Chemical Composition and Biological Activity of Birch Bark Extracts on Human and Murine Healthy/Melanoma Cell Lines

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Betulapendula R. presents an increased interest due to its chemical composition, rich in pentacyclictriterpenes, which are recognized for their remarkable biological activityespecially in the pathologies associated with the cutaneous organ. The present research work was purported to assess the biological activity of two type of birch bark extracts, namely total hydro alcoholic extract (BBE1) and betulin purified extract (BBE2) in terms ofviability ontwo healthy cell lines (human keratinocytes-HaCat and newborn mice epidermis - JB6Cl41-5a) and two melanoma cell lines (human-A375 and murine-B164A5). Both extracts did not exhibited any toxicity on healthy cells at concentrationsd ≤ 25 µg/mL. BBE1 showed greater efficacy than BBE2 on both melanoma cell lines.

Keywords:betulin, skin, cancer, biological activity

Skin tumors are by far the most common human malignancies, especially among the Caucasians, with over 1,000,000 cases detected every year [1]. Abnormal skin cell growth most often occurs in areas exposed to UV radiations, but there are some exceptions that may also occur in areas that have not been exposed. The types of skin cancer are named according to the cell of origin and their clinical behavior and the most common types of skin malignancies are basal cell carcinomas, squamous cell carcinomas and melanomas [2]. The risk of developing the malady is associated with both type factors, genetic and environmental,respectively. Exposure to intermittent UV rays(related with sunburn) is considered to have the major role in the development of melanoma, butother factors such as: the number of nevi, the characteristics related to pigmentation - hair, eyes, skin color, and family history also have an important contribution.

Standard treatment of skin melanoma involves the surgical removal of the primary tumor and surrounding normal tissue. Unfortunately, cases of advanced disease imply palliative surgery, chemotherapy and/or radiotherapy, newer targeted or immunotherapy drugs which despite new diagnostic and treatment developments remain with poor results [3]. Prior to 2011, there were only two treatments approved by the FDA for advanced melanoma: dacarbazine and interleukin-2 which are in the present known with low response rates and considerable toxicity [4,5]. Synthetic drug therapy utilized for melanoma imply alarming side effects and resistance fact that has led to an increased interest in bioactive compounds from natural sources.

Terpenes are a large group of secondary plants metabolites and are considered to be potentially useful in cancer pharmacology due to their selective cytotoxicity on numerous human carcinogenic cells as demonstrated in *in vitro* and *in vivo* studies [6].The birch bark is recognized for the increased content of terpenic compounds and its chemical composition is closely related to the preparation and purification methods used and influences the percentage of the major compound, betulin, that may range from 54 to 82% of the dry weight [6]. Betulinhave a potent anticancer effects and also a significant function in inflammatory processes and can play a future role in immune mediated cancer therapiesif the limitations(like poor solubility) are solved by developing new formulations or standardized extracts [7].

The present work was aimed to assess the biological activity of birch bark total hydro alcoholic and betulin purified extracts in terms of *in vitro*tests, namely: antioxidant activity (AOA) and cytotoxicity on two healthy cell lines (HaCat - immortalized human keratinocytes, JB6Cl41-5a - newborn mice epidermis) and two melanoma cell lines (A375 - human melanoma and B16 melanoma 4A5 - murine melanoma).

Experimental part

Materials and methods

Plant material and reagents

Birch bark from *Betula pendula*was collected in Caras Severin County (South West region of Romania) during summer season of the year 2016. Botanical identification of the plant was realized at the Pharmaceutical Botany Department, Faculty of Pharmacy, Victor Babes University of Medicine and Pharmacy Timisoara, Romania and a voucher specimen is deposited at the Herbarium of the Faculty. The collected vegetal material was dried at room temperature away from light and in dry place for two weeks until reached a constant weight.

All the reagents used were acquired from Sigma-Aldrich, Germany.

Extraction procedure

In order to obtain birch bark total hydro alcoholic extract (BBE1) 25 g of vegetal material, crushed and homogenized, was placed in the Soxhlet apparatus and 200 mL of ethanol 70% were used for eight cycles of extraction. The resulting extract was filtered, concentrated by a rotary evaporator (Heidolph Hei-VAP Advantage Rotary Evaporator package) under vacuumand the obtained precipitate was isolated, purified and stored at -20°C until further analysis.

For betulin purified extract, a solution of BBE1 (1g in 20 mL hot ethanol) was treated with calcium hydroxi-dehydroxide, boiled for half an hour,and filtered. The solvent was evaporated on a rotary evaporator, impurities were

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removed by successive washings withhydrochloric acid then with water. The precipitate obtained was disolved in benzene, refluxed for 15 min, cooled, filtered and isolated. Were removedbetulinic acid, lupeol and other polar impuritiesandtheresultingprecipitate (BBE2)was used for further analysis.

Extracts characterization

Quantification of betulin was performed using HPLC-MS analysis. An Agilent HPLC 1100 system equipped withbinary pump, degasser, auto sampler, and thermostat, analytical column Zorbax SB-C18 100 mm x 3.0 mm i.d., 3.5µm and mass spectrometer Agilent Ion Trap 1100 SL was utilized. Chromatographic determination were made by isocratic elution using formic acid 0.4%/methanol 14:86 (v:v). The HPLC conditions were: flow 1 mL/min, temperature 40°C, injection volume 2 µL.

MS conditions were: APCI, ionization mode positive, drying gas nitrogen, temperature 300°C, capillary voltage 2000 V.

Antioxidant activity

The DPPH test was employed in order to evaluate the radical-scavenging ability of the two types extracts. In brief, 250 µL of extract was diluted with 1000µL ethanol 70% and 250 µL of DPPH 1mM was added. The UV-Vis measurements were realized at 517 nm andthe antioxidant activity recorded was correlatedwith the one of ascorbic acid (positive control) and solvent (negative control). The values of antioxidant activity (%) of tested samples were calculated as reported in previous studies [8].

Cell lines, specific reagents, cell culture and viability assay

The cells utilized in this study were: HaCat - immortalized human keratinocytes, JB6Cl41-5a - newborn mice epidermis, A375 - human melanoma, aquired from ATCC (American Type Culture Collection), and B164A5 - murine melanoma aquired from Sigma Aldrich. All the lines were received as frozen items and stored in liquid nitrogen until the beginning of the experiments. The Alamar blue reagent was purchased from Sigma-Aldrich (Germany). The specific reagents necessary for the cultivation of cell lines were: Dulbecco's modified Eagle Medium (DMEM) purchased from ATTC, trypsin/EDTA solution, phosphate buffer saline (PBS), antibiotic mixture, fetal calf serum (FCS) and Trypan blue solution purchased from Sigma Aldrich (Germany).

HaCat, A375 and B164A5 cells were cultured in DMEM, supplemented with 10% FCS and penicillin/streptomycin mixture and JB6Cl41-5a cells were cultured in EMEM supplemented with 0.1% non-essential amino acids and 5% FCS. The culture plates were incubated in standard conditions for in vitro experiments (37°C, humidified atmosphere with 5% CO_2) and the counting of cells was realized with an automated counter (CountessTM II Automated Cell Counter), in the presence of Trypan blue.

Cell viability was determinated by using Alamar blue assay. In brief, $1x10^4$ cells/ well growned in 96-well plates were stimulated with different concentrations (5, 10, 25 and 50 µg/mL) of BBE1 and BBE2,respectively. 20µL of Alamar blue/well was added and incubated for 3h, andin the final step the reading of the absorbance at 570 and 600 nm was realizedby using a xMark™ Microplate Spectrophotometer (Biorad). Cell viability was calculated in the same manner described in our previous studies [9].

Statistical analysis

Graph Pad Prism 7 software was usedto describe and interpret the data. One-way ANOVA followed by Tukey's test was utilized to determine the statistical differences between the different experimental and blank samples (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$). The data were expressed as the means ± standard deviation (S.D.).

Results and discussions

Individual quantitative analysis of betulin found in the total hydro alcoholic extract of birch bark (BBE1) and in the betulin purified extract (BBE2) was realized by HPLC-MS analysis. The identity of the chemical compound was certified by comparison with standard compound. The data are presented in table 1. In theanalysis:the R_t was 2.75 min for betulin; the expected ion, correlated to betulin molecular weight (442.3), theoretically it is its proton aduct (m/z 443.3), practically has been identified a majority ion at m/z 425.3 which correspond to the adduct formed between $H^+$and dehydrated betulin.Liquid chromatography couplet with mass spectrometry was previously utilized for the quantification of this type of compoundspresent in natural plant extracts [10,11].

Table 1

CONCENTRATION OF BETULIN IN THE HPLC-MS TESTED BIRCH BARK TOTAL HYDRO ALCOHOLIC AND BIRCH BARK BETULIN PURIFIED EXTRACTS

Extract	*Weight (mg)*	*Concentration (ng/ml)*	*Total amount (mg)*
BBE1	16.36	1021.19	10.18
BBE2	9.12	887.33	8.879

Figure 1 describes the antioxidant activity of the birch bark total hydro alcoholic and birch bark betulin purified extracts which have been proven to have a significant activity compared to the one of ascorbic acid (control sample). Thereby, the percentage values of AOA were for BBE1 around 90%, for BBE2 around 76%, while for the solvent negative values and for ascorbic acid around 95%. The data showed that BBE1 possesses the highest antioxidant activity.

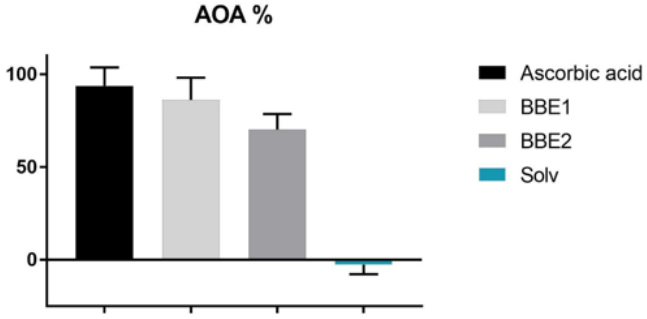

Fig.1. Antioxidant activity of birch bark total hydro alcoholic extract (BBE1), birch bark betulin purified extract (BBE2) and ethanol 70% (Solv)

Different methods are used to evaluate the radical scavenging effects of compounds with antioxidant properties. The DPPH assay possesses some advantages among which: quick, simple and reliable method, does not require special conditions. The free radical scavenging properties are related to the antioxidant capacity some compounds to lose H^+ and the DPPH free radical easily receive an electron or H^+ from the compound with antioxidant capacity and to become a stable molecule [12].

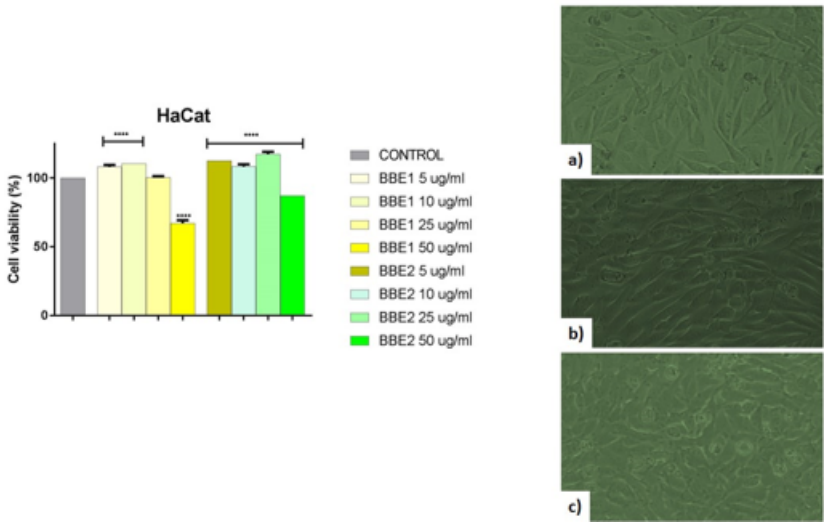

Fig. 2. Viability percentage of human keratinocytes cells at 24h post-stimulation with samples (5, 10, 25 and 50 μg/mL) and the morphology of cells: a- control cells (unstimulated), b - cells stimulated with birch bark total hydro alcoholic extract (BBE1) and c- cells stimulated with birch bark betulin purified extract (BBE2)

Alamar blue assayin the concentration range 5–50 μg/mL of the test compounds was chosen as a control test in order to assess the efficiency of the extracts on cell lines.

Regarding healthy cells, normal human keratinocyte, both type of birch bark extracts at concentration ≤ 25 μg/mL did not exhibited any sign of toxicity (fig. 2).Moreover, at the lowest concentrations, for BBE1 (5 and 10 μg/mL), and for the first three concentrations, for BBE2 (5, 10 and 25 μg/mL), a stimulatory effect can be observed. For both types of extracts the highest concentration tested was proven to be toxic (fig. 2). In case of human keratinocytes no significant morphological changes were observed after stimulation (with concentrations below 50 μg/mL), their morphology remained well defined, elongated and bounded to the culture plate as can be seen in the figure 2.

Data recorded on murine cells, newborn mice epidermis, showed that BBE1 at low concentrations tested exerts a slight stimulatory effect while, at highest concentration tested (50 μg/mL)the effect begins to become toxic (fig. 3).Regarding BBE2 for 5, 10 and 25 μg/mL lthe same stimulatory effect was observed and for last concentration tested the toxicity was lower compared to BBE1,as can be seen in the figure 3.Murine healthy cells revealed a good/normal confluence in the presence of BBE1 and a high confluence when were stimulated with BBE2 at concentrations below 50 μg/mL (fig. 3).

Evaluation of the effects induced by BBE1 and BBE2 on human (A375) and murine (B164A5)melanoma cells viability, at four concentrations (5, 10, 25 and 50 μg/mL) were tested in a 24 h exposure test. Data obtained revealed that BBE1 induced a significant decrease of A375 living cells percentage at highest concentrations tested -at 25 μg/mL the viability was around 67% while at 50 μg/mLl the viability was around 37% - the effect being dose-dependent (fig. 4). The smallest concentrations have been shown to have no influence on human melanoma cells.The assessments of BBE2 revealed a reduced viability percentage of A375 cells- at 5 and 10 μg/mL, respectively the viability of the cells was around 91% while at 25μg/mL the viability was ~ 81% and at 50 μg/m the reached value for cell viability was ~ 70%. These values are significant lower compared to BBE1 (fig. 4). Human melanoma control cells showed a normal epithelial morphology, spindle and cobblestone shapes, strongly bounded andadherent to the culture plate, and high confluence while, after stimulation with extracts, especially with BBE1, the cells became rounded and began the detachment process, fact that proven the apoptosis, data in agreement with the one recorded on viability experiments (fig. 4).

The antimelanoma activity of betulin has already been demonstrated in murine melanoma cells: against B164A5 cellswith an IC_{50}value of 1.9 μM and a dose-dependent pro-apoptotic activity against B164A5 and B16F10 murine melanoma cells [7,13,14].

In the experiments conducted in this study, the murine melanoma cells(B164A5 cells) also seemed to be influenced by the birch bark extract but only in the case of BBE1.Compared to human melanoma cells, murine melanoma cells showed a steady decrease in viability percentage in a concentration-dependent manner – at 5 μg/mL viability ~ 93%, at 10 μg/mL viability ~ 86%, at 25 μg/mL viability ~ 82% and at 50μg/mL viability ~ 64%. BBE2 induced a slight decrease in cell viability at the lowest

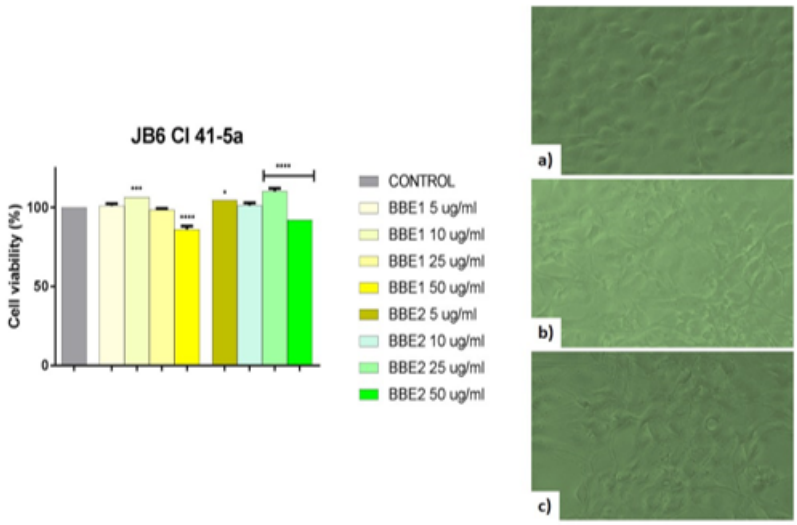

Fig. 3. Viability percentage of newborn mice epidermis cells at 24h post-stimulation with samples (5, 10, 25 and 50 μg/mL) and the morphology of cells: a- control cells (unstimulated), b – cells stimulated with birch bark total hydro alcoholic extract (BBE1) and c- cells stimulated with birch bark betulin purified extract (BBE2)

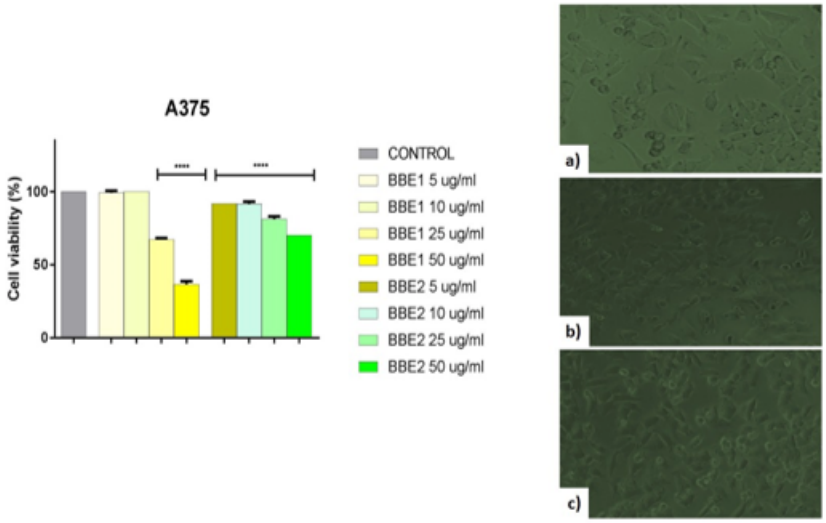

Fig. 4.Viability percentage of human melanoma cells at 24h post-stimulation with samples (5, 10, 25 and 50 µg/mL) and the morphology of cells: a- control cells (unstimulated), b – cells stimulated with birch bark total hydro alcoholic extract (BBE1) and c- cells stimulated with birch bark betulin purified extract (BBE2)

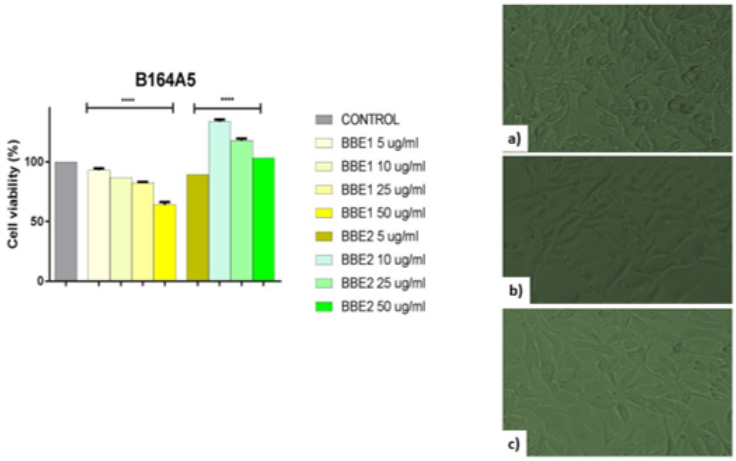

Fig. 5.Viability percentage of murine melanoma cells at 24h post-stimulation with samples (5, 10, 25 and 50 µg/mL) and the morphology of cells: a- control cells (unstimulated), b – cells stimulated with birch bark total hydro alcoholic extract (BBE1) and c- cells stimulated with birch bark betulin purified extract (BBE2).

concentration (5 µg/mL viability ~89%) but regarding the other three concentrations a stimulatory effect was observed (fig. 5). Considering murine melanoma cells, the one exposed to BBE1are the most affected, in terms of viability and morphology: changes of shapeand as expected, the detachment from the culture plate (fig. 5).

Conclusions

The originality of this research consists in the biological evaluation of total hydro alcoholic and betulin purified birch bark extracts, especially in comparative terms. The antitumor activity against tumor cells was significant, and no toxic activity on normal cells tested was observed at concentrations below 50 µg/mL. Birch bark total hydro alcoholic extract presented a greater efficacy as compared to betulin purified birch bark extract on both human melanoma and murine melanoma cells. The activity of the extracts evaluated was considerable at medium and high concentrations whereas at low concentrations the percentages of viability was quasi normal for human melanoma cells and increased for murine melanoma cells.

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