

***In vitro* experimental evaluations of birch bark extracts with selectivity in the obtaining of betulinol**

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Abstract

The modern and sedentary human organism exposed to excessive pollution often feels an acute need for antioxidant vitamins which can prevent various types of cancer. But once the disease has been established, it can be combated with a series of remedies made available by natural medicine, with many plants whose anti-cancer role has been proved. The aims of this research were to develop some birch bark extracts with different betulinol purity and to evaluate their activity on three cells line: the epidermoid carcinoma cells - A431 and the cells of mammary adenocarcinoma - MCF7 and MDA-MB-231; the cells viability evaluation was based on Alamar blue method. The results of this study indicate a dose-dependend stimulative activity of extracts on cancer cells.

Keywords: natural extracts, carcinoma, cells culture, viability.

1. Introduction

If more than 100 years ago, around 95% of the drugs came from nature, in the last period due to the development of chemistry syntheses, this percentage has changed radically. Natural remedies gradually recorded an important decline, and they were replaced with products based on different chemical syntheses, giving rise to a real pharmaceutical industry. Initially, the reproduction of existing chemical structures in nature was attempted in laboratories, and then in large industrial units [1].

In Romania, the green pharmacy admits the infusions of the birch flowers and leaves and the bark decoction, but it does not quite refer to the sap. In a country like Russia, birch sap is the panacea transmitted from father to son for the treatment of unexpected diseases.

Las Casas reports that the attention of Napoleon Bonaparte's personal physician who accompanied him in the Russian campaign was attracted by the curative qualities of a liquor that Russian peasants extract from the tree stem. Apparently, the emperor tasted the liquor, liked it and used it, as he noticed that he was re-invigorating the nervous system so demanded in the warrior adventure he attended, occupying Russia [2].

The birch extracts present proved effects on hypertension, cardiopathy, kidney disease, rheumatism, diabetes (especially as prevention), respiratory diseases, obesity control, melanoma and HIV prevention, based on the synergism of some active substances such as: betulinol (~85%), betulinic acid (5%), oleanolic acid (~3%), lupeol (almost 1%), and other triterpenes (~6%) such as erythrodiol etc. [3]. All these compounds present similar chemical structures based on lupine-type triterpenes (C₃₀H₅₂, Figure 1a).

Figure 1 presents the chemical structures of the main components found in different birch extracts:

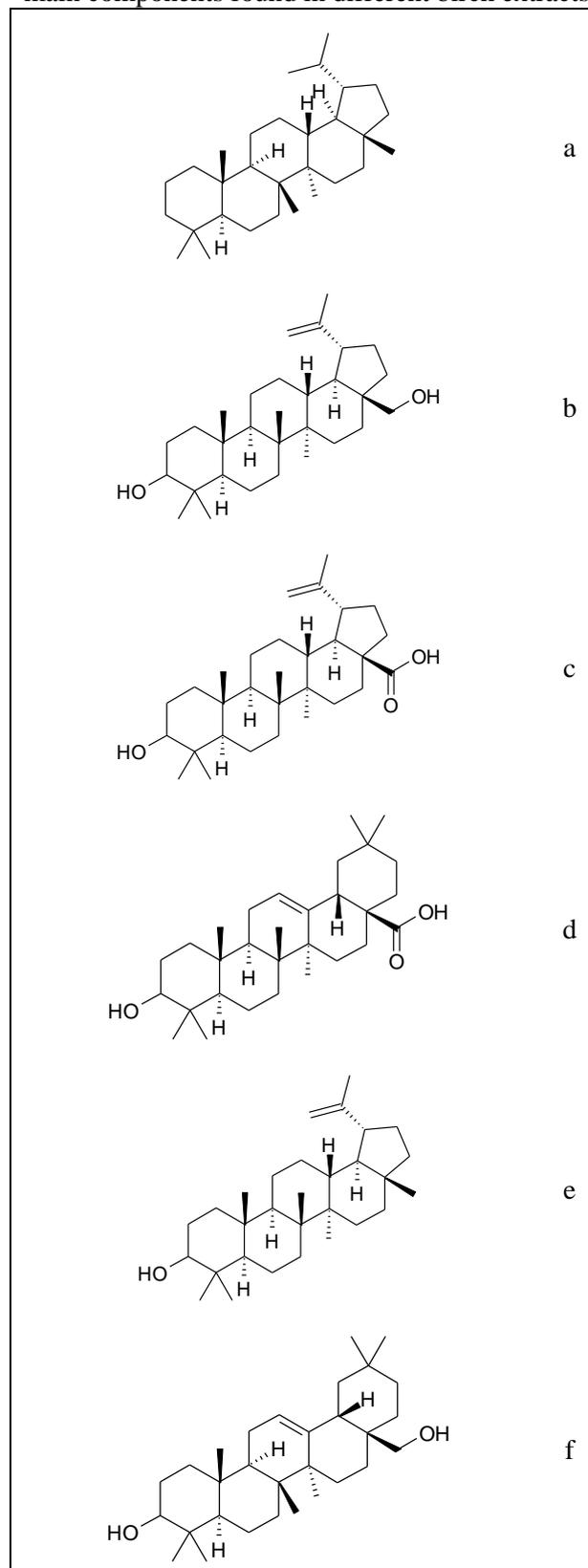


Figure 1. Chemical structure of (a) lupane, (b) betulinol, (c) betulinic acid, (d) oleanolic acid, (e) lupeol, (f) erythrodiol

Betulinol, betulin or Lup-20(29)-ene-3 β ,28-diol is a very abundant and naturally occurring triterpene, found in different species of *Betulaceae* family and also in *Ziziphus vulgaris* var. *spinosa*, *Zizyphus mauritiana*, *Diospyros leucomelas*, *Nelumbo nucifera*, and *Trochodendron aralioides* [4].

The main aim of this study was the *in vitro* experimental evaluation of different birch bark extracts on different cancer cells.

2. Materials and methods

2.1. Extracts obtaining

The obtaining of the natural extracts is described in our previous scientific paper [5]: the plant material has been collected from SW Romania in the summer period; it was dried slow (~2 weeks). 25.0 grams crushed vegetal material was mixed with 200 ml ethanol 70% in a Soxhlet for 8 extraction cycles. The resulted product was filtered and concentrated by a Heidolph Rotary Evaporator.

Table 1. Samples codes and descriptions

Code	Sample description
Control	Solvent (ethanol, 70%)
BBE0	First fraction after the Soxhlet extraction cycles
BBE1	BBE0 was treated with calcium hydroxide in hot pure ethanol
BBE2	BBE1 was treated with benzene and refluxed
BBE5	Previous extract was first treated with hot pure ethanol and then with distilled water. The product was dissolved in chloroform and passed through a silica gel column and in the last step the product was treated with hot ethanol and high purity crystals of betulinol were obtained

2.2. Identification and dosing of betulinol content

Betulinol identification in every obtained extract was performed by FTIR technique and its content was monitored using HPLC-MS analysis. An Agilent HPLC 1100 system equipped with binary pump, degasser, thermostat, Zorbax SB-C18 analytical column 100 x 3.0 mm, 3.5 μ m and Agilent Ion Trap

1100 SL mass spectrometer was used. Chromatographic determination was performed by isocratic elution using formic acid 0.4% acetonitrile / methanol 14:86 (v/v). HPLC conditions were: flow rate 1 ml / min, temperature 40 °C, injection volume 2 µl. The MS conditions were: APCI, positive ionization mode, nitrogen gas for drying, temperature 300 °C, capillary voltage 2000 V.

2.2. Cells cultures

The cells used in this study were: epidermoid carcinoma cells - A431, cells of mammary adenocarcinoma - MCF7 and MDA-MB-231. The cell lines were purchased from the American Type Culture Collection or ECACC (European Collection of Authenticated Cell Cultures). These were received as frozen samples and stored in liquid nitrogen until the beginning of the experiments. The Alamar blue reagent was purchased from Sigma-Aldrich (Germany). The specific reagents needed to cultivate cell lines purchased from the same companies, Sigma Aldrich and ATCC.

3. Results and Discussions

The epidermoid carcinoma line is a human hypertryloid cell line. The modal chromosome number was 74 to 36% of the cells. The higher ploidies cell rate was 1.0%. The epidermoid carcinoma cell line A-431 is derived from a 85 years-old woman. Cells in the presence of the test extracts (crude and purified birch bark extracts) did not show any significant changes and no effect could be detected that can lead to their cell death. On the contrary, a stimulating effect of the tested extracts on the cells was observed (Figure 2).

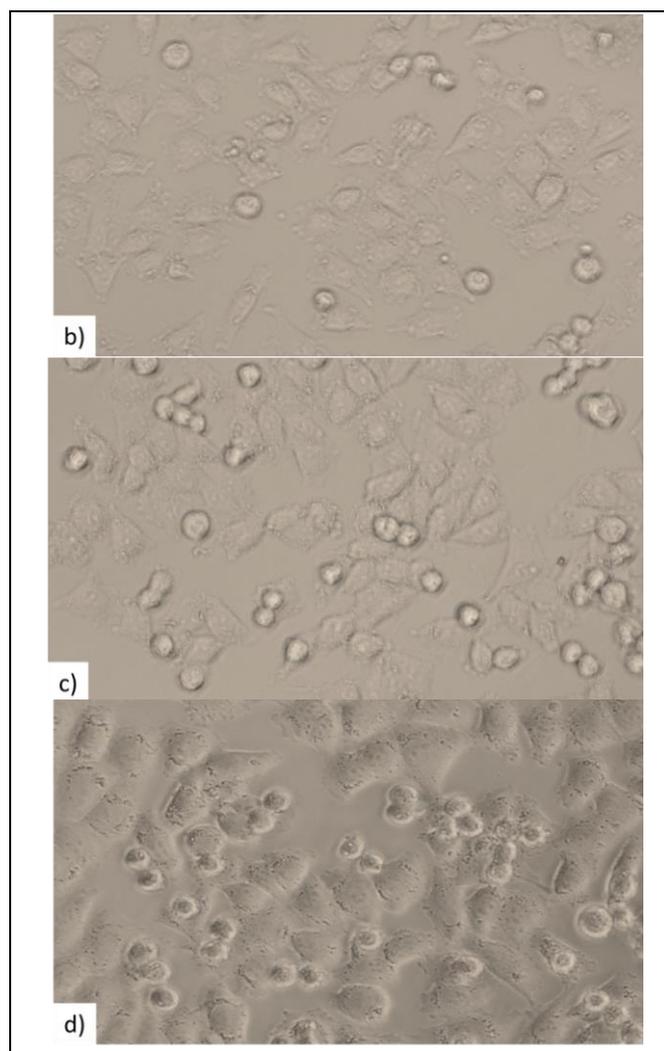


Figure 2. *In vitro* morphological aspect of A-431 cells: (a) unstimulated; (b) stimulated with BBE0; (c) stimulated with BBE1 and (d) stimulated with BBE5

In the case of the two human breast carcinoma lines, MCF7 and MDA-MB-231, some form differences were observed (an elongated form for MDA-MB-231 and a more rounded form for MCF7), although both are described as having epithelial morphology. Characterization studies of these lines were performed using MALDI-TOF and the results indicated that the level of distances between the MCF7 and MDA-MB-231 cell lines revealed by MSP analysis could be explained by the fact that even if these cell lines have the same origin (pleural effusions of patients with metastatic breast cancer), the MCF7 cell line (invasive cells) expresses specific markers for the luminal epithelial phenotype and is used as a template for estrogen receptor positive tumors, while the MDA-MB-231 cell line (invasive cells) express mesenchymal markers (vimentin) and

is used for estrogen receptors - negative breast cancer [6]. Moreover, significant differences were observed between the proteomic profiles MCF7 and MDA-MB-231 [7]. In the case of MCF7, the number of chromosomes varied from hypertryloid to hypotetraploidy, 2S component appearing to 1%. There were 29-34 chromosomes markers on metaphase S; 24 to 28 markers occurred in at least 30% of the cells and generally a large submetacentric marker (M1) and 3 large subtelocentric markers (M2, M3 and M4) were recognized in over 80% metaphases. Chromosome 20 was nullisomic and X was disomic.

The MCF7 line maintains several features of differentiated breast epithelium, including the ability to process estradiol through cytoplasmic estrogen receptors and the ability to form cupols. The cells express the WNT7B oncogene. The analysis of the growth curve showed that within 2 days after sowing at 4×10^4 viable cells / cm^2 , MCF7 cells appear as three-dimensional clusters attached to some floating cells. After 4 days, after sowing, the attached cells begin to spread to form a flattened monolayer. On day 5, the most cells appear as a flattened monolayer and approximately 70% confluent. However, it is not uncommon for MCF7 to display the delayed attachment until after the first or second subculture. If this occurs, the cells are treated as a semi-adherent line and the floating cells are not thrown, as previously stated. Clusters of suspended cells should be broken by light pipetting with a small pipette (5 ml or less). After a few days of incubation, cells should re-emerge as three-dimensional islands (there will be some non-rebounding groups). Ultimately, growth will spread from the islands, and culture should flatten over time.

The tested extracts did not exert any negative effects on cellular morphology; they showed stimulatory potential as can be seen in Figure 3.

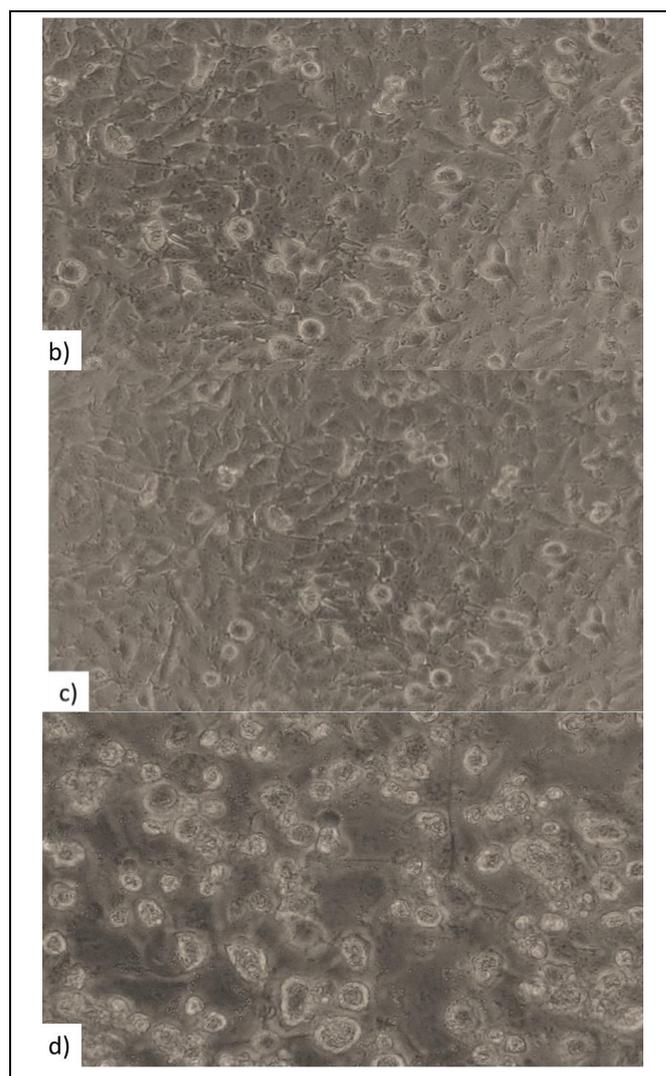
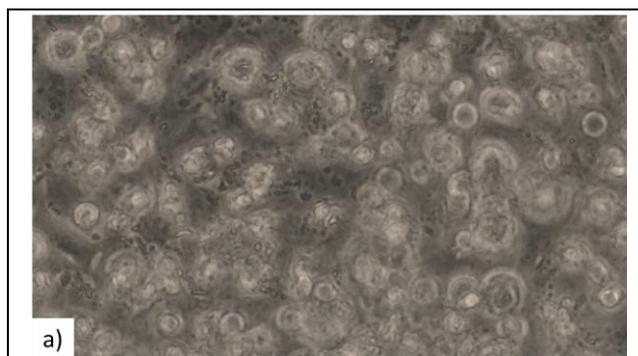


Figure 3. *In vitro* morphological aspect of MCF7 cells: (a) unstimulated; (b) stimulated with BBE0; (c) stimulated with BBE1 and (d) stimulated with BBE5

The MDA-MB-231 aneuploid cell line (modal number = 64, interval = 52-68) with chromosome number in almost triploid range. Normal N8 and N15 chromosomes were absent. Eleven rearranged marker chromosomes, as well as non-linear chromosomes, are observed in addition to most autosomes that are trisomic. Receptor expression: epidermal growth factor (EGF) - expressed, alpha growth factor (TGF alpha) transformed - expressed. Also, in the case of these cell types no major morphological changes were detected in the presence of the tested extracts. As can be seen in Figure 4, the cells retain much of their initial form without signs of apoptosis.

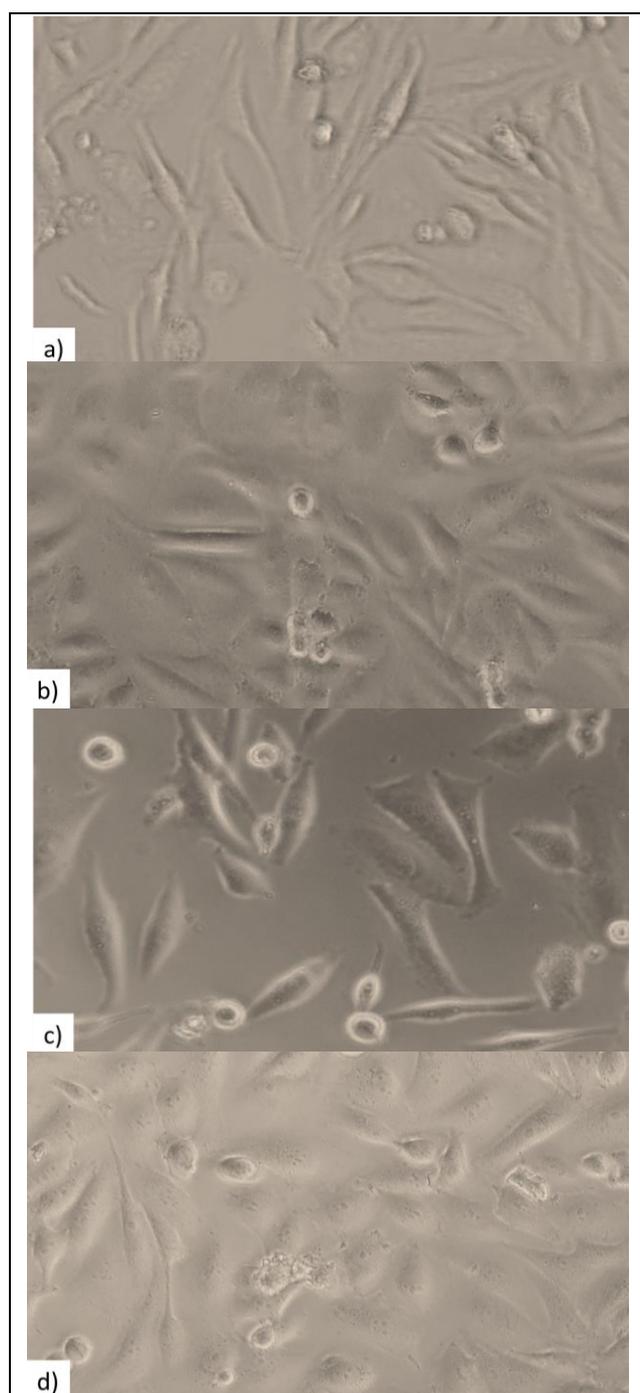


Figure 4. *In vitro* morphological aspect of MDA-MB-231 cells: (a) unstimulated; (b) stimulated with BBE0; (c) stimulated with BBE1 and (d) stimulated with BBE5

The evaluation of the effects of tested extracts on the viability of epidermoid carcinoma cells at different concentrations (5, 10, 25 and 50 µg / ml) was tested by exposure for 24 hours. Data obtained showed that BBE0, BBE1 and BBE2 extracts had stimulatory effects on cells, while betulinol standardized as BBE5 induced a slight decrease in cell viability (Figure 5).

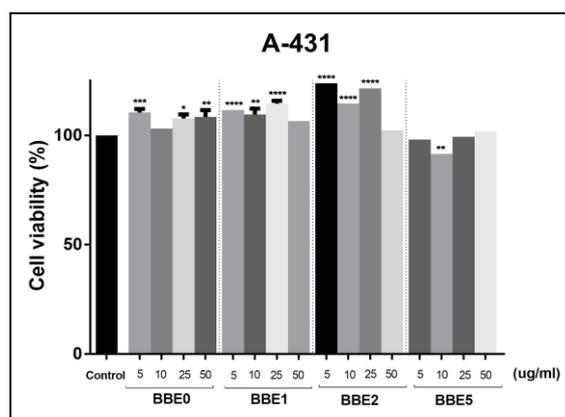


Figure 5. A-431 cells viability for tested extracts at different concentrations

In the case of breast cancer, on both types of cells used, stimulative effects were observed, especially on MDA-MB-231 cells. For these cells, the viability was significantly increased at BBE5 stimulation as follows: at 5 µg/ml viability ~128%, at 10 µg/ml viability ~140%, at 25 µg/ml viability ~141% and at 50 µg/ml viability ~150% (Figures 6 and 7).

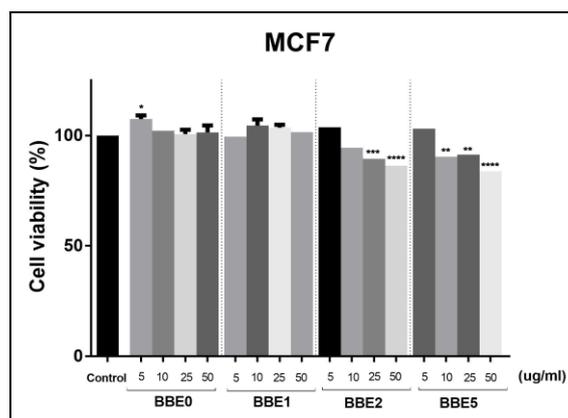


Figure 6. MCF7 cells viability for tested extracts at different concentrations

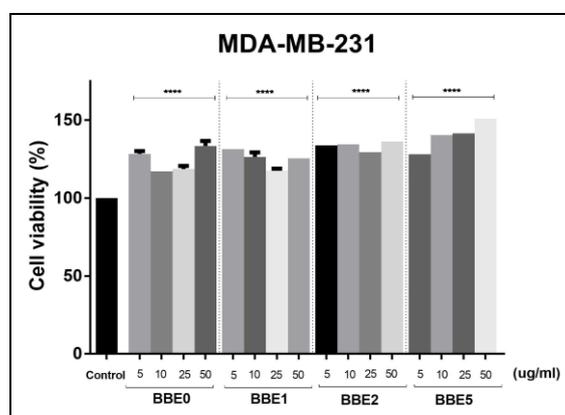


Figure 7. MDA-MB-231 cells viability for tested extracts at different concentrations

The literature [8] confirmed the betulinol broader cytotoxicity against several different cancer cell lines: it was found to be cytotoxic against all nine neuroblastoma cell lines (e.g., SKNSH, IMR-5, NBL-S, NBAS5 and LAN-5) and it is slightly more cytotoxic than lupeol on melanoma cell lines (MEL B16 2F2, G361 and SK-MEL-28), neuroblastoma (GOTO and NB-1) and leukemia (HL60, U937 and K562).

4. Conclusion

Phytotherapy, the treatment based on different plants, is the most widespread therapeutic method in the world. It is based on the folk medicine, and traditional therapies often appeal to it. This paper describes the obtaining of some birch bark extracts with a higher content of betulinol. The *in vitro* experimental evaluations of these obtained extracts on different cells lines such as A-431, MCF7 and MDA-MB-231 revealed different stimulative effects.

Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human / or animal subjects (if exist) respect the specific regulation and standards.

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