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Detecting the presence of antitumor terpenoids in bark of white birch from North-eastern Bulgaria

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Abstract
For the first time a chemical analysis of white birch *Betula pendula Roth.* bark samples from North-eastern Bulgaria region was carried out in order to demonstrate the presence of pentacyclic triterpenoids of ursane, lupane, and oleanane families. A liquid-phase extraction procedure was adapted. The results both from TLS and GC-MS analysis confirmed the presence of two compounds of interest: betulin and α-amyrin in ratio close to 7:1. Detectable quantity of betulinic acid in the samples was not found.

Keywords: natural antitumor terpenoids, α-amyrin, betulin, betulinic acid, lupane, ursane

Introduction
Since ancient times the therapeutic and antimicrobial properties of the bark of many tree species is known. Different infusions, decoctions and extracts are traditionally used by folk medicine. The advancement of analytical methods made possible the gradual particularization of the chemical composition of such products, and the isolation of specific bioactive compounds. Recently, particular attention was given to the group of non-steroidal terpenoid compounds contained in the bark of the white birch (*Betula pendula Roth.*) which antitumor activity is now considered to be proven. For example, betulinic acid (Fig. 1) inhibits cell growth in human melanoma [1], induces cellular apoptosis in patients [2, 3] and compromises the replication of the HIV-virus [4, 5]. Additionally, such compounds exhibit high antibacterial activity, inhibit the Escherichia coli and Staphylococcus aureus growth [6], and in general act as an anti-inflammatory agent [7].

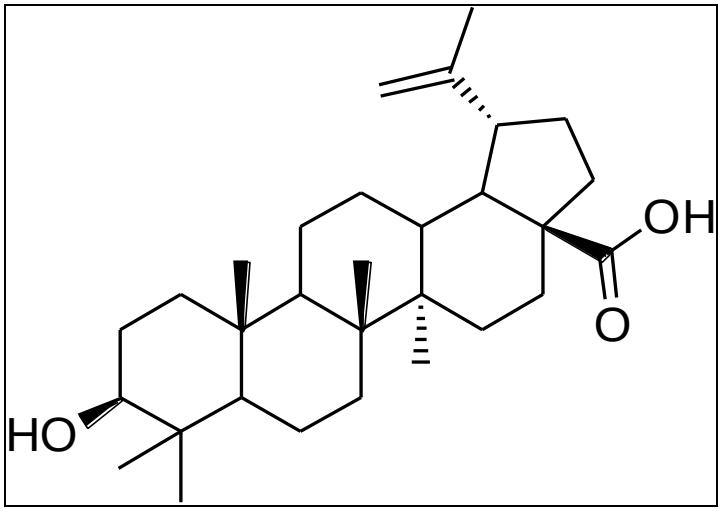
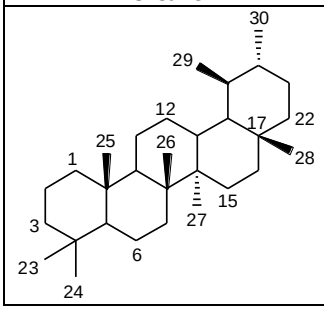
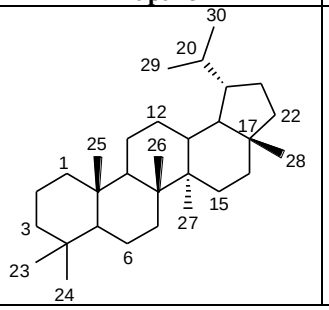
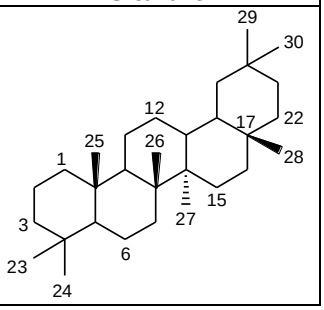


Fig 1: (3β)-3-Hydroxy-lup-20(29)-en-28-oic acid (betulinic acid)

From chemical point of view these substances are (poly) substituted pentacyclic triterpenes which can be categorized into several groups, depending on their hydrocarbon skeleton. The chemical structure of the more common progenitor triterpenes are presented in Table 1.

Table 1: Chemical structure of some non-steroidal pentacyclic triterpens

Ursane	Lupane	Oleanane
		

The significant interest in these compounds and their potential application makes one wonder whether such substances are a natural component of the white birch bark grown in Northeastern Bulgaria. This prompt the establishment of current preliminary study's purpose: (a) to determine whether these samples of birch bark contain pentacyclic triterpenoids, (b) to assess their content ratio and purity, and (c) to propose a procedure for isolation.

2. Materials and methods

Specimens from birch bark (*Betula pendula Roth.*) obtained from Northeastern Bulgaria were examined. The elected trees for harvesting the bark were with a minimum trunk diameter of 25 cm, since such trees recover better.

For the analysis by thin layer chromatography were used aluminum plates (Merck), 200 μm silica gel.

For the gas chromatography analysis with mass spectral detection following equipment was used: Agilent Technologies 7890B GC System + 5977A MSD. Data acquisition and processing were controlled by a specialized software package Agilent MassHunter; reference data from mass spectral library NIST version 2.0 g was used for comparison. GC-MS working parameters are listed in Table 2. All of the used chemical reagents were of analytical grade or better, unless otherwise indicated. The necessary solutions are prepared using distilled or purified deionized water (0,067-0,100 μS / cm, TKA ™ Pacific water purification system).

Table 2: GC/MS Operating parameters

Parameter	Value	Parameter	Value
Initial oven temp.	50°C	GC Column	HP-5ms
Initial time	0.5 min	Column dimensions	30 m × 0.25 mm
Oven ramp rate	3°C min^{-1}	Film thickness	0.25 μm
Oven final first ramp	200°C	Inlet mode	Splitless
Final time first ramp	0 min	Flow mode	Constant flow
Oven ramp rate	4°C min^{-1}	Flow rate	1.5 mL/
Oven final temp.	320°C	Carrier gas	He
Final time	10 min	Ion source temp.	230°C
Total run time	90.5 min	Inlet temp.	250°C

3. Results and discussion

Current research can be divided logically into three stages: collection of samples from white birch bark (sampling), extraction of target compounds from samples (extraction) and identification of existing substances (analysis).

3.1 Sampling

The birch bark is extracted by peeling. Material gathered is separated by size and larger pieces are immediately placed to dry in dark, well ventilated place. The bark's surfaces are cleaned from debris, including moss and lichens. The prepared samples were cut into small pieces and then crushed in a blender. In the following procedure the resulting mass is sifted. For the purposes of this work the prepared samples have average particle size of 0.8-1.0 mm.

3.2 Extraction

An 80 g of ground bark of birch sample was subjected to continuous 24 hour Soxhlet extraction. As an extraction solvent 400 ml of ethanol (Pharmacopoeia, 96%) was used. After completion of the procedure 1 L of distilled water was added. The resulting precipitate was filtered off at normal pressure through a "blue ribbon" filter and washed twice with distilled water. The drying was conducted at temperature not higher than 50°C. The obtained product was 18.6 g of fine powder (23% of the initial sample weight) with a white/cream color and faint but characteristic odor, practically insoluble in water, soluble in methanol and ethanol.

3.3 Analysis

Preliminary analysis of purity was performed on the extract by thin layer chromatography using three different mobile phases: methanol, acetone and chloroform. Visualization was done using iodine vapor. In the three developed chromatograms only two distinctly separate spots are observed.

To perform the initial GC-MS examination, a stock solution was prepared dissolving 11.5 mg birch bark extract without any further purification in 11.5 mL ethyl acetate, giving a total concentration of 1000 μg mL^{-1}. This stock solution was used for preparation of first aliquot dilution (1:100) in ethyl acetate (total concentration 10 μg mL^{-1}). From this dilution 200 μL were measured, 50 μL derivatizing agent BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) were added, and the mixture was heated at 78^0C for 30 minutes. After completion of the derivatization and cooling the mixture to room temperature, 1 μL (extract content of about 8 ng) was injected into the chromatographic column. The temperature conditions used were adapted from a procedure with similar methodology [8]. Operating parameters are described in Table

2. As a result a peak was identified at R_t = 77.15 min, which mass spectrum (Fig. 2) contains a set of characteristic ions *m/z* 586 $[M]^+$, 571 $[M–CH_3]^+$, 496 [M–TMS–OH]$^+$, 483 [M–CH_2–O–TMS]$^+$, 393 [M–CH_2–O–TMS, –TMS–OH]$^+$, etc., corresponding to the betulin silylated derivative, betulin × 2TMS (Table 3).

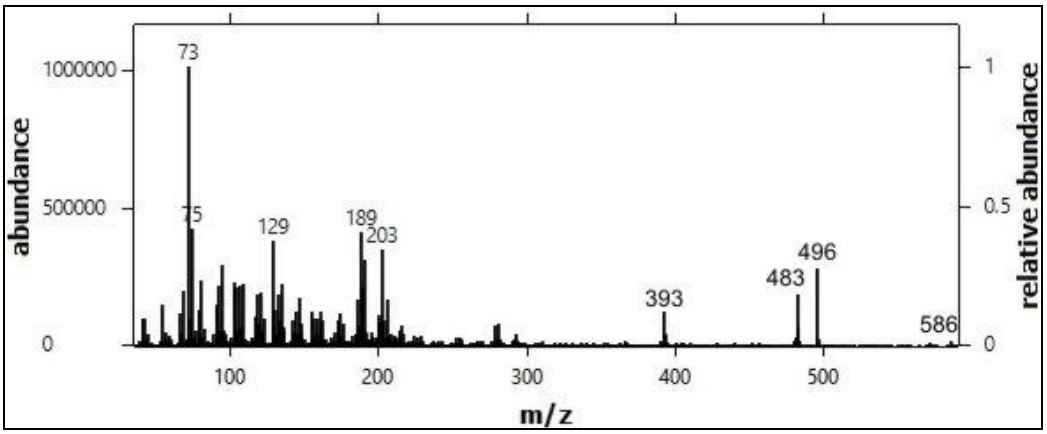
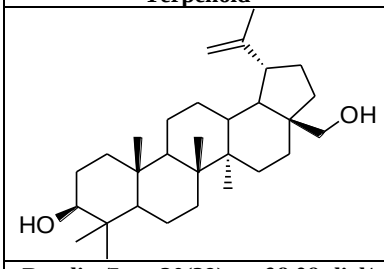
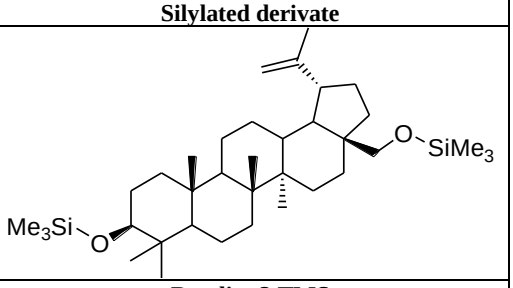
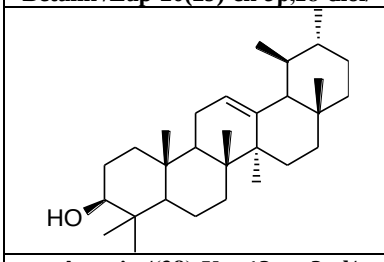
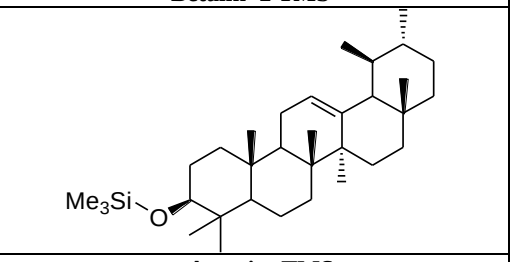


Fig 2: Mass spectrum of silylated derivate betulin×2TMS, R_t = 77.15 min

Table 3: Chemical structure of identified terpenoids and their silylated derivates

Terpenoid	Silylated derivate
	
Betulin /Lup-20(29)-en-3β,28-diol/	**Betulin×2 TMS**
	
α-Amyrin /(3β)-Urs-12-en-3-ol/	**α-Amyrin×TMS**

In order to increase the efficiency of analysis and to check the presence of accompanying substances in lower amounts, a second, more concentrated aliquot dilution (1:12.5) was prepared. The analysis was carried out in a similar manner. 1 µL of derivatized mixture (extract content of about 100 ng) was injected. With this quantity on the new chromatogram besides the expected peak at R_t = 77.15 min (betulin × 2TMS), a second one at R_t = 74.81 min was identified. The difference in retention times is significant and both peaks are fully resolvable without overlap (Fig. 3). The mass spectrum of the new peak (Fig. 4) contains a set of ions *m/z* 498 $[M]^+$, 483 $[M–CH_3]^+$, 393 [M–TMS-OH]$^+$, corresponding to the silylated derivative of the compound α-amyrin (Table 3). The integration of both peaks showed the ratio between α-amyrin and betulin in the sample extract close to 1:7.

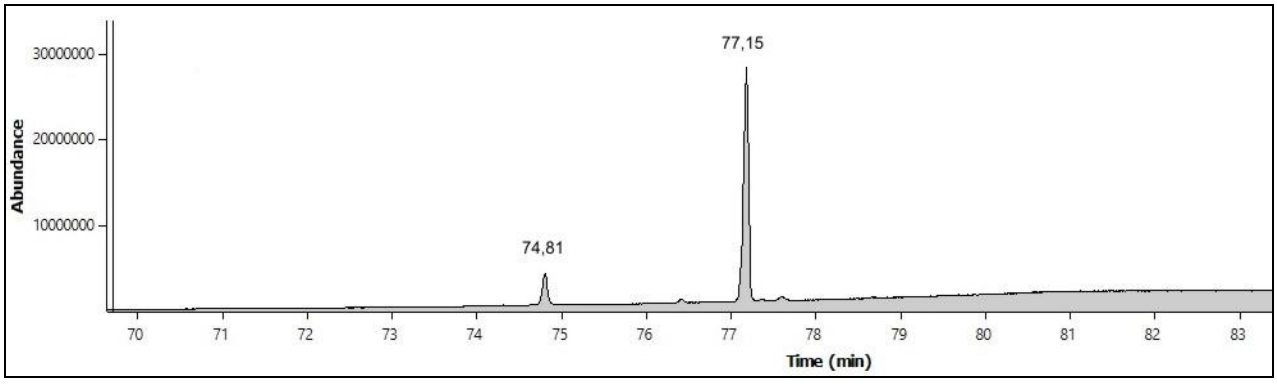


Fig 3: Chromatogram (fragment). α-Amyrin×TMS (74.81 min), betulin×2 TMS (77.15 min). Peak area ratio17:115 ≈ 1:7.

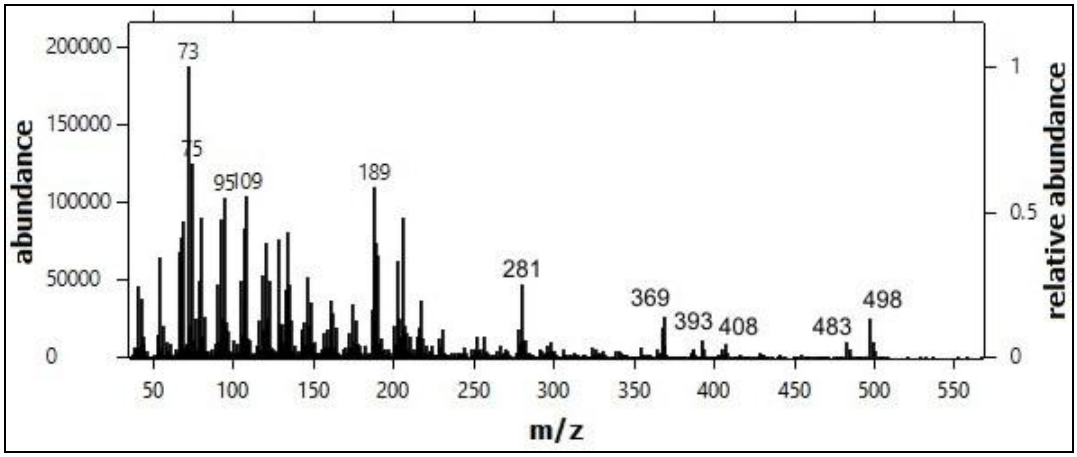


Fig 4: Mass spectrum of silylated derivate α-amyrin×TMS, R_t = 74.81 min

Analyzing chromatograms it was noteworthy that both diluted and concentrated sample lacked distinct peaks in the range 20-90 min, except the two already identified terpenoids and the peak of thiosalicylic acid (R_t = 23.45 min). This could be interpreted as clear evidence of the efficiency of the extraction procedure as it becomes obvious that organic impurities are not present in the final product. This conclusion well correlates with the preliminary thin layer chromatography study.

The origin of the thiosalicylic acid in the birch bark extract is unclear. However, its presence should be pointed out, as the compound is used as a precursor in the synthesis of drugs, which application in atherosclerosis and melanoma treatment is currently actively discussed [9, 10].

4. Conclusion

As a result of the conducted preliminary study the presence of two pentacyclic triterpenoids in a birch bark samples (*Betula pendula Roth.*) from Northeastern Bulgaria were confirmed. Identified compounds are: betulin (lupane type terpenoid) and α-amyrin (ursane type terpenoid). Their content in the resulting extract is expected to be significant, since the compounds are detected even in nanogram quantities of the assay sample. In the analyzed objects betulinic acid was not detected.

Adapted methodology used for the extraction of terpenoids from the bark of the white birch includes a liquid phase continuous extraction with ethanol as an extraction solvent. This type of extraction was shown particularly suitable, with high selectivity, since in the final samples other compounds were identified in a comparable amount neither by TLC nor by GC-MS analysis.

With the proven presence of terpenoids in the target group, the expansion of this research deems appropriate. For this purpose two main tasks should be solved: to optimize the temperature regime for gas-chromatographic analysis, as currently it is excessively time consuming, and to develop or adapt methods for the quantification of these compounds.

5. Conflict of Interest

We wish to confirm that there are no known conflicts of interest associated with this publication.

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