ХИМИЯ РАСТИТЕЛЬНОГО СЫРЬЯ. 2020. №1. С. 277–282.
DOI: 10.14258/jcprm.2020016503

UDC 582.632.1+543.054+547.914.4

SEARCH FOR A SOURCE OF TRITERPENE ACIDS IN RUSSIAN BIRCHES

© ***D.N. Vedernikov[1*], O.A. Eregina[1], A.V. Shemyakina[2]***

[1] St. Petersburg State Forest Technical University named after S.M. Kirov, Institutsky lane, 5, St. Petersburg, 194021 (Russia), e-mail: dimitriy-4@yandex.ru
[2] Far East Forestry Research Institute, Volochaevskaya st., 71, Khabarovsk, 680030 (Russia)

The composition of triterpene acids and triterpene alcohols of the outer bark of a damaged silver birch tree (*Betula pendula* Roth.) the outer bark of silver birch branches, the outer bark of *Betula davurica* Pall., *B. mandshurica* (Regel) Nakai, *B. platyphylla* Sucacz. and *B. costata* Trautv. is considered. Silver birch bark was harvested in the Leningrad region. The bark of other species of birches was harvested in the Khabarovsk region. The qualitative composition of triterpene compounds was determined by chromatography-mass spectrometry. The quantitative composition was determined by gas-liquid chromatography and absolute calibration. Butyl esters of acids were preliminarily prepared for GLC analysis of triterpene acids. The outer bark of the eastern species of birches contains significantly more betulinic and oleanolic acid than silver birch. The relative content of triterpene acids is greater in the outer bark of branches and in the bark of damaged silver birches. The increase in acid content is apparently associated with the desire to heal a wound. The main component among the triterpenoids of the investigated raw materials is betulin. For the separation of acids from other triterpenoids, various solubilities of the compounds in solvents and the ability of acids to form poorly soluble salts were used.

Keywords: birch outer bark, species of birch, extractive substances, triterpenoids, quantitative analysis, gas chromatography, separation of triterpene compounds.

Introduction

Betulinic acid (BA) belonging to the lupane type of triterpenoids is a natural biological active substance. BA contains in the bark and leaves of many plants, for example, the bark of birches. BA can be synthesized from betulin from the bark of white-birch bark. Synthesis usually takes three or more stages with a maximum betulinic acid yield of 55% [1]. BA has a wide range of anti-tumor effects and exhibits anti-HIV activity [2–6].

Oleanolic acid (OA) belonging to the oleane type of triterpenoids has a hepatoprotective effect, anti-inflammatory and anti-cancer effects. It can also stop the cell cycle of pancreatic cancer cells. OA and related pentacyclic triterpenes are natural glycogen phosphorylase inhibitors. OA may be useful for treating diabetes and its complications, ischemic cardiovascular or cerebrovascular diseases, hyperlipemia, obesity, atherosclerosis, metabolic syndrome or cancer [7, 8]. OA demonstrates high antimicrobial activity [9, 10].

OA ameliorates the toxic effects of 6-hydroxydopamine (6-OHDA) [11], OA demonstrates an antidiabetic effect [12].

Oleanolic acid acetate (AOA) inhibits rheumatoid arthritis [13].

The above compounds mentioned above found in the bark of the birch, which grows in the European part of Russia. But their content is insignificant [14]. The development of technology for the isolation of triterpenic acids from *B. pendula* bark is hindered by the betulin high content. The aim of this work was to search for raw materials for triterpene acids extraction from the bark of various birches, to develop a scheme for triterpene acids extracting from natural sources, to determine the composition of triterpenoids from the bark of wounded pendent birch *B. pendula* Roth.(1), *B. davurica* Pall.(5), *B. mandshurica* (Regel) Nakai (3) *B.* p*latyphylla* Sucacz. (4) and B. *costata* Trautv.(2)

Ведерников Дмитрий Николаевич – профессор, e-mail: dimitriy-4@yandex.ru
Ерегина Ольга Алексеевна – студент, e-mail: olya.eregina96@gmail.com
Шемякина Анна Викторовна – ведущий научный сотруднк, e-mail: Ashem777@mail.ru

* Corresponding author.

Experimental

For analysis, three samples were prepared from each species of birch in the Leningrad region (wounded birch (1), birch branches of (1)) and in the Krasnoyarsk region.

2b,3,4,5 were harvested in September 2018 in the Khekhtsir forestry of the Khabarovsk region (20 km from Krasnoyarsk), the age of (1,3,4,5) birches was 40–45 years. The age of (2a) was 25–27 years.

The wounded bark was removed from the trees, from which 2 years ago birch bark was removed for weaving, after separation of the dead phloem.

The crust from the branches of birch was removed manually. The average ratio of cork, phloem and xylem in the branches of a thickness of 3–11 mm: 14:17:69.

Outer bark samples were crushed to sizes 2.5–5 mm and thickness 0.5 mm and extracted with isopropyl alcohol (5 g) in a Soxhlet apparatus for 6 hours. The resulting alcoholic extracts was evaporated and dried for 3 hours at 105 °C. Sample extracts in the amount of 60 mg were transferred into a 10 ml volumetric flask and butylated with an ether solution of diazobutane. The resulting products were dissolved and adjusted to the mark with isopropanol. 1 μl of the resulting solution was analyzed by GLC. The GLC method was already used earlier in the quantitative analysis of triterpenoids at temperatures of 259 °C [15] and 300 °C [16]. For the quantitative assessment of triterpenoids, a gas chromatographic method (GLC) of analysis was developed using a Shimadzu GC-2014 chromatograph with a flame ionization detector, a SH-Rxi-5SilMS column 30 meters long, 0.25 mm inner diameter, and 0.25 μm thick coated film . The column temperature is the isotherm at 280 °C, the temperature of the detector is 290 °C, the temperature of the evaporator is 280 °C, the nitrogen carrier gas flow rate is 30 ml per minute, and the pressure is 80 kPa.

The chromate-mass-spectrometric analysis was carried out using a 6850A Agilent chromate-mass spectrometer (Agilent Technologies, Inc.) with a model G2629A gas chromatograph equipped with a model G2577A HP5973 Network selective mass spectrometry detector. The flow rate of carrier gas (He) was 1mL per min. The injector temperature was 280 °C. The transfer line were kept at 280 °C. The quadrupole temperature was 280 °C. The ion source was kept at 230 °C. To fractionate samples, a 0.25 μm HP-5MS quartz column (30000 × 0.25 mm) with a 5% phenyl-methyl siloxane stationery phase was used. The thermostat temperature was programmed to increase from 100 to 280 °C at a rate of 5 °C min^{-1} and temperature control at 280 °C for 40 minutes. The dosed volumes 1 μL were injected in the split mode at a ratio of 20 : 1. The ionizing energy was 70 eV. Mass range was from 55 to 550 m/z. Mass spectra were compared with the NIST 05 database and mass spectra of compounds previously isolated from the outer bark.

Determination of the amount of triterpenoids was carried out by the absolute calibration method using previously isolated compounds from birch outer bark: betulin, lupeol, betulinic acid, oleanolic acid acetate, oleanolic acid [14]. The purity of the standards was determined by GLC. The purity was from 95 to 99%. The structure of the compounds was determined by mass spectrometry and NMR spectroscopy.

Mass-spectrum (m/z) of betulin (B): 442 (15), 427(8), 411(43), 385(10), 288(10), 220(19), 205(20), 203(86),189(100), 175(44), 161(26), 147(42), 135(81), 121(65), 119(56), 107(68), 95(92), 81(83), 69(63), 55(61), 43(45);

oleanolic acid methyl ester: 512 (1), 497(<1), 452(5), 437(2), 409(1), 393(1), 377(1), 337(<1), 314(1), 301(<1), 285(<1), 262(51), 249(7), 233(2), 215(3), 203(100), 189(28), 175(9), 161(5), 147(7), 133(16), 119(15), 105(13), 93(10), 81(11), 69(16), 55(11), 43(37).

methyl ester of oleanolic acid acetate: 512(1), 497(<1), 452(5), 437(2), 409(1), 393(1), 377(1), 337(<1), 314(1), 301(<1), 285(<1), 262(51), 249(7), 233(2), 215(3), 203(100), 189(28), 175(9), 161(5), 147(7), 133(16), 119(15), 105(13), 93(10), 81(11), 69(16), 55(11), 43(37);

betulinic acid methyl ester: 470 (6), 452(4), 411(11), 281(5), 262(27), 249(11), 233(11), 220(14), 207(23), 203(31), 189(100), 175(32), 161(16), 147(18), 133(26), 119(24), 105(34), 91(26), 81(27), 69(18), 55(19);

lupeol (L): 426 (36), 411(15), 393(4), 315(10), 218(52), 207(76), 189(100), 135(81), 121(76), 109(80), 95(88), 81(175), 69(58), 55(51).

Standards in the amount of 10–50 mg were transferred into a 10 ml volumetric flask and butylated with an ether solution of diazobutane.

$$C = K\frac{S \times Y}{m},$$

where *C* – content triterpenoid in bark, *Y*-yield of isopropanol extract (%), *m* – the amount of extract in the flask per 10 ml (mg), *S* is the peak area of the compound in the GLC chromatogram. *K*- tangent of the slope of the calibration line. *K*=1.92 for betulin, 0.89 for lupeol; 1.24 for betulinic acid butyl ester; 1.24 for oleanolic acid butyl ester; 0.88 for oleanolic acid acetate butyl ester.

Diazobutane was obtained by the method of producing diazomethane using urea, sodium nitrite [17]. Instead of methylamine, equimolar amounts of butylamine were used. The acid butylation reaction takes a longer time than methylation. At butylated stood the reaction mixture for 2 hours. Peak resolution of betulonic aldehyde and BA butyl ester in the GLC analysis of triterpenoids containing butyl esters of acids was better than in the analysis of a mixture containing BA methyl ester. Figure 1 shows a typical chromatogram of an extract obtained from *B*. *mandshurica* after butylation and analysis by GLC.

NMR spectra were recorded using a NMR spectrometer Jeol ECX-400A (100MHz for ^{13}C spectra), and $CDCl_3$ as a solvent.

Results and discussion

All species contained neutral substances: lupeol, betulin (table 1). Lupeol has also recently become interesting for its biological activity [18]. In most species of birches, betulonic and betulinic aldehydes, lupenon, were also identified. In this study, attention was given to acids.

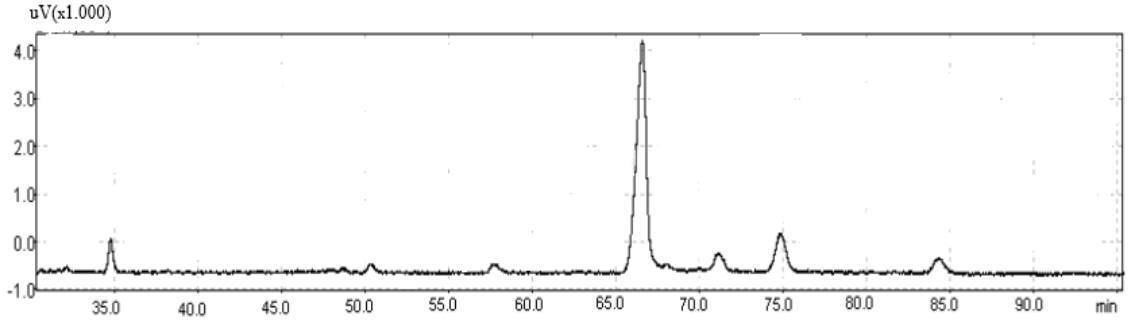


Fig. 1. GLC chromatogram of triterpenoids isolated from *B*. *mandshurica* after synthesis of butyl esters

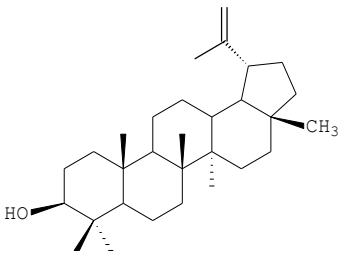
Table 1. The content of the main triterpenoids in the outer barks of various birches

Wood species	Extract yield, % of dry matter	L	B	BA	OA	AOA
Retention time		34.8	66.6	74.8*	71.1*	84.4*
B. *mandshurica* (3)	32.8	3.1	20.0	2.2	0.6	0.6
B. *platyphylla*(4)	45.1	2.3	28.0	3.6	1.0	–
B. pendula (1)	25.7	0.9	20.8	1.1	–	<0.1
B.costata ** (2a)	6.5	0.3	0.4	0.3	1.6	0.2
B.costata *** (2b)	12.0	14.0	54.8	9.7	<0.1	1.7
B.davurica(5)	10.9	–	<0.1	0.2	0.3	0.6
Wounded (1)	6.9	0.4	1.0	1.0	0.3	0.2
Outer bark of (1) branches	14.0	1.2	1.8	0.5	1.0	1.0
Inner bark of (1) branches	23.0	0.1	0.1	–	–	0.01

* butyl ester.

** the only specimen of birch betula pendula from the Primorsky region. Age 22 years old.

*** samples of birch from the Krasnoyarsk region. Age 40-45 years' old.



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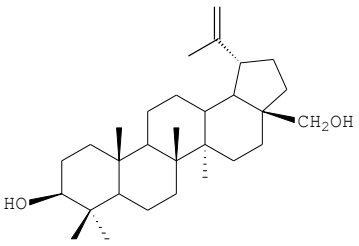
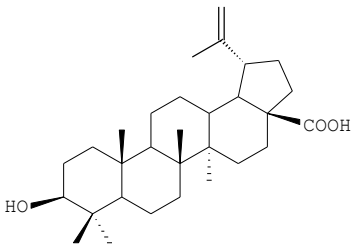
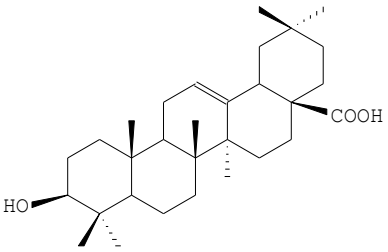


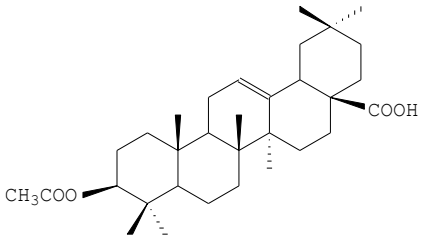
B



BA



OA



AOA

(1) has the smallest amount of triterpene acids in birch bark. An increase in the relative acid content in (1) is observed in the bark of the branches and in the bark, which grows to replace the damaged one after removing the birch bark for weaving. In this case, the tree appears to respond to injury by synthesis of triterpene acids. In the crust of branches, the content of triterpene acids, relative to betulin, is much higher, which facilitates the separation of triterpenoids. But when processing the whole branches, the content of triterpene acids in the extracts is significantly reduced. The separation of the crust of branches from bark and wood after grinding by known methods (blowing birch bark, sifting) does not help in the case of separation of crushed branches [19, 20]. The outer bark of the East Siberian species contain significantly more triterpene acids. Of the studied species, only (2) has no industrial significance and is not used to produce cellulose, plywood and building materials.

For the separation of triterpenoids and salts of triterpene acids, the different ability of triterpenoids to dissolve in solvents was used (Fig. 2). To isolate AOA, a hydrocarbon solvent (gasoline, petroleum ether (PE)) and an aqueous alkali solution are required. AOA is extracted with a hydrocarbon solvent from the birch outer bark or from the alcohol extract of the bark quantitatively together with neutral triterpenoids: lupeol, lupenon, betulinic and betulonic aldehyde.

B is also partially extracted with a hydrocarbon solvent, but most of it is precipitated by cooling the extract. AOA is isolated in the form of insoluble in water and hydrocarbons, salt when washing the hydrocarbon extract with an aqueous solution of alkali. Filtration of the salt and subsequent acidification with an aqueous (up to pH3) or alcoholic solution of sulfuric acid leads to the production of AOA with 90% purity. An alcoholic solution of the obtained acid is poured into a 5-fold volume of water to form a precipitate of AOA. The NMR data of the obtained crystalline product coincided with those known in the literature [21]. NMR ^{13}C data ($CDCl_3$): 38.1 t (C-1), 27.7 t (C-2), 81.0 d (C-3), 37.8 s (C-4), 55.3 d (C-5), 18.3 t (C-6), 32.5 t (C-7), 39.4 s (C-8), 47.6 d (C-9), 37.0 s (C-10), 23.5 t (C-11), 122.5 d (C-12), 143. 6 s (C-13), 41.7 s (C-14), 27.7 t (C-15), 23.6 t (C-16), 46.6 s (C-17), 41.0 d (C-18), 45.9 t (C-19), 30.8 s (C-20), 33.2 t (C-21), 32.5 t (C-22), 28.1 q (C-23), 16.8 q (C-24), 15.5 q (C-25), 17.1 q (C-26), 26.0 q (C-27), 184.1 s (C-28), 33.1 q (C-29), 23.7 q (C-30), 171.2 s (COO), 21.4 q ($OOC\underline{C}H_3$)

BA and OA are isolated from the outer bark or from the alcohol extract by a more polar solvent: diethyl or methyl tert-butyl ether (MTBE) together with betulin. Acids, similarly to AOA, are extracted from the extracts with an aqueous solution of alkali, but contain up to 30% betulin. Recrystallization from isopropanol, acetone or ethanol allows you to clean the product only from other triterpenoids, but not from B. Purification from B, it may be more appropriate to carry out using turpentine as a solvent [22].

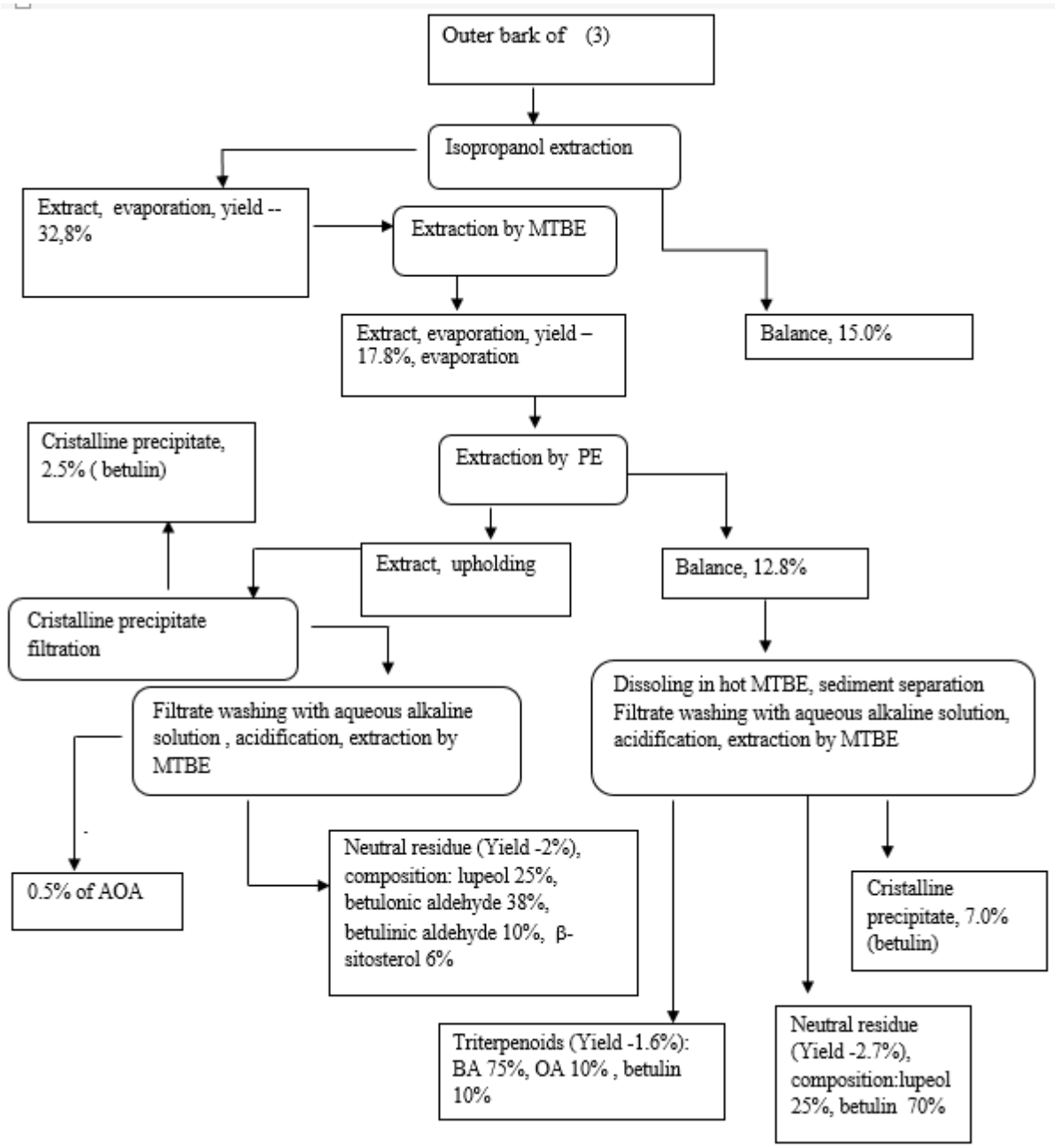


Fig. 2. Triterpenoid separation scheme and material balance

Conclusions

B. mandshurica (Regel) Nakai, and *B.costata* Trautv. outer bark are suitable for the industrial isolation of triterpene acids. There is a high relative content of triterpene acids in the crust of branches and in the bark of wounded *B. pendula*. Existing methods for separating birch outer bark from the inner bark (steeping, sifting, various, blowing) do not help when separating the outer bark from the birch branches.

The main stages of the isolation of triterpenoids include: extraction with various solvents, isolation of acids from extracts in the form of salts, filtration, acidification.

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Received September 26, 2019

Revised November 10, 2019

Accepted November 28, 2019

For citing: Vedernikov D.N., Eregina O.A., Shemyakina A.V. *Khimiya Rastitel'nogo Syr'ya*, 2020, no. 1, pp. 277–282. (in Russ.). DOI: 10.14258/jcprm.2020016503.