STUDIES OF THE CHEMICAL COMPOSITION OF BIRCH BARK EXTRACTS (*Cortex betula*) FROM THE *Betulaceae* FAMILY

A. Z. Abyshev,[1] É. M. Agaev,[1] and A. B. Guseinov[1]

Translated from Khimiko-Farmatsevticheskii Zhurnal, Vol. 41, No. 8, pp. 22 – 26, August, 2007.

Original article submitted February 15, 2005.

This report presents the results obtained from chemical studies of the composition of dried birch bark extracts (DBBE) from *Betula pubescens* Ehrh. analyzed by GLC, HPLC, and IR and PMR spectroscopy. The extract contained the following groups of natural compounds: terpenoids (75.2%) and their esters (fatty acid esters of betulinol and lupeol, 4.4%), ether oils (0.08%), hydrocarbons (6.3%) and their epoxides (1.0%), steroids (β-sitosterol, 2.7%), tannins (2.1%), flavonoids (1.56% – mainly kaempferol, its 7-methyl ester, quercetin, the 4-methyl ester of naringenin, etc.), hydroxycoumarins (0.85% – umbelliferone, esculetin, etc.), and a number of unidentified compounds (about 4.0%). The major components of DBBE were triterpenoids and hydrocarbons. Preparative chromatographic separation of fractions containing triterpenoids and hydrocarbons yielded isolated samples which allowed the following compounds to be identified: the terpenoids betulinol, isobetulinol, lupeol, lupenone, betulonic aldehyde, betulonic acid, betulinic acid, and platanic acid, and the hydrocarbons α-santalene, β-*trans*-bergamotene, and α-*trans*-bergamotene, and their epoxy derivatives. The chemical composition of DBBE varied depending on the preparation method used, this particularly affecting the content of the major component betulinol, which varied from 54 to 82%. These studies led to the development of a contemporary preparative technology for betulinol with a yield of 95%, along with production of many of its acyl derivatives (for example, the diacetate, succinate, benzoate, etc.) with quite high yields (95 – 98%); betulinic acid was synthesized in mild conditions with a yield of at least 99.0%. These compounds are currently subject to pharmacological studies.

Various birch species are widely distributed in the forest zones of the northern and western areas of Europe. More than 20 birch species grow in the territory of the Russian Federation, in particular the European part and western Siberia [1]. Their outer and inner bark layers contain a variety of biologically active compounds, which are of practical interest for the pharmaceutical industry and medical practice. As aqueous and ethanolic infusions, birch leaves, buds, and young branches are used in folk medicine as diuretics and sudorific agents in gout and rheumatic disorders. In scientific medicine, various therapeutic preparations made from birch buds are used in liver and biliary tract disorders, as expectorants, for avitaminosis, atherosclerosis, and gastric ulcers, and birch tar is used in a number of creams (for example, Wilkinson and Vishnevskii creams) and as an antiseptic [2].

Substances extracted from birth bark, particularly betulinol, betulinic and platanic acids, extracted with organic solvents from different birch species and other plants, have been used in recent years either as biologically active ingredients (BAI) or individually as antitumor, antiviral, immunomodulatory, and hepatoprotective substances [3 – 12]. Thus, birch bark, which is a large-scale waste product of wood-processing concerns and the agrochemical industry, provides an inexhaustible source of a variety of individual biologically active substances which might be used as the basis for developing new, highly effective, therapeutic agents.

The chemical composition of the bark of many birch species, namely *B. pendula* Roth. (*B. verrucosa* Ehrh.), *B. pubescens* Ehrh. (*B. alba* L.), *B. davurica* Pall., *B. maximowicziana* Regel, *B. mandshurica* (Regel) *Nakai*, *B. platyphylla* Sukacz, and others have received quite detailed study [1, 3 – 16]. These investigations have demonstrated that extracts of the outer bark contain different classes of natural compounds – ether oils, saponins, tannins, hydrocarbons, carbohydrates, flavonoids, coumarins, carotenoids, terpenoids, etc., the major bark components among these groups being derivatives of the pentacyclic triterpenoid lupane (lupeol, lupeol-3-acetate, betulinol, betulinol diacetate, betulinol aldehyde, betulinic acid, platanic acid,

[1] St. Petersburg Science Research Institute Vaccines and Sera.

etc.). Analysis of the results obtained in these studies indicates that the qualitative composition and quantitative contents show some variation depending on the conditions, the growth site, and the age of the species studied. This is particularly marked in relation to triterpenoid compounds; for example, the major component of the triterpenoid fraction – betulinol – can, depending on the birch species, location, growth conditions, and tree age, vary from 10 to 40% relative to bark weight.

Substances extracted from the bark of *Betula pubescens* Ehrh. (*B. alba* L.) with organic solvents are now used by the company SNS-Farma in a specially patented technology [17] as dry birch bark extract (DBBE) which is used as a BAI and as a component in perfumery-cosmetic products as major active ingredients.

Thus, the main aim of the present work was to study the chemical composition of this extract, i.e., to determine the qualitative composition and quantitative contents of the main active components, to allow standardization of the composition and increased quality of this BAI, as well as pharmacological analysis of individual triterpene compounds extracted from DBBE.

This requires consideration of the possible development of various therapeutic forms based on the most potential components of the extract of interest (see Tables 1 and 2) for introduction into medical practice.

EXPERIMENTAL SECTION

Study samples of DBBE were obtained from the production facility of the company SNS-Farma. The qualitative and quantitative composition of DBBE was determined by thin-layer chromatography (TLC), gas-liquid chromatography (GLC), and high-performance liquid chromatography (HPLC). Extract (5 mg) was dissolved in 20 ml of acetone, as were solutions of corresponding standards. Chromatography columns were sequentially loaded with 50 μl of extract solution and standards and chromatograms were recorded using a computerized integrator; at least five chromatograms were obtained in the conditions described below.

GLC analysis was performed using a Hewlett-Packard model 5890 gas-liquid chromatograph and a Hewlett-Packard model 5970A mass selective detector. The chromatograph was fitted with a capillary column (J&W Scientific, 10 × 0.25 mm) coated with DB-5 (film thickness 0.25 μm) and was operated at temperatures of 100 – 300°C with a heating rate of 10°C/min. The injector and detector temperatures were maintained at 300°C.

HPLC analyses were performed using a Gold System liquid chromatograph (Beckman) with a model 168 UV diode matrix detector. The working wavelength was 200 nm. The stainless steel column (250 × 4.6 mm) was packed with Nucleosil C_{18} octadecyl silica gel, granule size 5 μM and was run at a temperature of 30 ± 1°C. The mobile phase consisted of acetonitrile (TU 6-09-14-2167–84) and deionized water (1:1) and was run at a flow rate of 1 ml/min. Sample volume was 50 μl. The chromatogram recording time was 40 min.

Quantities of study compounds were assessed using an internal standard by normalization by peak area. The internal standard, for example for assay of hydrocarbons and their epoxides, triterpenoids and their esters, consisted of standard samples of α-santalene (melting point 263 – 264°C, $[\alpha]_D$ 92.5°), β-bergamotene ($[\alpha]_D$ 44.1°), and betulinol (Table 3) respectively, produced by ourselves and Aldrich.

TLC was performed on Sorbfil UV-254 nm plates (Silica gel CTX-1A) of size 5 × 15 cm or Silufol UV-254 nm plates (Silpearl) of size 10 × 20 cm in a solvent system consisting of benzene:ethyl acetate (3:1).

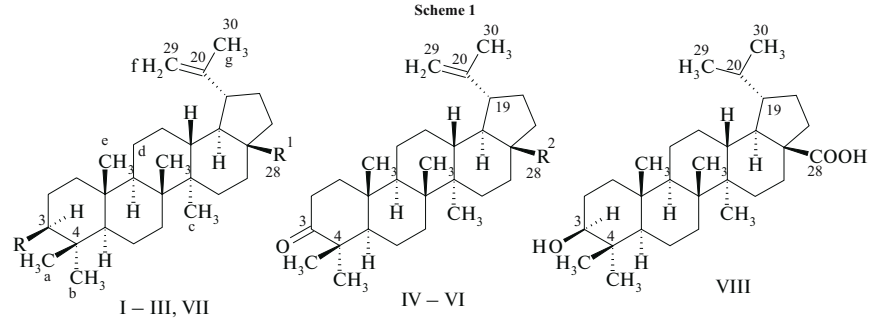
Preparative fractionation of DBBE (500 g) to individual compounds or groups of compounds was performed by column chromatography on column of size 6 × 100 cm filled with silica gel L100/250 μm (800 g) or aluminum oxide (1.2 kg) with Brockman activity II, the eluant being petroleum ether (boiling point 40 – 70°C) (fraction A), mixtures of petroleum ether with chloroform (4:1) (fraction B), 2:1 (fraction C), 1:1 (fraction D), chloroform (fraction E), and acetone:chloroform (1:3) (fraction F). Each fraction had a volume of 400 ml. A total of 140 fractions were obtained. Fraction composition and substance purity were monitored by TLC, GLC, and HPLC. Some of the results are presented in Tables 1 and 2.

Individual substances obtained by chromatography of the extract and after recrystallization from various solvents were

TABLE 1. Determination of the Contents of the Major Triterpenoids of Dry Birch Bark Extract from *Betula pubescens* Ehrh. (% of DBBE Weight), HPLC

Fraction	Betulinol	Isobetulinol	Betulonic acid	Lupeol	Lupenone	Betulinic acid	Betulonic acid	Platanic acid
A	–		–	–	–	–	–	–
B	–		–	–	–	–	–	–
C	–	0.2 ± 0.1	4.3 ± 2.1	–	5.0 ± 0.2	–	–	–
D	50.2 ± 0.4		–	2.8 ± 3.1	0.6 ± 0.2	–	–	–
E	7.3 ± 0.1		–	0.5 ± 0.2	–	0.2 ± 0.3	0.5 ± 0.4	0.22 ± 0.3
F	1.6 ± 0.2		–	–	–	1.0 ± 0.4	0.2 ± 0.4	0.7 ± 0.2

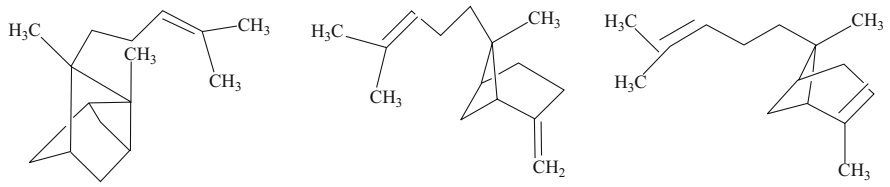
Scheme 1



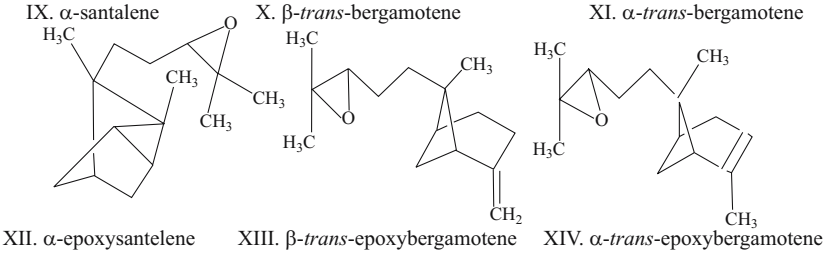
I – III, VII IV – VI VIII

I: R = OH, $R^1 = CH_2OH$;
II: R = OH, $R^1 = CH_2OH$;
III: R = OH, $R^1 = CH_3h$;
VII: R = OH, $R^1 = COOH$

IV: $R^2 = CH_3h$
V: $R^2 = CHO$
VI: $R^2 = COOH$



IX. α-santalene X. β-*trans*-bergamotene XI. α-*trans*-bergamotene



XII. α-epoxysantelene XIII. β-*trans*-epoxybergamotene XIV. α-*trans*-epoxybergamotene

identified from physicochemical and spectroscopic data (see Table 3).

The melting points of all crystalline compounds were measured using an Electrothermal apparatus. IR spectra were recorded using a Specord-75 instrument using pastes in Vaseline. PMR spectra were taken using Bruker AC-200 and AM-500 spectrometers with a working frequency of 200.13 kHz; the internal standard was TMS or HMDS.

Analysis of fractions A-F showed that the major components of DBBE from *Betula pubescens* were terpenoids (75.2%) and their esters (fatty acid esters of betulinol and lupeol, 4.4%), which is consistent with published data [13 – 16]. Ether oils (0.08%) were also found, along with hydrocarbons (6.3%) and their epoxides (1.0%), steroids (β-sitosterol, 2.7%), tannins (2.1%), flavonoids (1.56%, mainly kaempferol, its 7-methyl ester, quercetin, the 4-methyl ester of naringenin, etc.), hydroxycoumarins (0.85% – umbelliferone, esculetin, etc.), and a series of unidentified compounds (about 4.0%). From the applied point of view, fractions containing triterpenoids and hydrocarbons have the greatest potential, as the contents of these substances in DBBE are high, allowing them to be obtained in adequate quantities for production of synthetic analogs. These fractions were therefore subjected to further physicochemical study (see Tables 1 – 3 and the structures of study compounds).

Our studies also established that depending on the birch bark extraction method, the contents of the potentially important betulinol component in extracts varied from 54% to 82% (see Table 4), while the contents of other triterpenoids

TABLE 2. Determination of the Contents of the Major Hydrocarbons of Dry Birch Bark Extract from *Betula pubescens* Ehrh. (% of DBBE Weight), GLC

Fraction	α-Santalene	α-*trans*-Bergamotene	β-*trans*-Bergamotene	α-epoxysantelene	α-*trans*-Epoxy-bergamotene	β-*trans*-Epoxy-bergamotene
A	0.3 ± 0.2	–	0.2 ± 0.41	–	–	–
B	0.7 ± 0.2	2.0 ± 0.35	0.60 ± 0.32	0.2 ± 0.1	0.15 ± 0.2	0.2 ± 0.15
C	1.0 ± 0.1	1.5 ± 0.21	–	0.1 ± 0.1	0.15 ± 0.1	0.2 ± 0.15

TABLE 3. Physicochemical Characteristics of Triterpenoids from Dry Birch Bark Extract from *Betula pubescens* Ehrh.

Compound	Yield %	M. p., °C	Empirical formula	IR spectra, ν_{max}, cm^{-1}	PMR spectra, $CDCl_3$ or DMSO, δ, ppm
Betulinol (I)	59.1	253 – 255, aqueous* ethanol	$C_{30}H_{50}O_2$	3420, 1605, 1430, 1370, 1280, 1170, 1090, 1070, 1000, 960, 850, 690	0.7 – 1.0; 1.7 (s, 18H, a – e, g), 1.1 – 2.4 (m, 26H, –CH– and $–CH_2–$ in ring), 3.1 (m, 2H, 2OH), 4.2 (m, 2H, $–CH_2–O$), 4.6; 4.8 (s, 2H, f)
Isobetulinol (II)	0.2	219 – 221, aqueous ethanol	$C_{30}H_{50}O_2$	3430, 1600, 1430, 1350, 1210, 1080, 1000, 850, 760, 680	0.7 – 1.0; 1.7 (s, 18H, a – e, g), 1.1 – 2.4 (m, 26H, –CH– and $–CH_2–$ in ring) 3.1 (m, 2H, 2OH), 4.2 (m, 2H, $–CH_2–O$), 4.6; 4.8 (s, 2H, f)
Lupeol (III)	3.3	211 – 213, ethyl acetate	$C_{30}H_{50}O$	3300, 1600, 1450, 1370, 1280, 1170, 1080, 1010, 990, 980, 950, 900, 880, 830, 790, 680, 490	0.6 – 1.1 (s, 21H, a – e, h, g), 1.2 – 2.5 (m, 26H, –CH– and $–CH_2–$ in ring), 3.2 (m, 1H, –OH), 4.6; 4.8 (s, 2H, f)
Lupenone (IV)	5.6	Benzene	$C_{30}H_{48}O$	1685, 1605, 1450, 1370, 1280, 1160, 1080, 1000, 980, 960, 950, 905, 880, 830, 790, 680	0.6 – 1.1 (s, 21H, a – e, h, g), 1.2 – 2.5 (m, 25H, –CH– and $–CH_2–$ in ring), 4.6; 4.8 (s, 2H, f)
Betulonic aldehyde (V)	4.3	165 – 166, ethyl acetate	$C_{30}H_{46}O_2$	1690, 1670, 1605, 1450, 1380, 1280, 1160, 1070, 1000, 980, 960, 950, 900, 880, 870, 790, 670	0.6 – 1.1 (s, 18H, a – e, g), 1.2 – 2.6 (m, 25H, –CH– and $–CH_2–$ in ring), 4.6; 4.8 (s, 2H, f), 10.02 (s, 1H, –CHO)
Betulonic acid (VI)	0.7	246 – 248, aqueous methanol	$C_{30}H_{46}O_3$	1690, 1680, 1600, 1450, 1380, 1290, 1170, 1070, 1010, 990, 950, 900, 860, 800, 790, 630	0.6 – 1.1 (s, 18H, a – e, g), 1.2 – 2.6 (m, 25H, –CH– and $–CH_2–$ in ring), 4.6; 4.8 (s, 2H, f), 11.3 (s, 1H, –COOH)
Betulinic acid (VII)	1.2	307 – 310, aqueous ethanol	$C_{30}H_{48}O_3$	3430, 1680, 1605.1420, 1350, 1200, 1000, 840, 680	0.6 – 0.9; 1.7 (s, 18H, a – e, g), 1.1 – 2.6 (m, 26H, –CH– and $–CH_2–$ in ring), 2.9 (m, 1H, –OH), 4.6; 4.8 (s, 2H, f), 11.5 (s, 1H, –COOH)
Platanic acid (VIII)	0.72	277 – 279, aqueous acetone	$C_{30}H_{49}O_3$	3200 – 3450, 1690, 1610, 1440, 1370, 1200, 1000, 850, 690	0.6 – 1.1; 1.7 (s, 21H, a – e, f, g), 1.1 – 2.8 (m, 26H, –CH– and $–CH_2–$ in ring), 3.0 (m, 1H, –OH), 11.8 (s, 1H, –COOH)

* Solvents used for recrystallization are given.

were significantly lower (Table 1). The highest and most stable yields of betulinol from DBBE were obtained by direct recrystallization from isopropanol (IP) and, particularly, from dimethylformamide (DMF). Some of the experimental data are presented in Table 4.

TABLE 4. Preparative Extraction of Betulinol from Different Series of Dry Birch Bark Extract from *Betula pubescens* Ehrh.

Extraction sample	Extraction solvent	Betulinol yield, %	R_f (Silufol UV-254 nm, ethyl acetate:benzene, 1:3)	M. p., °C	Color of final powder*
DBBE-26	IP	54	0.32	252 – 255	Cream
DBBE-26	IP	60	0.33	257 – 259	Cream
DBBE-26	DMF	69	0.33	257 – 259	Cream
DBBE-28	IP	50	0.34	256 – 259	White
DBBE-28	DMF	75	0.33	255 – 257	White
DBBE-28	DMF	73	0.34	255 – 257	Cream
DBBE-32	IP	63	0.33	256 – 258	White
DBBE-32	DMF	75	0.33	255 – 257	White
DBBE-41	IP	59	0.34	256 – 258	White
DBBE-41	DMF	82	0.32	253 – 255	White
DBBE-41	DMF	80	0.34	256 – 258	White

Given that the literature [3 – 16] contains good experimental data showing that such components of *Betula pubescens* Ehrh. as betulinol, betulinic acid, and platanic acid have high antitumor, immunomodulatory, and anti-HIV activities, they are naturally of interest for experimental and clinical pharmacology as therapeutic substances. However, despite the fact that chemicopharmacological studies of these compounds have been conducted both in this country and abroad for more than 200 years, these substances, particularly betulinol, which has a potential raw materials supply, have not entered clinical practice. Nonetheless, we believe that continuation of the study of this compound and its synthetic analogs is very relevant for using it to create a whole series of extremely important antitumor, immunomodulatory, and, particularly, anti-HIV compounds, as there are as yet no such highly active agents in the world's medical arsenal.

Thus, these data have allowed us to develop the first preparative method for obtaining betulinol, betulinic acid, and several of their analogs, which are needed for preparation of the corresponding therapeutic forms (as tablets or capsules). Betulinol was obtained at a yield of 99.5%; techniques for

synthesis of many of its acyl derivatives (for example, the diacetate, succinate, benzoate, etc.) with quite high yields (95 – 98.5%) were developed; a betulinic acid synthesis in mild conditions was developed, with a yield of at least 99.0%.

The scientific-industrial complex "Chemistry and Technology of Medicinal Preparations", St. Petersburg Science Research Institute Vaccines and Sera, has developed corresponding standard documents (Pharmacopeia Articles, Commercial Pharmacopeia Articles, and Manufacturing Specifications) for National Reference Samples for the substance and therapeutic forms of betulinol, betulinic acid, and platanic acid; production in the form of tablets containing doses of 0.04 and 0.08 g is planned, along with capsules containing 0.2 g for pharmacological studies as antitumor, immunomodulatory, and anti-HIV agents.

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