The betulinic acid (BA) was used for structural analysis and evaluation of resultant cancer cell growth inhibitory activity [1].

BA has anticancer, antibacterial, antifungal, and antiviral activity and other biological properties. The structural modifications provide derivatives known that have improvements in these areas [2].

Extracts of birch bark (Betula species) have been used in traditional medicine from ancient times because contain BA as a major component. So betulinic acid (BA) is a plant derived molecule that can increase apoptosis specifically in cancer but not in normal cells for this reason making it an attractive anticancer agent [3].

Pentacyclic triterpene with lupan skeleton is interesting in research of antitumor, antibacterial and anti-inflammatory therapeutic properties. These compounds have a limited bioavailability due to their hydrophobicity and limited solubility in an aqueous medium. Some triterpene/cyclodextrine with increased bioavailability can lead to obtaining therapeutic results at a greatly improved rate of efficacy/toxicity.

Triterpenes are secondary plant metabolites that are widespread in plants, mainly located in the peel, leaf and stem bark [4]. Particularly, high amounts of bioactive triterpenes have been identified in heather (Calluna vulgaris L.) and marigold (Calendula officinalis L.) such as ursolic acid, oleanolic acid, betulinic acid, α- and β-amyrin, lupeol and faradiol. These triterpenes have demonstrated a wide range of biological activities, including anti-inflammatory antioxidant and apoptotic activity [5].

Pentacyclic triterpene research began with the publication by Pisha et al. in 1995 [3] of the anticancer activity of betulinic acid, tested on melanoma cell lines. Antitumor activity of betulinic acid is currently known to be manifested on numerous tumor cell lines (breast, colon, lung, neuroblastoma mainly by inducing apoptosis). Data concerning biological activity of betulin are rather poor, betulin being much less studied compared to its relative activity, betulinic acid. Still in the last years, many researchers focused on the potential use of betulin in the pharmacological field, probably due to an increasing and continuous need for new cancer fighting agents [6].

The pharmacological relevance of the triterpene family [4] has increased during the last two decades and antitumor effects, combine demonstrating multitarget properties such as wound healing, anti-inflammatory, antibacterial, antiviral, hepatoprotective and antitumor effects, combined with low toxicity. Triterpene plants are source of actives for phytopharmaceutical development. Knowledge of the occurrence of triterpene plants is extensive, but little is known about their quantitative distribution. The aim is to search for rich sources of triterpene from lupane, oleane and ursane group as materials for extraction. A new kind of one-step plant extract with 78% betulin (BE) was prepared from birch bark [7]. Were investigated whether this kind of extraction procedure could be adapted to other triterpene plants in order to gain highly concentrated extracts with triterpene leading substances other then BE.

Various galenic possibilities are known for the preparation of triterpenes. As ingredients of medicinal plants, triterpene are used in traditional herbal medicine. The galenic possibilities of triterpene rich plant extracts are wide ranging and preparation of lupine, oleane and ursane extracts can be done like that [7]. Triterpenes are secondary plant metabolites that are widespread in plants, mainly located in the peel.

The outer bark of birch tree is rich in pentacyclic triterpene compounds such as betulinic acid (BA, 3β-hydroxy-20(19)-lupan-28-oic acid), betulin (BE, lup-20(29)-ene-3β,28-diol), lupeol (L, Lup-20(29)-en-3-ol) and other minor components, such as oleanolic acid, ursolic acid and betulinic aldehyde [8]. The pentacyclic triterpenes are known to have a wide-range of pharmaceutical activities, among them possessing anti-virus, anti-inflammatory, anticancer and other properties [9]. Betulin reaches the highest percentage within the composition of triterpenes from the birch bark [10] and exhibits significant therapeutic activity, acting as antitumor, antiviral and antiseptic agent. Betulinic acid, the oxidation product of betulin, reveals important anti-HIV-1 activity and specific cytotoxic activity against different tumor cell lines [8]. Betulin and betulinic acid have been involved in chemical modulation, leading to highly active derivatives, some of them comparable to clinically used drugs.
Betulinic acid is a chemical compound obtained from the outer bark of white birch tree *Betula alba* [3]. Biological activities were reported when betulinic acid was used, on anti-inflammatory, anti-tumor, anti-angiogenesis, anti-viral, anti-HIV, anti-neoplastic, anti-plasmodial. There were reported too selective anti-tumor activity on cultured human melanoma [11], neuroblastoma malignant brain tumor [12] and leukemia cells (HL60, U937 and K562) and neuroblastoma (GOTO and NB-I) cell growth [13]. Now BA is used in phase II of clinical trials to treat melanoma. There were reported the lack of toxicity towards normal cells, that suggested BA as an attractive anti-tumor agent [3]. That makes BA special in comparison to compounds that are currently used in cancer therapy, such as taxol, camptothecin, elipticine, etoposide, vinblastine and vincristine, compounds which are very toxic and inhibit replication of both cancer and normal cells [15]. This study represents the first try to obtain organic crystals of betulinic acid with some organic solvents.

**Experimental part**

**Materials and Methods**

**Raw materials**

The reagents (analytical grade): active substance (betulinic acid, BA), solvents (methanol, isopropanol, ethanol and NaOH solution) were provided by Merck and Sigma-Aldrich (Germany).

**Synthesis procedure**

0.1912 g NaOH in 2.4 mL distilled water was used to obtain a 2M solution. From this stock solution there were taken 100 µL and were mixed with 300 µL distilled water. 2.1 mg BA were mixed with 200 µL methanol and after that was added 300 µL methanol for another 3 times. The mixture was stirred using a Vortex and it was warmed up to 38 °C in a bath water.

The following samples containing betulinic acid were obtained:

- Sample 1: solution containing 0.8 mg BA, 8.5 µL NaOH 0.2 M and 100µL methanol
- Sample 2: 54 µL Sample 1 (BA+ methanol+ NaOH 0.2M) mixed with 20 µL distilled water. This sample is a mixture 2/3 methanol and 1/3 distilled water
- Sample 3: 20 µL Sample 2 mixed with 20 µL distilled water. This sample is a mixture 1/3 methanol and 2/3 distilled water.
- Sample 7: solution containing 2.1 mg BA dissolved in 1100 µL methanol.

**Characterization methods**

The aspect of samples was investigated using a microscope with fluorescence type - Optika Microscopes Optikam Pro Cool 5 and Optika View.

The evaluation of the sizes and the stability of BA crystals was done using a Cordouan Zetasizer system (Cordouan Technol., France) consisting of a Vasco Particle Size Analyzer and a Wallis Zetapotential Analyzer. There were set the following Vasco Particle Size Analyzer parameters: temperature (25°C), time interval (30 µs), number of channels (425), laser power (40%), acquisition mode (continuous), and analysis mode (Pade-Laplace). The following Wallis Zetapotential Analyzer parameters were chosen: cuvette type (plastic, with a wavelength between 380 and 780 nm), temperature (25°C), laser power (35%), applied field (automatic), resolution (medium, 0.8 Hz), 3 measures/sequence, and Henry function (Smoluchowski).

9 weeks old SKH1 hairless male mice were used in this research. The animals were achieved from Charles River Lab. (Hungary) and they were the subject of the skin carcinoma animal model presented in the literature by our team [16].

All the experimental procedures were carried-on according to all NIAH-National Institute of Animal Health rules: mice were fed *ad libitum* and kept under standard conditions (12 hours light/dark cycles, an almost constant temperature, 21±2 °C, and humidity between 50 and 60%); the procedures were first studied and approved by the Committee for Ethics Research of Victor Babes University of Medicine and Pharmacy Timisoara (Romania).

Previously presented samples were prepared as emulsions for an easier application on the mouse skin, following a procedure presented by A. Scheffler: 4% BA, 29.3% avocado oil, 14.7% almond oil and 52% water [17]. The mice were divided in 6 groups (n=3/mice group):

- group 0 (negative control group) - mice were exposed to a solvent mixture;
- group 1 - mice were exposed to suspension of Sample 1;
- group 2 - mice were exposed to suspension of Sample 2;
- group 3 - mice were exposed to suspension of Sample 3;
- group 7 - mice were exposed to suspension of Sample 7;
- group 9 (positive control group) - mice were exposed to a solution of sodium lauryl sulfate (1 M);

200 µL of every emulsion were applied on the dorsal side once a day at the same moment by the same operator, 2 times weekly for 5 weeks. During all the experiments, the emulsions were applied 30 min before any evaluation. For the evaluation of the skin’s response to the samples, two physiological skin parameters (erythema and the hydration level of stratum corneum) were measured, by the means of a non-invasive techniques (mexametry and corneometry) using a Multiprobe Adapter System (MPAS) from Courage-Khazaka Electronics (Germany), equipped with the following probes: Mexameter®MX18 and Corneometer®CM 825. The requirements of the equipment manufacturer (room temperature 20-23°C and the humidity 40-60 %) have been met during theses evaluations.

The determined values are presented as differences between a value recorded on a skin area exposed to a sample and a value recorded on a skin area unexposed according to the protocol already presented by our team [18].

**Statistics**

Measurements were repeated three times on every mouse and mean values were used. All statistical analyses were performed using a trial version of IBM SPSS. Numerical data were presented as mean ± standard errors. The results were analyzed using one-way ANOVA method. p<0.05 were considered statistically significant. * ** and *** indicate p<0.05, p<0.01 and p<0.001.

**Results and discussions**

In this study, there can be seen the first attempt of obtaining BA organic crystals with different solvents. In this present preliminary study different solutions (betulinic acid with methanol and different concentration of NaOH, respectively with isopropanol or ethanol) were tried for growing organic crystals.

The crystals of Sample 2 after 48 h are presented in figure 1. The image was obtained on an optical microscope with a magnification of 40x. The crystals from the other samples were obtained only after 600 h (approx. 25 days).
The crystals of Sample 2 can be observed even at a small magnification like 10x (fig. 1). Figure 2 presents big crystals with dimensions of 5x2x 2 mm. The small sizes of crystals of BA+methanol from Figures 3 and 4 are due to the small concentrations of solvent. In figure 5, the crystals of BA+methanol can be observed very well defined using a magnification of 20x, respectively 40x.

Diluted suspensions of BA crystals (1:200, w/v) were analyzed by a zetasizer to determine the size distribution (Table 1). The zetasizer results implied that the mean particle size was between nano- and micro-scale (around 100 nm). An easier aggregation of these crystals is shown by the recorded Zeta Potential value of Sample 7 [19].

The capacity to treat a damaged skin can be evaluated by visual observations, but some investigation tools are currently available to reduce the inter-observer variability. In this research, mexameter, and corneometer measurements were used as non-invasive techniques to evaluate the changes of some skin parameters (figs. 8 and 9).

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Particle size (nm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>Sample 1</td>
<td>63 ± 12</td>
<td>0.3</td>
</tr>
<tr>
<td>Sample 2</td>
<td>57 ± 8</td>
<td>0.2</td>
</tr>
<tr>
<td>Sample 3</td>
<td>59 ± 10</td>
<td>0.3</td>
</tr>
<tr>
<td>Sample 7</td>
<td>183 ± 29</td>
<td>0.4</td>
</tr>
</tbody>
</table>

The efficacy of suspensions based on BA crystals was evaluated on an experimental skin animal model, after the exposure of mice to UVB irradiation and skin damage.
promoters such as DMBA [16]; there were chosen mice with similar changes of skin aspect (almost the same degree of injury).

Two skin parameters (level of erythema/haemoglobin and the level of hydration of stratum corneum) were evaluated in the present study using non-invasive methods. The results are expressed as differences (Δ, delta parameter) between the values measured in the exposed area and the values measured in a non-exposed area of the same mouse.

Figure 8 shows that the initial level of erythema was around 50-60 arbitrary units, which can be associated to a damaged skin and the treatment with empty suspension or with SLS solution keep the values at the same level. On the other hand, the applications of suspensions containing BA crystals reduce the inflammation (the erythema level expressed as differences decreases from around 55 to 25 arbitrary units). The most efficient sample seems to be the suspension from mice group g_7, based on BA dissolved in methanol.

Figure 9 presents the evolution of the hydration level. The experiment began with a strong dehydration treatment representing by the exposure to aggressive agents such as UVB and tumor promoters; after this period, the hydration difference between an exposed and an unexposed area of the same mouse was around 4 arbitrary units. It can be observed that no important changes of hydration level were obtained in the case of positive and negative control groups. Once again, the beneficial effects of samples containing BA were observed in the evolution of hydration level recorded in the case of the other mice. The hydration levels back to almost normal for the mice treated with suspension based on BA crystals (from around -4 arbitrary units to -1). Sample 7 (BA dissolved in methanol) presents the most significant effect.

Conclusions
Betulinic acid, a natural pentacyclic triterpene with lupan skeleton, presents an important anti-inflammatory and chemopreventive activity at skin level; it can reduce noxious processes such as irritation, inflammation and skin degradation when it is topically applied as prophylactic agent. Unfortunately, this compound has a very low aqueous solubility, like the others representatives of its class (betulin, lupeol, ursolic acid, oleanolic acid etc.). In this research, betulinic acid crystals with a medium stability and sizes between 57 and 183 nm were obtained and they were tested on mice skin in order to evaluate the protective activity against UVB skin exposure. The observations strongly recommend topical administration of betulinic acid crystals as a protective and regenerative strategy in skin inflammation and damage.

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