reconstructed skin tissues purchased from MatTek Corporation.

The impact of BA-loaded proniosomes versus BA-DMSO solution on healthy human cell lines was assessed by the means of standardized techniques as MTT (cell viability), LDH (cytotoxicity), immunofluorescence (cellular and nuclear morphology), methods that were described by Coricovac et al., IJMS 2021 (article that was partially funded from this project).

The *in vitro* experiments using 3D human skin tissues were performed according to the protocols provided by the manufacturer and the following parameters were analyzed: the impact of proniosomal suspensions (blank and BA), but also of BA (powder) on tissue viability, and the irritant and phototoxic potential. Standardized techniques have been used, adapted to the specific

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conditions of 3D tissues, such as: MTT ((3- (4,5-Dimethyl-2-thiazolyl) -2,5-diphenyltetrazolium bromide) - measures the viability and LDH (lactate dehydrogenase) - measures cytotoxicity. The methods are detailed in the publication of Pinzaru et al., Antioxidants (Basel) 2021 (partial funding from this research project).

Results

- BA in DMSO showed a selective cytotoxic action in healthy cells (HDFa, HEKa, HEMa, and HepaRG), the effect being dose- and cell-type dependent; the most significant cytotoxic effect was noticed at the highest concentrations tested - 50 and 75 µM.
- BA-loaded proniosomes also showed a reduction of healthy cells viability, but at a lower extent as compared to BA in DMSO
- The BA powder does not reduce the viability of 3D tissues and has no irritant or phototoxic potential
- the BA proniosomal suspension had a stimulatory effect on 3D tissues viability, exerted no irritant potential and a protective effect against the phototoxic UVA impact
- the blank proniosomal suspension had no negative impact on tissues' viability and no irritant potential, still its protective capacity against UVA exposure was lower as compared to BA proniosomal suspension.

All the graphs and images obtained during the studies will be used as parts for the following articles, and in order to avoid auto plagiarism, we decided not to include them in this report.

O.4. *In vitro* pharmacological profile of hollow and BA-loaded proniosomes

A.4.1. Assessment of test compounds impact on skin cancer cell lines: A431 – human epidermoid carcinoma, TE354.T – human basal cell carcinoma and SK-MEL-31 – human primary melanoma.

A.4.2. In vitro metabolism of BA loaded niosomes using Hepa RG cells

Applied methodology

Assessment of the pharmacological profile of BA was performed using consecrated cell viability and cytotoxicity methods, as: MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) and LDH (lactate dehydrogenase assay). To evaluate the changes

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induced at nuclear level was applied the Hoechst 33342 staining and the cellular morphological changes were highlighted using immunofluorescence. The tumor cell lines (A431 – human epidermoid carcinoma, TE354.T – human basal cell carcinoma and SK-MEL-31 – human primary melanoma – Figure 3) were purchased from ATCC (American Type Culture Collection) and were cultured according to manufacturer's recommendations.

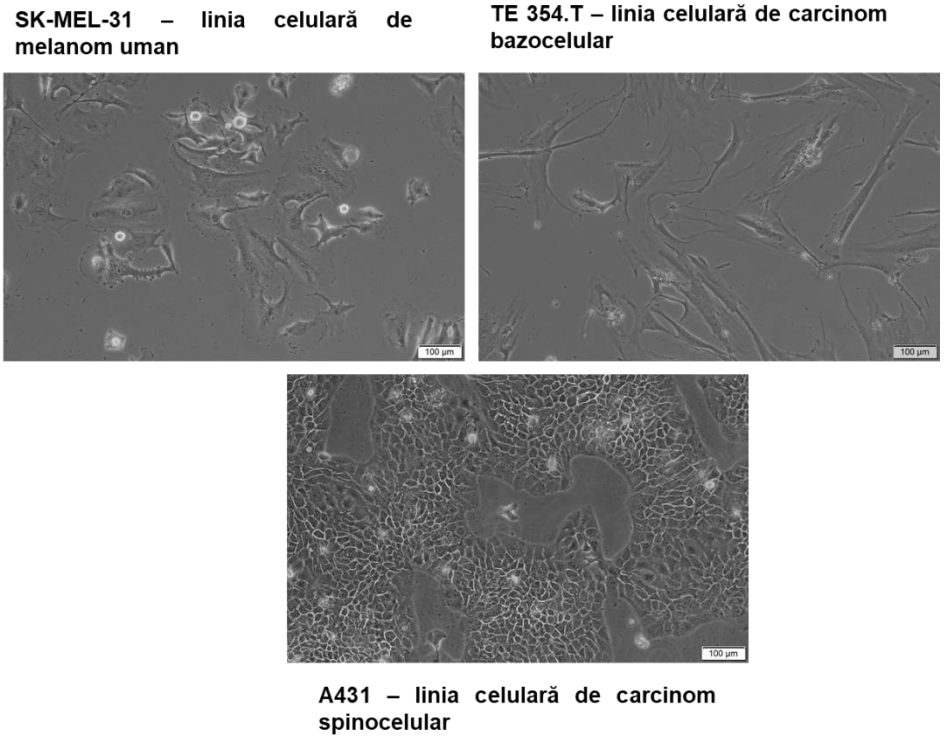

Figure 3. Human tumor cell lines studied.

The following parameters were monitored: cell viability (MTT and Alamar blue), changes in cell morphology (bright field and immunofluorescence), cytotoxicity (LDH) and changes in the nucleus (specific signs of apoptosis / necrosis) - Hoechst 33342. In order to avoid the obtention of false positive results due to the potential interference of proniosomes with the MTT absorbance measurements, were also applied techniques other kits such as Cell Titer Glo and Neutral Red.

The experimental design consisted of the assessment of both BA proniosomal suspension and BA in DMSO using different concentrations (1-75 μM) for a 24h treatment. The blank proniosomal suspension and DMSO solution were also tested in the same conditions as the test compounds.

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Results

- BA in DMSO induces a dose-dependent toxicity in tested tumor cell lines - TE 354.T, A431 and SK-MEL-31, toxicity characterized by decreased cell viability, cytotoxicity, morphological changes (round cells floating in the environment, reduced confluence, cell debris) and apoptotic changes at the nucleus level (fragmentation of the nucleus, condensation of chromatin, etc.)
- BA-loaded proniosomes exerted a similar effect as BA in DMSO, still the toxicity was recorded starting with the lowest concentration tested – 1 µM, what suggests an increased efficacy of BA after formulation as proniosomes.

All the graphs and images obtained during the studies will be used as parts for the following articles, and in order to avoid auto plagiarism, we decided not to include them in this report.

The results obtained at objectives 1 to 4 will be disseminated in the form of a scientific article (in the drafting phase) which will be published in an international journal with high impact factor (over 5).

O.5. Preparation and characterization of BA-loaded proniosomes as topical formulation for transdermal delivery

A.5.1. Selection and obtention of the type of topical formulation (emulgel, hydrogel, cream, ointment) appropriate for BA-loaded proniosomes delivery

A.5.2. Evaluation of topical formulation physico-chemical properties: rheological characteristics (color, odor, pH, ease of absorption, spreadability, homogeneity), stability, in vitro release capacity.

Applied methodology

The topical formulation selected for BA incorporation was proniosomal gel, that was prepared by coacervation phase-separation method. The surfactants used to obtain the proniosomal gel formula are GRAS (Generally Recognized as Safe) substances, as follows: cholesterol, sorbitan monostearate (Span 60) and soy lecithin (Lipoid S 75).

The surface morphology of the proniosomal gels was assessed by the means of scanning electron microscopy – SEM (Hitachi SU8230 electronic microscope provided with a STEM module and EDX X-Max^N 80 detectors). The pH was measured by the means of Sension ™ 1

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portable digital pH meter (Hach Company, U.S.A). The encapsulation efficiency was determined spectrophotometrically. The rheological features (flow behavior, viscosity, consistency, penetration degree and spreadability) were analyzed by a stress-controlled rheometer (RheoStress 1, HAAKE, France) equipped with a cone-plate geometry (1/60), a penetrometer (PNR 12, Petrolab, Germany) equipped with a micro-cone and suitable container, and the Pozo Ojeda-Sune Arbussa extensometer. RAMAN studies were also performed for the confirmation of BA incorporation within the gel.

The toxicological profile of the obtained proniosomal gels (blank and BA-loaded) was evaluated using 3D human skin tissues by applying the same methodology as described in objective 3.

Results

- the obtained proniosomal gels are non-Newtonian systems with a pronounced pseudoplastic-thixotropic behavior (Figure 4)

Figure 4. The visual appearance of the obtained pronisomal gels – Blank and BA (0.3%) after 6 months of storage.

- The pH value of the proniosomal gel containing betulinic acid was slightly lower (6.556±0.04) as compared to the neutral pH, due to the acidic character of the drug, still the determined pH values were within the provided range for semisolid preparations (4.5-8.5) by the national pharmacopoeia suggesting that will be well tolerated by the skin
- characterization of the rheological properties of the BA-based gel indicated a higher viscosity and thixotropy as compared to blank gel, a higher degree of penetration, but lower spreading capacity. All these properties fall within the specific parameters of semi-solid pharmaceutical preparations.
- the encapsulation efficiency of BA in the gel exceeded 50%.

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- the blank gel presents slightly spherical agglomerated vesicles, being somewhat evenly distributed
- BA-based proniosomal gel showed a rather high viscosity compared to the blank, which may mean that the betulinic acid has been successfully encapsulated in the structure of the gel
- RAMAN studies confirmed the presence of BA within the proniosomal gel
- The blank proniosomal gel showed a slight irritating effect (tissue viability approx. 80-85%), while BA gel slightly stimulated the viability of other tissues
- UVA exposure decreased the viability of tissues exposed to blank gel, while BA gel provided protection against the phototoxic effect induced by UVA.

The methods used to obtain the proniosomal gel and those applied for its characterization were standardized and described in the article published by Pinzaru et al., Antioxidants (Basel) 2021 (partial funding from this research project).

O.6. Quality assurance assessment of the final product by means of non-invasive methods

Applied methodology

The quality assurance assessment of the BA-loaded proniosomal gel was performed on healthy human volunteers in accordance with the European legislation concerning the involvement human healthy volunteers in testing the therapeutical agents for human use. The experiment protocol and the informed consent were approved by the Bioethics Committee of UMFVBT.

The methods applied were non-invasive using the Courage Khazaka equipment MPA 7 and were applied on both female (n=10) and male (n=10) volunteers. The physiological skin parameters were monitored as: erythema, melanin content, skin hydration, transepidermal water loss, sebum, skin elasticity.

Results

- the BA proniosomal gel was well tolerated on the skin, no signs of erythema or edema were noticed
- an increase in skin hydration was observed after BA proniosomal gel application and a mild increase in skin elasticity.

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- the other skin physiological parameters were not significantly influenced.

The results obtained at O5 and O6 will be disseminated as an original article (at the moment is in the final drafting phase) that will be submitted to an international journal with a high impact factor (> 5).

O.7. Patent proposal for BA-encapsulated in proniosomes topical formulation, media and scientific dissemination

A.7.1. Results dissemination by participating to national and international conferences and publication of articles in international ISI-indexed journals after the accomplishing the activities scheduled in O3, O4 and O5

A.7.2. Patent proposal elaboration.

Results

- participation to a national conference – 3 oral presentations
- participation to an international conference – 1 poster
- 2 scientific articles that are in the final draft phase
- elaboration of the patent proposal draft
- a bachelor thesis
- publication of 6 scientific articles in international journals with an impact factor > 4, the research was partially funded by the present project
- project description on UMFTVB site

Results dissemination

Conferences:

1. Pinzaru I, Marcovici I, Coricovac D, Macasoi I, Dehelean C. In vitro toxicological profile of rutin as antimelanoma agent. Farmacia: de la inovare la buna practica farmaceutica, Ed. Universitatii din Oradea, Congresul National de Farmacie editia a XVIII-a, 15-17 Septembrie 2021, pp.82. ISBN 78-606-10-2144-4 – oral presentation
2. Dehelean C. Coricovac D. Natural compounds a promising alternative for SARS-CoV-2 treatment. Farmacia: de la inovare la buna practica farmaceutica, Ed. Universitatii

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3. Coricovac D, Marcovici I, Pinzaru I, Macasoi I, Vlaia L, Soica C, Dehelean C. Betulinic acid formulated as proniosomal gel for topical use in skin disorders. Farmacia: de la inovare la buna practica farmaceutica, Ed. Universitatii din Oradea, Congresul National de Farmacie editia a XVIII-a, 15-17 Septembrie 2021, pp.99. ISBN 78-606-10-2144-4. – oral presentation
4. Coricovac D, Pinzaru IA, Marcovici I, Iftode OA, Moaca A, Macasoi I, Vlaia L, Dehelean C. Betulinic acid formulated as proniosomal gel – a promising candidate for skin cancer management. Toxicology Letters 368S1 (2022) S84–S283, pp. S195, Abstracts of the XVIth International Congress of Toxicology (ICT 2022), UNITING IN TOXICOLOGY, Maastricht, The Netherlands, September 18–21, 2022, doi: 10.1016/j.toxlet.2022.07.530 – poster presentation

Published articles:

1. Pinzaru I, Tanase A, Enatescu V, Coricovac D, Bociort F, Marcovici I, Watz C, Vlaia L, Soica C, Dehelean C. Proniosomal Gel for Topical Delivery of Rutin: Preparation, Physicochemical Characterization and In Vitro Toxicological Profile Using 3D Reconstructed Human Epidermis Tissue and 2D Cells. Antioxidants (Basel). 2021 Jan 10;10(1):85. doi: 10.3390/antiox10010085. **IF = 7.675**
2. Coricovac D, Dehelean CA, Pinzaru I, Mioc A, Aburel OM, Macasoi I, Draghici GA, Petean C, Soica C, Boruga M, Vlaicu B, Muntean MD. Assessment of Betulinic Acid Cytotoxicity and Mitochondrial Metabolism Impairment in a Human Melanoma Cell Line. Int J Mol Sci. 2021 May 4;22(9):4870. doi: 10.3390/ijms22094870. **IF = 6.208**
3. Pinzaru I, Sarau C, Coricovac D, Marcovici I, Utescu C, Tofan S, Popovici RA, Manea HC, Pavel IE, Soica C, Dehelean C. Silver Nanocolloids Loaded with Betulinic Acid with Enhanced Antitumor Potential: Physicochemical Characterization and In Vitro Evaluation. Nanomaterials (Basel). 2021 Jan 9;11(1):152. doi: 10.3390/nano11010152. **IF = 5.719**

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4. Pinzaru I, Chioibas R, Marcovici I, Coricovac D, Susan R, Predut D, Georgescu D, Dehelean C. Rutin Exerts Cytotoxic and Senescence-Inducing Properties in Human Melanoma Cells. Toxics. 2021 Sep 19;9(9):226. doi: 10.3390/toxics9090226. **IF = 4.472**

5. Simu S, Marcovici I, Dobrescu A, Malita D, Dehelean CA, Coricovac D, Olaru F, Draghici GA, Navolan D. Insights into the Behavior of Triple-Negative MDA-MB-231 Breast Carcinoma Cells Following the Treatment with 17β-Ethinylestradiol and Levonorgestrel. Molecules. 2021 May 8;26(9):2776. doi: 10.3390/molecules26092776. **IF = 4.927.**
6. Dehelean CA, Coricovac D, Pinzaru I, Marcovici I, Macasoi IG, Semenescu A, Lazar G, Cinta Pinzaru S, Radulov I, Alexa E, Cretu O. Rutin bioconjugates as potential nutraceutical prodrugs: An in vitro and in ovo toxicological screening. Front Pharmacol. 2022 Sep 23; 13:1000608. doi: 10.3389/fphar.2022.1000608. **IF = 5.988**

Bachelor thesis:

1. Lupan B, Coricovac D. Evaluarea efectelor farmacologice ale glicozidelor cardiotonice versus efecte toxice – June 2022

Project manager: Conf. Univ. dr. Dorina Coricovac