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Birch tree outer bark, a natural source of bioactive pentacyclic triterpenes with an antitumor activity

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Abstract

Birch tree is widely spread in Romania being known for a very long time for its healing properties: antiviral, anti-inflammatory, antiallergenic, antihypoxic, hepatoprotector, hypolipemic. The main compound of the birch bark is betulin, a pentacyclic triterpene, which can be easily converted to betulinic acid, a pharmacological more active compound. The purpose of the present study was to underline 2 different possibilities of extraction, well known even on agro-alimentary field and the evaluation of their in vitro activity on a specific cell line: A2058 (human melanoma) comparing with a known antimelanoma compound betulinic acid.

Keywords: birch bark, betulinic acid, in vitro, melanoma

1. Introduction

Birch tree and the variety spread in Romania, *Betula pendula* Roth - Betulaceae family is found both in the fields and mountain areas [1,2]. The healing properties of birch tree have been known for a long time, birch bark being applied in the past in folk medicine for the treatment of skin diseases (eczema, psoriasis) [1,3]. Parts of birch tree are used in different formulations such as tea, shampoos, creams [3,4]. The biological properties of main components, betulinic acid and betulin, of lupanic structure (Fig. 1) are very important: antiviral (antiflu, anti-HIV), anti-inflammatory, antiallergenic, antihypoxic, hepatoprotector, hypolipemic [5-8]. By C-28 oxidation of hydroxyl group to carboxylic group, betulin can be easily converted to betulinic acid, a more active compound that showed important biological activity and is now included in preclinical trials [9,10]. Betulin's extraction from the bark of the birch tree starts at around 200 °C and lead to an isolation of active compound. [11,12].

A suitable solvent should be methanol that is often cited in the literature [11-13] and the most common procedures are Soxhlet continuous extraction and ultrasonication [1]. The purpose of the present study was to underline two different possibilities of extraction, well known even on agro-alimentary field and to evaluate their in vitro activity on a specific cell line: A2058 (human melanoma) comparing with betulinic acid, a well known antimelanoma compound that is in preclinical trials.

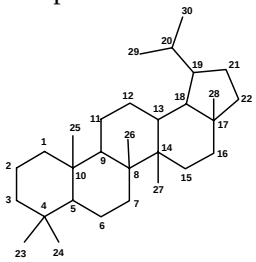

Figure 1. Lupan skeleton structure

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*Preparation of the extract.*The analysed extracts were prepared using the following techniques: E1: 4.92 g of vegetal powder were extracted by ultrasonication for 3 hours in 20 minutes intervals using methanol (250 ml) at a 1:10 w/w ratio bark to extraction solvent;E2: 4.90 g of vegetal powder were taken from natural exterior bark form the birch tree and extraction was performed on Soxhlet device for three hours, using methanol as a solvent (250 ml), at a 1:10 w/w ratio bark to extraction solvent.The extracts were brought to siccum using the Büchi rotary evaporator R-200 with "V" assembly (vertical water condenser).Betulinic acid for *in vitro* tests was purchased from Sigma-Aldrich (Taufkirchen, Germany). Cell line was purchased from ECACC and Sigma Aldrich stored UK .

2. Materials and Methods

*Vegetal products.*The vegetal product used was the exterior bark of the common birch tree, *Betula pendula* Roth, taken from Anina's Mountains (Banat Region, Romania) on September, 2009. Voucher probes are kept in the Herbarium of the Faculty of Pharmacy form the University of Medicine and Pharmacy Timisoara after the vegetal material was positively identified at the Department of Pharmaceutical Botany. The vegetal part was only the detachable outer bark of the tree [13,16]. Vegetal material was crushing with a mill till became a vegetal powder.

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*HPLC/MS analysis:*The initial data were based on literature [14]

Apparatus: HPLC coupled with mass spectrometer: HP 1100 Series binary pump, Degasser online 1100 Agilent, auto sampler HP 1100 Series, thermostat HP 1100 Series, mass spectrometer Agilent Ion Trap 1100 VL; HPLC working conditions: Analytical column : Zorbax SB-C18 100 mm x 3.0 mm i.d., 3.5 µm (Agilent), Filter on-line 0.2 microns (Agilent), Mobile phase: isocratic elution using formic acid 0.4% (V/V) / methanol 15/85 (V/V), Debit: 1 ml/min, temperature: 40º C, Injection volume: 5 µl;

Detection: Betulin - MS – ion m/z 425.3 monitoring, corresponding to protonated betulin dehydration product $[M-H_2O]^+$;Betulinic acid - MS – ion m/z 439.3 monitoring, corresponding to protonated betulinic acid dehydration product $[M-H_2O]^+$; MS working conditions: Ions source: APCI+ (atmospheric pressure chemical ionisation), Ionization mode: positive, Electrospray: nitrogen, pressure 60 psi, Vaporiser: 400º C, Drying gas: nitrogen, debit 5 L/min, temperature 300º C, Capillary voltage: 2000 V, Analysis mode: SIM-MS, m/z 425.3 (betulin) and 439.3 (betulinic acid); All chromatographic operations were carried out at ambient temperature; 29.95 mg of the dried extract was dissolved in 50 ml methanol. The mixture was filtered through a membrane filter before injecting into the chromatograph.

*Standards:*For quantitative determination betulin/betulinic acid standards were used (Extrasynthese, France). The standard solution was prepared by dissolving 8266.9040 µg betulin / betulinic acid in 100 mL methanol, thus having 8.2669 mg/100 mL concentration.

*Scanning electronic microscopy.*Particle morphology was examined using electronic scanning microscope Hitachi S4700 (Hitachi Scientific Ltd, Japan). A thin-layer covering device (Polaron E 5100, Bio-Rad Microscience Division, England) was used to induce an electric conductivity to the surface of the sample. Air pressure was 0,1 Torr (13332 mPa).

*MTT in vitro analysis.*Two types of cancer cells A2058 (human melanoma) were seeded onto a 96-well microplate and attached to the bottom of the well overnight. After 24 hours 200 µL of new medium containing (Dulbecco's) the test substances were added and incubated for 72 h; the medium was supplemented with 10% fetal calf serum and 100U/ml penicilin G and 100µg/ml streptomycin sulfate.

Melanoma cells were passaged at confluence after treatment with 5mM EDTA [15]; the living cells were then assayed by the addition of 20 µL of 5 mg/mL MTT solution. The intact mitochondrial reductase converted and precipitated MTT as blue crystals during a 4 h contact period. The medium was then removed, and the precipitated crystals were dissolved in 100 µL of dimethyl sulfoxide (DMSO). Finally, the reduced MTT was spectrophotometrically analysed at 545 nm, using a microplate reader; wells with untreated cells were used as controls. All *in vitro* experiments were carried out on two microplates with at least five parallel wells. DMSO was used to prepare stock solutions of the tested substances and the highest DMSO concentration (0.3%) of the medium did not have any significant effect on the cell proliferation. The amount of birch bark extract was 0.5 mg/ml solvent and for the test were used 3 dilutions (100x – D1, 200x- D2 and 300x- D3. The stock solution for betulinic acid was 0,5 mg/ml.

3. Results and discussions

HPLC analysis was carried out using a Zorbax SB-C18 column (100 mm x 3.0 mm i.d., 3.5 µm, Agilent); we used as mobile phase a mixture of formic acid 0.4% (V/V) and methanol 15/85 (V/V); the literature mentions a mixture of phosphate buffer – methanol which was replaced in the present study in order to minimise the background noise. As mentioned before all chromatographic operations were carried out at ambient temperature. The chromatographic peaks of betulin/betulinic acid were confirmed by comparing their retention times with betulin/ betulinic acid standards of 96 % purity (*Extrasynthese*, France).Quantification was carried out by the integration of the peak using the external standard method. The standard and the sample solutions were injected one at a time into the chromatographic system and peak areas were recorded.

The retention time was 2.75 minutes for betulin and 3 minutes for betulinic acid. The detection limits were calculated as minimal concentration producing a reproductive peak with a signal-to-noise ratio greater than 3. The linearity was verified by preparing five betulin solutions of different concentrations having the same volume, using a stock solution of 8.2669 mg/100 ml concentration and methanol (1:9, 3:7, 5:5, 7:3, 9:1 v/v ratios).

The correlation coefficient of the calibration plot indicated a good linearity ($r^2 = 0.9996$).

In Table 1 are presented the quantitative results found in the analysed extract.

Table 1. The content in betulin and betulinic acid of the analysed birch bark extracts samples

Sample	Extract weight (mg)	% of betulin	% of betulinic acid
E1	34.6	38,69	**2,76**
E2	43,8	49,72	**3,72**

SEM analysis has been performed in order to present a full characterisation of birch bark extracts. Pictures show an amorphous structure with a different aspect for the 2 samples (Fig. 2).

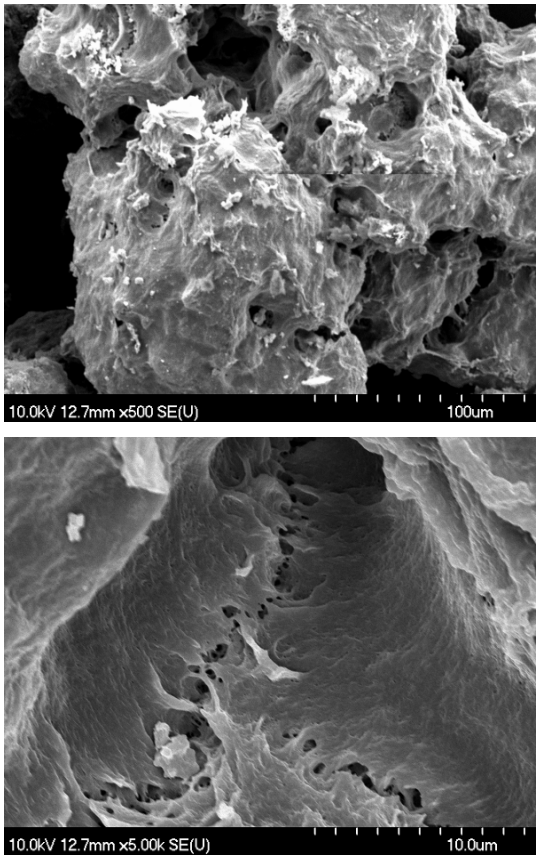

Figure 2. SEM pictures of E1 and E2 extracts

In the following images is presented the in vitro activity of the 2 types of extract, E1 and E2. The results indicated a different biological activity for the 2 extracts and this could be correlated to their composition in lupan skeleton compounds.

The extract E2 that contains a higher amount of betulin and betulinic acid is more effective on tumor cell inhibition then extract 2 (Fig. 3, 4). Comparing to betulinic acid alone both extracts presented a lower antiprolifertive activity but still very significant (over 70%) (Fig.5). It is well known that betulinic acid possessed a higher and selective antimelanoma activity [4,5] and this aspect is reflected even on A2058 cell line. Betulin is important for its antitumor activity also but is not very effective on specific melanoma cell lines [7,8]. Combinations with other triterpenes and higher amounts of compound could increase its activity on melanoma cells.

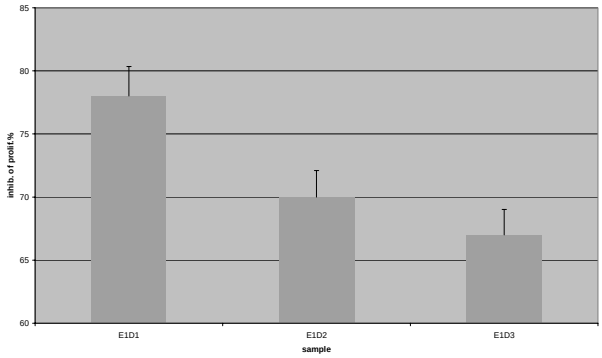

Figure 3. Antiproliferative activity of extract E1 at different types of dilutions

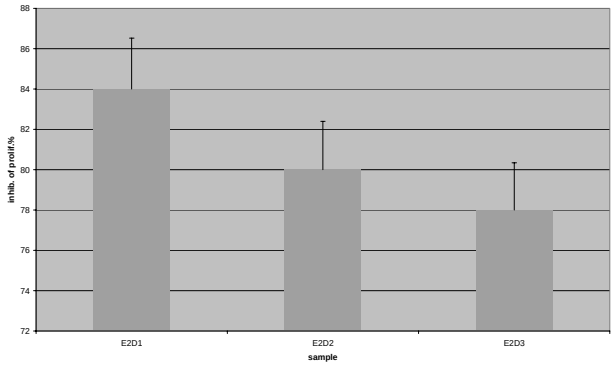

Figure 4. Antiproliferative activity of extract E2 at different types of dilutions

Isolation of betulin, a pentacyclic triterpene, was reported by several authors from white birch species, along with its carboxylic acid derivative, betulinic acid. Dry extract from birch tree outer bark, in topical applications as ointment contain up to 40% of pentacyclic triterpenes [2,4]. However, few studies have been made on the Romanian specie, Betula pendula Roth, regarding its content of betulin/betulinic acid in the outer bark. We used two extraction methods: Soxhlet and ultrasonication and different types of solvents in order to reach the maximum concentration in lupan skeleton triterpenes in the final extracts. Solvents were chosen considering literature data [1,11,12] which suggest organic substances such as methanol, dichloromethane and chloroform are preferred for a proper extraction.

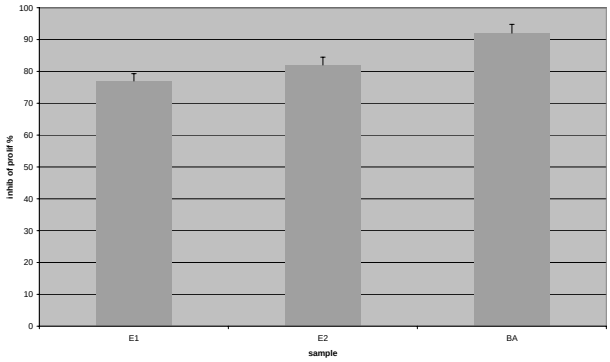

Figure 5. A comparative analysis for inhibition of proliferation between the 2 types of extracts (E1 and E2) at the dilution equivalent with quantity of betulinic acid (BA) in its stock solution on A2058 cell lines

The HPLC/MS method was used to determine the quantity of the two pentacyclic triterpenes in the outer bark of birch tree. It proved to be an excellent technique for simultaneous determination of betulin and betulinic acid with a good sensitivity and reproducibility. Ultrasonication is also a good method for the extraction of betulin and betulinic acid, comparable to Soxhlet continuous extraction but not superior to this. All species of Betula contain a high amount of betulin in the outer bark, quantity which goes from 26 to 35% [4,15]. The Romanian specie presented a high percentage of betulin, over 35% much higher amount than other species, which recommends *Betula pendula* Roth as a powerful resource of natural betulin. It also contains an amount of betulinic acid that increased the antitumor activity and the importance of vegetal source.

4. Conclusions

Betulin and betulinic acid were present in the birch tree bark extract according to HPLC/MS analysis and methanol proved to be an appropriate solvent which leads to the extraction of these compounds. The continuous Soxhlet extraction leads to a better quantity of active compounds.

Betulin may represent more than 35% (weight percentage) of a Betula spp. bark extract.

Outer bark of *Betula pendula* Roth, a plant species that can be found in Romania is an important source of pentacycilic triterpenes especailly the alcohol known as betulin. That aspect explains the new applications of total extract as therapeutic agent in preclinical and clinical studies. The activity of total extracts of birch tree outer bark is very important on melanoma cells even the higher amount in the composition is betulin.

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