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Evaluation of Betulin and Betulinic Acid Content in Birch Bark from Different Forestry Areas of Western Carpathians

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Abstract

In order to evaluate the content of betulin and betulinic acids in Birch barks originating from Western Carpathians, Transylvania (Romania) forests, in relation with the location of birch trees, it was recorded the geographic and vegetation conditions at the specific locations of each type of sample. Two groups of birch tree bark (totally 10 samples) were collected, from two regions, pasture surfaces where birch trees appeared spontaneously (samples 1 to 5) and another group from forests of combined trees, including mainly pine trees (samples 6 to 10). The identification and quantitation of betulin and betulinic acid were made by High Performance Liquid Chromatography with UV detection. In the first region it has been found mean concentrations of 126.85 ± 12.56 mg/g betulin bark while acid betulinic had average values of 12.78 ± 1.26 mg/g bark. In the second region we found mean concentrations of 89.84 ± 8.43 mg/g betulin of bark while acid betulinic had average values of 10.60 ± 0.97 mg/g bark. Statistically, significant differences were noticed between the two regions, the first region being richer in these molecules. Generally, the percentage of betulinic acid was around 11.23% against betulin, higher in the second region, even the absolute concentrations were lower for both molecules. These data are useful indicators of the potential offered by birch bark sources found in Transylvania region, to obtain extracts enriched in betulin and betulinic acid.

Keywords: *Betula pendula*, betulin, betulinic acid, HPLC, Western Carpathian forest

Introduction

Romania is a rich country considering the variety of birch forests. It can be found four main species (*Betula pendula* Roth., *Betula pubescens* Ehrh., *Betula humilis* Schrank. and *Betula nana* L.) out of them, the „common birch" (*Betula pendula* Roth.) being the most known, mainly present in Western Carpathians, inside forests or spread in the pasture areas (Dehelean *et al.*, 2007; Kovac-Besovic *et al.,* 2009 a; Stănescu, 1979). Having a pronounced „pioneer" character, due to its „rusticity", the common birch appears spontaneously, filling the empty surfaces after cuttings of other trees or after calamities (Șofletea and Curtu, 2000). It is characterized by high ecologic amplitude, developing without any special conditions. By its unique shape and white appearance of the bark, by its peculiar foliage, it is also a very decorative tree (Conway, 2002; Haralamb, 1956). The birch wood has hard proprieties, generally used for carpentry or even in aquatic industry. It is a good source of methanol, vinegar, coal and the bark, rich in tannins was mainly used to obtain heavy hydrocarbons and lubricant oils (Zhao *et al.,* 2007). The concentration of tannins (around 1.6%) especially located in the suberosis part recommend it for tanning industry. By dry distillation, the oil extracted is used as skins moisture or to be used as lighting fuel (especially in the North of Europe) (Wan *et al.,* 2012).

The birch bark is formed of a white suberos epidermal with multiple layers which exfoliates in narrow foils. Almost half of it contains a sort of resin which contains betulins, which confer a high capacity of preservation for longtime, demonstrated by its occurrence in the turba and coal (lignite) deposits. The foliage is useful for other significant substances, including flavonoids (Lahtinen *et al.*, 2006; Tamas *et al.*, 1978), which can be valorized for different products (EMA, 2007; Hoppe, 1975, Ladynina and Morozova, 1987; Vermeulen, 1999). The borch sap is also a useful product containing carbohydrates (8.7%), a mature tree producing in 24 hours, around 170-240 liters of sap (Fulda, 2008). The birch bark is also known as an antipyretic agent (Hoppe, 1975), containing 4-5% tannins and essential oils. Betulin, a resinous triterpene similar to lupeol, and the glycoside betuloside with the aglycone betuligenol are found in bark (Gessner, 1974; Kovac-Besovic *et al.*, 2009 b).

Betulinic acid is a minor bio-compound but with a high efficacy in treating different diseases. Interest in betulin has increased recently as certain derivatives from this composition are considered to be a potential agent against tumor cancer (Alakurtti *et al.*, 2006; Oh *et al.*, 2006; Sami *et al.*, 2006) tested on brain, skin or other tumor cells (Fulda *et al.*, 1999; Pezzuto *et al.*, 1999; Rajendran *et al.*, 2008), as well in the chronic hepatitis therapy (Shikov *et al.*, 2011).

Betulinic acid has anti-malarial, anti-inflammatory antiviral, anti-HIV activities and cytotoxicity against a variety of tumor cell lines comparable to some clinically used drugs (Cichewicz and Kouzi, 2004; Fulda, 2008; Kvasnica *et al.*, 2005). Betulinic acid has also been found that it can retard the progression of HIV-1 infection, which eventually leads to AIDS, by preventing the formation of syncytium (cellular aggregates) (Crevelin *et al.,* 2006; Zuco *et al.*, 2002).

The identification was made by spectrometry (O'Connell *et al.*, 1998), by HPLC (Ossipov *et al.*, 1996; Zhao *et al.*, 2007) or by vibrational spectroscopy (Cântă Pânzaru *et al.*, 2002; Falamas *et al.*, 2011).

Outer bark of birch (*Betula alba* cortex) contains pentacyclic triterpenes, mainly betulin (BE, up to 34%), but also betulinic acid (BA), oleanolic acid (OA), lupeol (LU) and erythrodiol (ER). They can be extracted as a triterpene rich dry extract (TE) which is able to form a topically applicable oleogel (Ekman, 1983; Laszczyk *et al.*, 2006; Lavrjonov and Lavrjonova, 2003).

Betula alba is used also in homeopathy, basically as a natural diuretic as well as against gastric aches (Mihailov, 2002). The buds contain 4-6% of essential oil (Gessner, 1974), and is used as a diuretic drug (Turova, 1974) (*Gemmae Betulae*). *Oleum Betulae empyreumaticum rectificatum* is the oil obtained by the dry distillation of the bark and wood of *Betula alba* and rectified by steam distillation. The external application of betulin is recommended in parasitic infestation of the skin with subsequent hair loss, rheumatism and gout, dry eczema and dermatoses, psoriasis and other chronic skin diseases (Ladynina and Morozova, 1987; Muravjova *et al.,* 2002; Turova, 1974). The dry distillation of birch wood yields about 6% phenols (cresole, quajacole, xylenole, coesole). In veterinary medicine the oleum, *Betulae empyreumaticum rectificatum*, in known as vermifuge (Gessner, 1974).

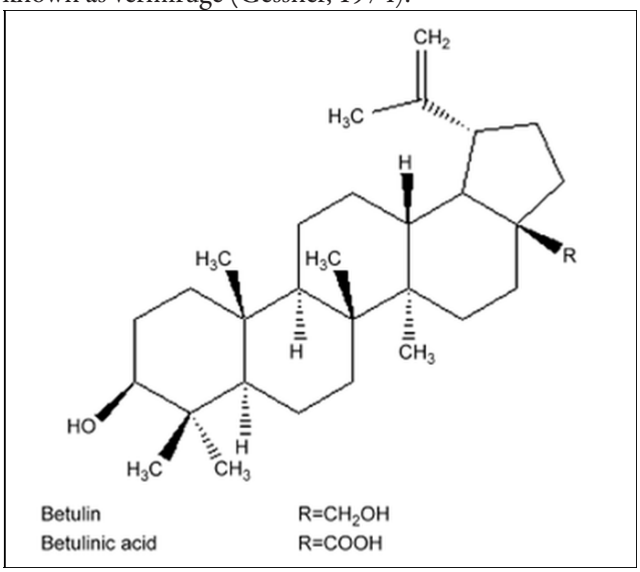

Fig. 1. Chemical structures of betulin and betulinic acid found, main triterpenoids with diverse bioactivities from the birch bark

Betulin and betulinic acid are pentacyclic triterpenes present in the bark of the birch tree and other vegetal sources. Quantitatively, in birch bark betulin is more significant than betulinic acid; therefore, birch can be a large and feasible source of raw material for betulin extraction (Soica *et al.*, 2012; Yin *et al.*, 2008).

The common birch found in Western Carpathians (Transylvania region of Romania) is a middle size tree (rarely taller than 25 m) with a thin trunk, larger at bottom, sometimes with irregularities in growth. The young trees have white bark which exfoliates easily, while in the old trees bark have harder dark black "ritidom", especially at the bottom. The branches crown is not well developed, irregular, with oval-pyramid shape and thin branches inserted obliquely in the trunk.

Betulin (3-lup-20(29)-en-3,28-diol) and betulinic acid (3-hydroxy-lup-20(29)-en-28-oic acid) are pentacyclic lupane type triterpenoids (Fig. 1) which can be easily isolated and purified. The birch tree (Betula spp., Betulaceae) is one of the substantial sources for both the molecules (Hayek *et al.*, 1989).

In the present study it was investigate the composition in betuline and betulinic acid of different birch tree barks found in forestry areas of Western Carpathians, bioactive molecules which can be valorized by biorefinery techniques.

Materials and methods

Specific areas for harvesting the birch bark samples

In order to correlate the content of betulin and betulinic acids found in samples, with the location of birch trees, it has been recorded the geographic and vegetation conditions at the specific locations of each type of sample. There were collected bark samples of similar ages, from different areas and trunk diameters, from different mountain areas of Western Carpathians.

Two groups of birch tree bark samples were collected: one group (samples 1 to 5) from pasture surfaces where birch trees appeared spontaneously and another group (samples 6 to 10) from forests of combined trees, including mainly pine trees. The average age of trees was 20 years in all cases and the trunk diameter of 10-12 cm at 1.3 m height from the soil level. The samples were transversal cuttings of 10 cm length and 10-12 cm diameter. All the collected barks were immediately dried at 60°C and stored in a dry and dark place.

The first lot of samples (1 to 5) was harvested from birch tree found in pasture areas, in the villages Beliş, Râşca, Dângău, Mărişel and Paraul Porcului. The trees were situated in the high hilly and pre-mountain areas, is situated at 46°65' latitude North, 23°03' longitude East and a altitude between 900 m and 1050 m a.s.l., with sunny exhibition and different slopes (Fig. 2). The environmental and vegetation conditions were similar. According to classification Koppen, the general climate is „D.f.c.k." meaning "humid,

rainy with cold winters", and with annual average temperatures in the warmest season, above 10°C. The dryness index ranged between 57 and 63 (mean value = 60).

The second lot of samples (6 to 10) (Tab. 1) were collected from birch trees found in the basin „Valea Ierii", is situated at 46°65' latitude North, 23°35' longitude East and a medium altitude of 1000 m a.s.l. with similar conditions, but with different slopes from the area (nr. 7, 8 and 9) or outside the forests (nr. 6 and 10) (Fig. 3). According to Koppen climate classification, the tress are situated in boreal, D.f.k., characterized by cold and humid winters, with average temperatures, in warm seasons, above 10°C, and -4°C in the cold season. It is a specific pre-mountain and mountain vegetation with pines, larch, spruce fir etc., as detailed in Tab. 1.

Chemical analysis

The betulin and betulinic acid were extracted from dried birch barks and analyzed at the Department of Chemistry and Biochemistry at the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, using High Performance Liquid Chromatography with ultraviolet detection (HPLC-UV). Pure standards of betulin and betulinic acid (provided from Roth, GmbH Germany) were used to build a calibration curve, in order to make quantitative evaluations. From each white bark sample, were used aliquots of 0.5 g in triplicate, which were mixed with 10 ml methanol 95% containing 1% HCl conc. and homogenized under sonication 15 min. After 30 minutes, the extract was filtered by paper and Millipore PTFE membrane (0.45 μm). The filtered sample was injected (20 μl) in the HPLC column (Supelcosil LC 18, 250 mm × 4.6 mm × 5 μm) of an Agilent 1200 HPLC device, with UV detection, applying an isocratic mobile phase consisting of acetonitrile: water, 9:1, flow 1 ml/min. The run temperature at 25°C and the detection at 210 nm were set as optimum.

The concentrations of each component (BA and B) were calculated according to the calibration curve and expressed in mg per gram barks. The ratios of betulinic acid to betulin was calculated and expressed as percentage (BA/B × 100).

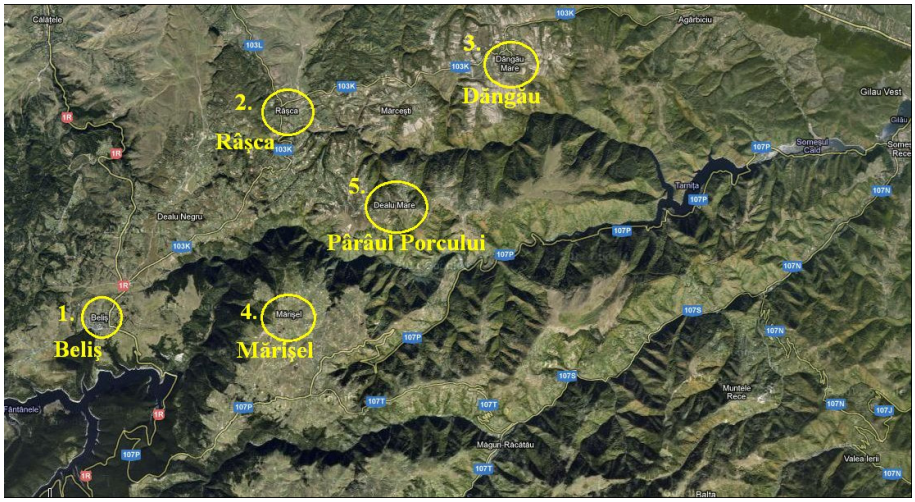

Fig. 2. Geographic position of the harvesting places (satellite picture) for samples 1-10

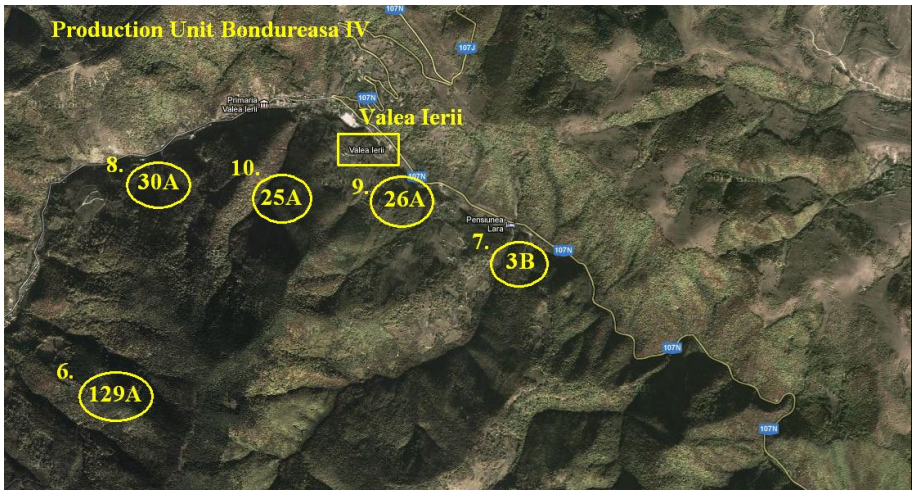

Fig. 3. Details about the surfaces with birches from where the samples 6-10 were harvested (Bondureasa region)

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Tab. 1. Samples of birch tree originating from Production Unit Bondureasa IV (samples 6 to 10)

Nr.	Characterization of the specific areas from the Production Unit-region Bondureasa IV
1.	Inferior versant, kneaded soil, South exhibition, altitude 980 m and slope angle 38 degree. Indicative flora: *Asperula-Oxalis*. Birch trees are disseminated in the empty areas between forests of pine tree (P) and beech tree (B) (ratios of 8P + 2B) with an average consistency of 0.5.
2.	Middle versant, kneaded soil, North exhibition, altitude 1210 m and slope angle 40 degree. Indicative flora: *Vaccinium*. Birch trees (Bi) are disseminated in forests of spruce fir (S) (composition 6S + 4 Bi) with a consistency of 0.3.
3.	Inferior versant, waved soil, North-West exhibition, altitude 920 m and slope angle 20 degree. Indicative flora: *Asperula-Dentaria*. Birch trees are disseminated in the empty areas between forests of pine tree (P) and beech tree (B) (ratios of 8P + 2B) with an average consistency of 0.6.
4.	Middle versant, waved soil, West exhibition, altitude 960 m and slope ange 25 degree. Indicative flora: *Asperula-Dentaria*. Birch trees (Bi) are disseminated in pure forests of spruce fir (S) (composition 10S) with an average consistency of 0.7.
5.	Inferior versant, kneaded soil, South exhibition, altitude 980 m and slope angle 38 degree. Indicative flora: *Asperula-Oxalis*. Birch trees are disseminated in the empty areas between forests of pine tree (P) and beech tree (B) (ratios of 4P + 1B) with an average consistency of 0.6.

Results and discussion

HPLC-UV analysis for the identification of betulin and betulinic acid

To identify accurately the betulin (B) and betulinic acid (BA), a mixture of pure standards was separated after a previous optimization of the separation protocol, established in the laboratory.

The standard curve for each compound was built in the concentration region of 0.01 to 0.1 mg/ml (data not shown) as well in a mixture ratio of 2:5, as shown in Fig. 4.

Betulinic acid (BA) and Betulin (B) concentrations in birch bark, depending on the region of harvest

The betulin peak was identified at 5.02 min. for sample 4, from Marisel area (Fig. 5.). To identify correctly and accurately the peak position of betulinic acid (BA) (t_R=4.45 min), a minor component in this sample, it has been made a co-chromatography with pure BA. Actually the two close peaks in Fig. 5A correspond to 2 isomers of BA.

The mean values of the concentrations of betulinic acid (BA) and betulin (B) concentrations (expressed mg/g bark) determined in birch bark samples, originating from different regions and areas were presented in Tab. 2.

The extraction solvent was chosen as 95% ethanol because betulin and betulinic acid could be extracted from white birch bark with a highest content. Results showed that all of the bioactive triterpenoid in white birch bark were strikingly dependent on the location (Tab. 2).

The results showed that content of the compounds were strongly dependent on the solvents with different polarities and similar studies (Zhao *et al.*, 2007; Zhang *et al.*, 2008). Ninety-five percent ethanol was a good extraction solvent that allowed extraction of triterpenoid with a highest content due to the co-solubility effect (Zhao *et al.*, 2007).

In the first region (samples 1 to 5) it was found mean concentrations of 126.85 ± 12.56 mg betulin per g of bark, while acid betulinic had average values of 12.78 ± 1.26 mg/g bark. In the second region (samples 6 to 10) it was found mean concentrations of 89.84 ± 8.43 mg betulin

Tab. 2. The mean values ± SD of betulinic acid (BA) and betulin (B) concentrations (expressed mg/g bark) determined in triplicate from birch bark samples, originating from different regions and areas. The percentage of BA against B was calculated

Nr.	Region of sample harvest	Betulinic Acid (BA) (X±SD) (mg/g)	Betulin (B) (X±SD) (mg/g)	Percentage (BA/B × 100) %
1	Belis	11.64 ± 1.23	165.60 ± 14.23	7.00
2	Rasca	14.68 ± 1.50	126.91 ± 11.56	11.56
3	Dangau	12.31 ± 1.07	109.14 ± 10.03	11.27
4	Marisel	11.15 ± 1.11	130.81 ± 12.78	8.52
5	Paraul Porcului	14.15 ± 1.39	101.82 ± 14.23	13.89
Average values for regions 1-5		12.78 ± 1.26	126.85 ± 12.56	10.44
6	PU* IV ua 129 A	11.80 ± 1.11	81.99 ± 7.83	14.39
7	PU IV ua 3 B	7.34 ± 0.73	57.44 ± 5.02	12.77
8	PU IV ua 30 A	9.76 ± 0.88	86.17 ± 8.62	11.32
9	PU IV ua 26 A	8.60 ± 0.75	77.98 ± 7.83	11.02
10	PU IV ua 25 A	15.44 ± 1.39	145.63 ± 12.86	10.60
Average values for areas 6-10 (region 6)		10.60 ± 0.97*	89.84 ± 8.43**	12.02
Average values for all samples		11.69 ± 1.15	108.34 ± 10.49	11.23

*p<0.1; **p< 0.05

* UP – production unit

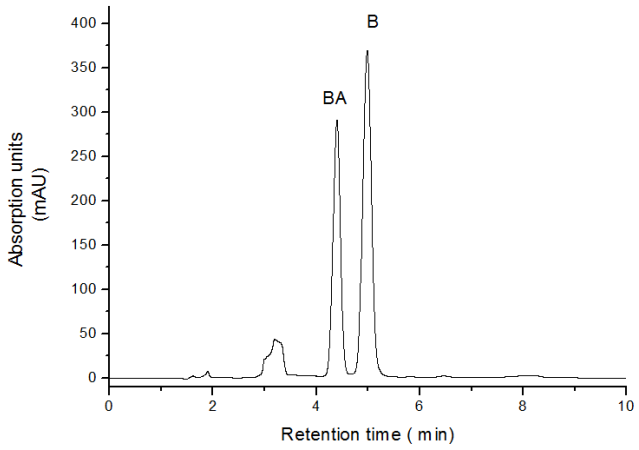

Fig. 4. The HPLC-UV chromatogram of a mixture of two pure standards of betulinic acid (BA) and betulin (B) at 0.02 mg/ml and 0.05 mg/ml, respectively. Retention times: t_R=4.45 min for BA and t_R=5.02 min for B. The detection was set at 210 nm

per g of bark, while acid betulinic had average values of 10.60 ± 0.97 mg/g bark.

The content of betulin and betulinic acid varied significantly in the white birch bark in different locations. In the first region, Belis sample proved to be the richest in betulin concentrations of 165.60 mg/g, and sample Rasca for betulinic acid with a concentration of 14.68 mg/g. Sample PU IV 25A second region, recorded the highest concentration of betulin (145.63 mg/g) and betulinic acid (15.44 mg/g). Statistically, significant differences were noticed between the two regions of bark origin, the first region being richer in these molecules. The ratio of betulinic acid to betulin was calculated and expressed as percentage (BA/B × 100). Generally, the percentage was around 11.23%, higher at the second region, even the absolute values were lower for the BA and B.

Betulin is a natural lupane-class triterpene derived from plants with a wide variety of biological activities. This compound group thus has wide medical potentials, and in fact has been shown to be active against intracellular pathogens (Salin *et al.*, 2010).

Betulin can be used as extracted or, after chemical modification, as a starting compound for its acid, betulinic acid, with both substances possessing various pharmacological properties (Soica *et al.*, 2012).

The data obtained are useful indicators of the potential offered by these birch bark sources found in Transylvania region (Romania), to obtain extracts enriched in betulin and betulinic acid. Further studies will focus on the bioconversion of betulin to betulinic acid using specific microorganisms and the study of the pharmacologic effects (as hepatoprotective and anticancer agents) of standardized.

Conclusions

The HPLC method mentioned here represented an excellent technique for simultaneous determination of betulin and betulinic acid in the extract of white birch bark, with good sensitivity, precision and reproducibility. The method gives a good resolution among betulin and betulinic acid with a short analysis. Significant variations in the content of betulin and betulinic acid in birch bark samples were recorded at Belis and Rasca in the first region and the sample PU IV 25 A second region. These data are useful indicators of the potential offered by birch bark sources found in Transylvania region, to obtain extracts enriched in betulin and betulinic acid.

A.

B.

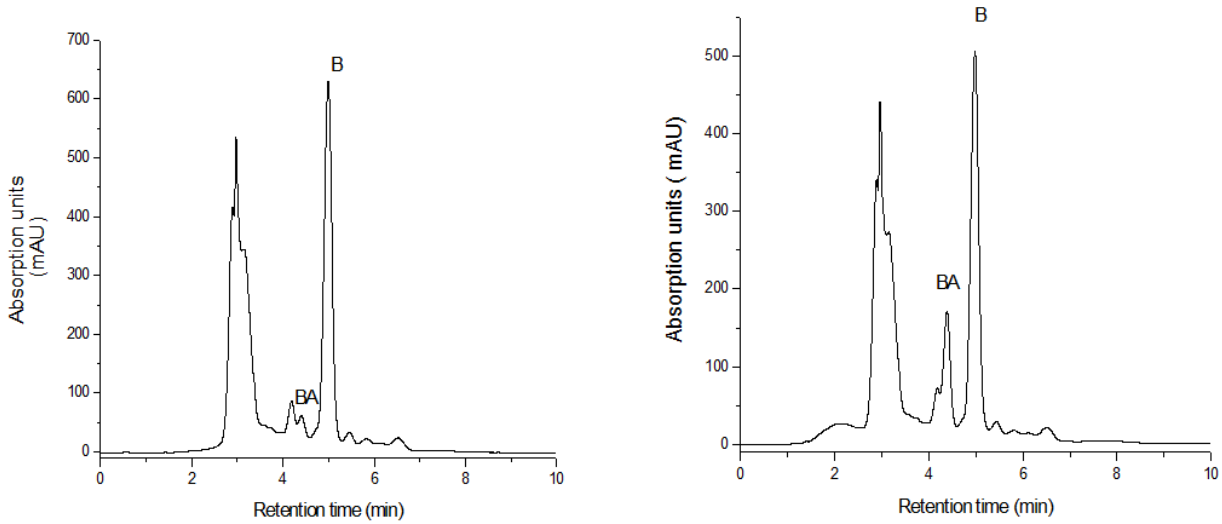

Fig. 5. A. HPLC-UV chromatogram of birch bark extract-sample 4 (from Marisel). B. Co-chromatogram of the sample 4 with pure standard of BA. Retention times: t_R=4.45 min for BA and t_R=5.02 min for B. The detection was set at 210 nm

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