Introduction

Birch (Betula L.) is one of the main arboreal plants in the forests of the boreal and temperate zones as well as the mountain regions of the Northern Hemisphere [1]. It belongs to a group of medicinal plants which have been used in traditional medicine since ancient times. Leaves, buds, tar and essential oils are used to treat a wide spectrum of diseases, including inflammations, infections, urinary-tract disorders, skin and hair disorders [2]. The application of birch essential oils in aromatherapy by direct inhalation for respiratory disinfection and against bronchitis has also been reported [3].

An integral part of folk culture of many nations is the use of steam bath-houses such as Finnish sauna, Russian bath and sweat lodges of American Indians for sanitation (as well as for physical and mental relaxation). According to traditions of northern people, bathing is associated with a particular use of birch: previously steamed in very hot water, bunches of dried leafy, fragrant boughs of birch are used for massage and stewing (intensive stimulation of skin). In this case, the exposure of man to birch volatile and water-extractable compounds results from both inhalation and direct contact [4]. To intensify the effectiveness of inhalation, the water used for steaming of birch boughs is sprinkled onto hot stones from time to time.

In spite of a wide usage of birch in folk and conventional medicine [5], the existing information on the chemical composition of its tissues is insufficient for medical purposes. The best investigated products are essential oils obtained by hydrodistillation of leaves or buds of Betula species that were the subject of numerous studies [6–10]. Undoubtedly, birch tissues contain many extractable polar compounds which cannot be isolated by hydrodistillation. On the other hand, some volatile organic compounds (VOCs) can be lost during hydrodistillation. Meanwhile, only few works deal with individual groups of compounds extracted from
birch buds and leaves [11,12], while there is virtually no information on VOCs which can play a distinctive role in aromatherapy. Biological activity (antibacterial properties, antioxidant capacity, etc.) of a plant material is determined by a mixture of substances with different mechanisms of action, including synergistic interactions. Hence, for medical purposes it is important to investigate the whole complex of these substances.

This communication describes the chemical composition of volatile and extractable compounds from Litwinow birch (Betula litwinowii Doluch.) buds. This birch belongs to species whose essential oils were comprehensively investigated by gas chromatography–mass spectrometry [7,9]. To the best of our knowledge, the volatile and extractable compounds of B. litwinowii buds have not been previously investigated. To isolate as many substances as possible from the plant material, we used headspace solid-phase microextraction (HS-SPME) and successive extraction with solvents of different polarities.

2 Experimental procedure

2.1 Chemicals and equipment

Triterpenoids, dipterocarpol, oleanolic acid, betulinic acid and betulinol, as well as pyridine and bis(trimethylsilyl) trifluoroacetamide (BSTFA) with addition of 1% trimethylchlorosilane were purchased from Sigma-Aldrich (Poznań, Poland). Bud extraction was carried out by diethyl ether and methanol (POCH SA, Gliwice, Poland). The SPME holder and DVB/CAR/PDMS (50/30 µm) fiber used in this study were obtained from Supelco (Bellefonte, PA, USA).

2.2 Plant material

Betula litwinowii birch buds were collected at the beginning of October 2012 from the trees planted in the Institute of Dendrology of the Polish Academy of Sciences (Kórnik, Poland). The voucher specimen is kept in this Institute’s Herbarium. The collected buds were covered with a layer of dry rubber-like matter (exudate). VOCs composition was investigated within 36 h from the samples collection. The rest of the buds were kept at a temperature of -18°C until assayed.

2.3 HS-SPME determination of VOCs

Freshly harvested buds (1.5 g) were transferred into a head-space vial of 16 mL in volume and immersed into temperature-controlled water bath (40°C). The septum of screw-cap was picked by the needle protecting the SPME fiber, and the fiber coating was exposed to a headspace gas phase for 50 min. The volatiles collected on the fiber were desorbed by introducing SPME fiber for 10 min into the injection port of the GC–MS apparatus. Analytes were separated and analyzed on a HP 6890 gas chromatograph with the mass selective detector MSD 5973 (Agilent Technologies, USA). This device was fitted with the HP-5MS fused silica column (30 m × 0.25 mm i.d., 0.25 µm film thickness), with electronic pressure control and split/splitless injector. Helium flow rate through the column was 1 mL min⁻¹ in a constant flow mode. The injector (250°C) worked in a splitless mode. The initial column temperature was 40°C rising to 220°C at 3°C min⁻¹. The MSD 5973 detector acquisition parameters were as follows: transfer line temperature 250°C and the detector temperature 270°C. The EIMS spectra were obtained at 70 eV of ionization energy. Detection was performed in a full scan mode from 29 to 600 a.m.u. To determine the retention times of reference compounds and to calculate linear temperature programmed retention indices (Iₜ) of separated volatiles, a SPME fiber was inserted for 2–3 s into the vial with mixture of C₅–C₁₈ n-alkanes. The separation of alkanes was performed under the above conditions.

2.4 Determination of extractable compounds

Buds (1.5 g) were milled and immediately transferred into a retort of 25 mL in volume and extracted by three portions of 15 mL of diethyl ether, constantly stirred. Next, the residue was extracted 3 times with 15 mL of methanol. The duration of each extraction cycle at room temperature was 30 min. The joint ether and methanol extracts were filtered through a paper filter and the solvent was removed on a rotor evaporator. The residue left on the walls was washed out (after its mass had been determined) by 5 mL of diethyl ether or methanol, and 0.5 mL of this solution was put into a vial of 2 mL in volume. After evaporation of the solvent, 220 µL of pyridine and 80 µL of BSTFA were added into the vial. The reaction mixture was sealed and heated during 0.5 h at 60°C to obtain trimethylsilyl (TMS) derivatives. The whole procedure was performed in triplicate.
Obtained solutions were separated and analyzed with the aid of the GC–MS apparatus and above-mentioned capillary column HP-5MS. Injection of 1 μL of the sample was performed with the aid of HP 7673 autosampler. The injector (250°C) worked in a split mode (1:50). The initial column temperature was 50°C rising to 310°C at 5°C min⁻¹. Detection was performed in the full scan mode from 41 to 600 a.m.u. After integration, the fraction of each component in the total ion current (TIC) was calculated.

Hexane solution of C_{10}–C_{40} n-alkanes was separated under the above conditions. Linear temperature programmed retention indices (∆Iₜ) were calculated from the results of the separation of this solution and silanized bud extracts.

The method precision was studied by three replicate extractions and analyses. The precision was expressed by relative standard deviation (R.S.D.). The peak areas of the extract components obtained by replicate analyses were used for calculation of their R.S.D. values, which amounted to 9% (ether extracts) and 16% (methanol extracts) in average. Fairly high values of R.S.D. are apparently conditioned by multi-staging procedure of compound extraction.

### 2.5 Component identification

To identify the components, both mass spectral data and the calculated retention indices were used. Mass spectrometric identification was carried out with an automatic system of GC–MS data processing supplied by NIST and home-made mass spectra libraries (the latter contains more than 550 spectra of TMS derivatives prepared from commercial preparations of flavonoids and other phenolics, as well as terpenoids). The retention indices of the registered components were compared with those presented in a home-made computer database containing more than 18 000 ∆Iₜ values for more than 5300 compounds. This database contains our previous results [13–17] and literature data from NIST collection [18]. The identification was considered reliable if the results of computer search at the mass spectra library were confirmed by the experimental ∆Iₜ values, i.e., if their deviation from the home-made database values (∆IₜDB) did not exceed ±5 u.i.

### 2.6. Microorganisms and culture media

The diethyl ether extracts of buds were tested against a set of microorganisms including bacteria from international and Polish collections, such as Gram-positive *Staphylococcus aureus* ATCC 29213 (ATCC, American Type Culture Collection) and *Bacillus cereus* ATCC 10987, Gram-negative *Escherichia coli* PCM 2268 (PCM, Polish Collection of Microorganisms) and *Pseudomonas aeruginosa* PCM 2270, as well as fungi *Candida albicans* PCM 2566. All the microorganisms were kept at -80°C in the storage medium (LB broth and glycerol at a ratio of 1:1) and then they were inoculated onto nutrient agar (bacteria) or Sabouraud agar (fungi) and incubated overnight at 37°C. The microbiological media used in the study were supplied by Oxoid (Oxoid Ltd, Basingstoke, England). The antimicrobial activity of the extracts was assessed by determining the minimal inhibitory concentration (MIC) in accordance with the Clinical and Laboratory Standard Institute (2011) protocols.

### 3 Results and discussion

#### 3.1 Choice of sample preparation procedure

In this work, different groups of components were investigated using different sample preparation procedures. Volatiles emitted from buds into the gas phase were collected by solid phase microextraction (HS–SPME) and determined by GC–MS. Successful application of HS-SPME method is considerably conditioned by the choice of fiber coating. Hence, the preliminary studies were performed to select the SPME fiber. Isolation of volatile compounds was carried out with the help of fibers with 100% polydimethylsiloxane (PDMS-100), Carboxene suspended in PDMS (CAR/PDMS, 85 µm) and dual coating of divinylbenzene and Carboxene (50/30 µm) suspended in PDMS (DVB/CAR/PDMS). Extraction was performed at extraction times from 30 to 70 min, and an extraction temperature 40°C. The best results were obtained for the extraction with DVB/CAR/PDMS fiber. It was determined that PDMS-100 fiber absorbs monoterpenes and their oxygenated derivatives well, but does not absorb compounds with low boiling points. Lower alcohols and carbonyl compounds were registered in the same samples with the use of CAR/PDMS fiber. On the other hand, this fiber does not completely return high boiling sesquiterpenoids. It was determined that for selected DVB/CAR/PDMS fiber the extraction time of 50 min was sufficient to reach equilibrium at 40°C.

Extractable components were isolated by successive extraction with two solvents, low polar
diethyl ether and high polar methanol. The latter approach allows the exclusion of many uncertainties and erroneous identification caused by “overloading” of the chromatographic column and “overlapping” of chromatographic peaks.

The previous experiments showed that the chromatograms of non-derivatized extracts lacked the peaks of some high boiling and polar compounds, such as sesquiterpene phenylpropenoids, kaempferol and catechine. Besides, some other polyphenols were eluted from a capillary column as broad and overlapping chromatographic peaks which significantly worsened the quality of the chromatograms. Hence, to achieve the above mentioned aims we used derivatization of extracted compounds.

As a result, 50 substances were registered in volatile emissions of buds, ether and methanol extracts contained 80 and 39 compounds, respectively. Each of these fractions overlapped only in a small range. On the whole, the buds showed 150 compounds of different classes. The chromatograms of volatile and extractable compounds of B. litwinowii buds are presented in the Fig. 1. Below, there are presented the qualitative and averaged semi-quantitative data on the chemical composition of these fractions.

### 3.2 Volatile components of buds

Apart from the semi-quantitative composition (TIC fraction) of birch buds volatiles, Table 1 contains some analytical parameters that were used to confirm the results of identification: \(I_1\) values, \(m/z\) values of the most intensive ions in the mass spectra (in order of decreasing intensity), as well as the mass number of molecular ions \(M^+\), if detected in the mass spectra. It can be seen that buds of B. litwinowii emitted into the gas phase mainly (93% of TIC) sesquiterpene compounds, both \(C_{15}H_{24}\) hydrocarbons and their relatively volatile \(C_{15}H_{20}O\) and \(C_{15}H_{26}O\) oxygenated derivatives. The analysis revealed that \(\beta\)-caryophyllene (26.6%) and \(\alpha\)-humulene (45.0%) were among the major constituents of buds volatiles. The volatiles contained also significant amounts of des-methyl-caryophylla-8(14)-en-5-one (3.9%). For comparison, TIC fraction of 13 monoterpenes is equal to 2.9%.

As seen in the literature data in the last column of Table 1, the sesquiterpenes form the main fraction of the hydodistilled essential oil from buds of this birch species. However, the major constituents of the oil are the oxygen-containing compounds: 14-hydroxy-\(\beta\)-caryophyllene (21.9%) and 14-hydroxy-4,5-dihydro-\(\beta\)-caryophyllene (36.8%) [9]. Despite our efforts, we failed to identify three sesquiterpenoids that are presented in the last rows of Table 1 and reported in the cited work.

### 3.3 Extractable components of buds

Averaged \((n = 3)\) relative composition of ether and methanol extracts are presented in Table 2. From the substances presented there, only 11 were previously identified in B. litwinowii buds. The identified compounds may be divided into groups as follows: sesquiterpene \(C_{15}H_{24}\) hydrocarbons, sesquiterpenoids (mainly \(C_{15}H_{20}O\) and \(C_{15}H_{26}O\) sesquiterpene alcohols), sesquiterpene phenylpropenoids, triterpenoids and flavonoids.

In addition to the terpenoids of essential oils, ether extracts from B. litwinowii buds contain many polar and high boiling compounds which cannot be distilled with water vapor. A distinguishing feature of the extracts is a high content (38.1% of TIC) of sesquiterpene phenylpropenoids. Esters of cinnamic acids with terpene alcohols belong to phenolic substances which were discovered in the plant kingdom rather late. The first compound from this class of substances, geranyl \(p\)-coumarate, was identified in buds of the North American balsam poplar (Populus balsamifera) [19]. Later, the same authors found the second monoterpene phenylpropenoid, geranyl caffeate, in the exudate of Himalayan poplar (P. ciliate) buds, as well as in the “poplar type” propolis collected in Oxfordshire, UK [20]. Russian authors [12] have recently isolated \(p\)-coumarates of sesquiterpene alcohols: 6-hydroxy-\(\beta\)-caryophyllene, 14-hydroxy-\(\beta\)-caryophyllene, 14-hydroxy-\(\alpha\)-humulene and 9,9-dimethyl-2,5-dimethylenebicyclo[6.2.0] decane-4-methanol from the typical for the Eurasian continent silver birch (Betula pendula) buds.

According to our data, buds of B. litwinowii contained eight sesquiterpene phenylpropenoids. However, the proportion of the triad formed by \(p\)-coumarate of 14-hydroxy-\(\beta\)-caryophyllene and by \(p\)-coumarates of two 14-hydroxy-4,5-dihydro-\(\beta\)-caryophyllene isomers (previously unknown compounds) was equal to 35% of TIC. It is remarkable that the corresponding alcohols \(I_{1729}^p, I_{1734}^p\) and 1760, Table 2) are the main components of the sesquiterpenes fraction: they formed about 13% of the total ion current.

Apart from the above mentioned \(p\)-coumarates, the ether extracts from B. litwinowii buds demonstrated the presence of other previously unknown phenylpropenoids, esters of ferulic and caffeic acids and caryophyllene-type sesquiterpene alcohols. Their identification was based on the MS data. Fig. 2 presents EIMS spectra of two TMS
Chemical profile and antimicrobial activity of extractable compounds of *Betula litwinowii* buds

Figure 1: GC-MS chromatograms of volatile (A) and extractable (B & C) components of *B. litwinowii* buds. (A): 1, (E)-β-caryophyllene; 2, α-humulene; 3, des-4-methylcaryophyll-8(14)-en-5-one; 4, caryophyllene oxide; 5, humulene epoxide II; 6, 14-hydroxy-β-caryophyllene; 7, 14-hydroxy-4,5-dihydrocaryophyllene. (B, ether extract): 1, (E)-β-caryophyllene; 2, α-humulene; 3, des-4-methylcaryophyll-8(14)-en-5-one; 4, humulene epoxide II; 5, 14-hydroxy-β-caryophyllene; 6, 14-hydroxy-4,5-dihydrocaryophyllene; 7, pectolinaringenin; 8, 14-hydroxy-β-caryophyllene p-coumarate; 9, 14-hydroxy-4,5-dihydrocaryophyllene p-coumarate; 10, betulinol; 11, oleanolic acid; 12, betulinic acid. (C, methanol extract): 1, α-xylopyranose; 2, α-fructofuranose; 3, β-fructofuranose; 4, α-glucopyranose; 5, β-glucopyranose; 6, myo-inositol; 7, catechine.
Table 1: Relative composition (% of TIC) of the volatile compounds (according to HS-SPME analysis) and essential oil from *Betula litwinowii* buds.

| Compound | CAS | Analytical parameters | % |
|----------|-----|-----------------------|---|
| **Ethanol** | 67-17-5 | $I_1^{exp}$ 448 | 31,45,46,43 | 46 |
| **Acetone** | 67-64-1 | 500 498 | 43,58,42,39,44,59 | 58 |
| **CS_2** | 75-15-0 | 518 518 | 76,44,32,78,38 | 78 |
| **n-Hexane** | 110-54-3 | 600 600 | 57,43,41,29,56 | 86 |
| **Chloroform** | 67-66-3 | 615 616 | 83,85,87,47,49 | 118 |
| **Tetrachloromethane** | 56-23-5 | 660 661 | 117,119,121,82,47 | - |
| **2,5-Dimethylfuran** | 625-86-5 | 711 712 | 96,95,43,53,81 | 96 |
| **α-Pinene** | 80-56-8 | 936 936 | 93,91,92,77,79 | 136 |
| **Camphene** | 79-92-5 | 949 953 | 93,121,79,107,67 | 136 |
| **4-Octanone** | 589-63-9 | 974 970 | 43,58,71,85,58 | 128 |
| **β-Pinene** | 127-91-3 | 976 978 | 93,41,121,69,91 | 136 |
| **6-Methyl-5-hepten-2-one** | 110-93-0 | 989 986 | 43,108,41,69,55 | 126 |
| **Myrcene** | 123-35-3 | 992 991 | 93,41,69,121,79 | 136 |
| **n-Decane** | 124-18-5 | 1000 1000 | 43,57,71,41,85 | - |
| **3-Carene** | 13466-78-9 | 1008 1010 | 93,91,79,92,77 | - |
| **α-Terpineene** | 99-86-5 | 1015 1016 | 121,93,136,91,77 | 136 |
| **p-Cymene** | 99-87-6 | 1023 1022 | 119,134,91,120,117 | 134 |
| **Limonene** | 138-86-3 | 1026 1031 | 68,93,39,67,53 | 136 |
| **(E)-β-Ocimene** | 3779-61-1 | 1047 1050 | 93,41,79,39,91 | 136 |
| **n-Butyl benzene** | 104-51-8 | 1053 1054 | 91,92,134,105,65 | 134 |
| **γ-Terpinene** | 99-85-4 | 1056 1059 | 93,91,136,121,77 | 136 |
| **cis-Linalool oxide (furanoid)** | 5989-33-3 | 1070 1074 | 59,94,43,68,55 | - |
| **Terpinolene** | 586-82-9 | 1080 1087 | 93,121,136,91,79 | 136 |
| **Linalool** | 78-70-6 | 1098 1098 | 71,41,93,43,55 | - |
| **1,3,8-p-Menthatriene** | 18368-95-1 | 1117 1113 | 91,119,134,77,105 | 134 |
| **α-Cubebane** | 17699-14-8 | 1351 1353 | 105,161,119,41,81 | 204 |
| **Aciphyllene** | 87745-31-1 | 1353 1355 | 95,189,204,147,93 | 204 |
| **Eugenol** | 97-53-0 | 1357 1358 | 164,131,149,103,77 | 164 |
| **α-Copaene** | 3856-25-5 | 1376 1376 | 119,161,105,93,91 | 204 |
| **β-Elemene** | 515-13-9 | 1392 1391 | 93,67,81,107,147 | 204 |
| **α-Cedrene** | 469-61-4 | 1410 1409 | 119,105,93,41,161 | 204 |
| **(E)-β-Caryophyllene** | 87-44-5 | 1418 1418 | 41,91,79,93,105 | 204 |
| **α-Humulene** | 6753-98-6 | 1455 1453 | 93,80,91,121,147 | 204 |
| **γ-Selinene** | 515-17-3 | 1475 | 189,204,133,105,93 | 204 |
| **β-Selinene** | 17066-67-0 | 1486 1484 | 41,79,93,105,67 | 204 |
| **α-Selinene** | 473-13-2 | 1495 1495 | 41,91,93,189,79 | 204 |
| **des-methyl-Caryophylla-8(14)-en-5-one** | - | 1538 1538 | 79,82,41,55,124 | 206 |
| **Caryophyllene oxide** | 1139-30-6 | 1582 1582 | 41,43,79,93,95 | 220 |
| **α-Humulene epoxide** | 19888-33-6 | 1599 1599 | 43,67,93,109,81 | 220 |
| **Humulene epoxide II** | 19888-34-7 | 1609 1606 | 43,67,93,109,138 | 220 |
| **Caryophylladien-5α-ol?** | - | 1629 | 91,136,41,119,79 | 220 |
| **Caryophylladien-5α-ol?** | 19431-79-9 | 1633 1640 | 136,79,91,41,69 | 220 |
| **6-Hydroxy-caryophyllene** | - | 1637 1635 | 41,69,136,109,79 | 220 |
| **4,5-Dihydro-β-caryophyllen-14-al** | - | 1640 1640 | 82,79,41,67,43 | 220 |
| **β-Betulenal** | - | 1652 1650 | 91,69,41,105,133 | 218 |
| **14-Hydroxy-β-caryophyllene** | - | 1667 1664 | 91,41,79,69,133 | 220 |
derivatives of the phenylpropenoid esters found in the buds, 14-hydroxy-β-caryophyllene ferulate and 14-hydroxy-β-caryophyllene caffeate. The main diagnostic ions at \( m/z \) 249 and 307 were derived from the phenylpropenoic moieties (as a result of ester bond cleavage) in the spectra of feruloyl and caffeoyl esters, respectively. The loss of acyloxy parts from \( M^+ \) with synchronous H-rearrangement gives rise to additional characteristic ions at \( m/z \) 266 and 324 (more comprehensive GC and MS characterization of phenylpropenoids found in plant material, as well as their synthetic analogues is presented by [17]).

Terpene phenylpropenoids deserve attention, since cinnamic acid esters show antioxidative properties and other biological activities [21]. Experiments on murine macrophage RAW264.7 cells demonstrated that caffeates of two terpene alcohols (geraniol and farnesol) displayed an inhibitory effect on nitric oxide production, which induces inflammation. These phenylpropenoids are also potential cytotoxic agents, thus they may exhibit antitumor activity [22]. Indeed, in vitro experiments showed that synthetic esters of ferulic and caffeic acids have the ability to inhibit the development of colon, gastric and breast cancer cells.

It can be assumed that pharmacological action of phenylpropenoids from \( B. \) litwinowii buds may be enhanced due to synergistic interactions of flavonoids. There is much evidence that flavonoids prevent various age/inflammation-related chronic diseases [23]. The fraction of flavonoids extracted by diethyl ether from buds of the investigated birch species is equal to 13.4% of TIC and about 10% of TIC is formed by flavones, mostly by kaempferol derivatives.

The other distinguishing feature of the ether extract is a high content (25% of TIC) of tetra- and pentacyclic triterpenoids of lanosterine and lupane series. Not all triterpenoids can be identified at present by GC–MS owing to the lack of available analytical parameters, electron impact mass spectra and chromatographic retention indices. In our investigation, five commercially available substances were positively identified, tetracyclic β-sitosterol and dipterocarpol, as well as pentacyclic betulinol \( \text{[lup-20(29)-α,28-diol]} \), betulinic \( [3\text{-hydroxylup-20(29)-en-28-oic]} \) acid and oleanolic \( \text{[olean-12-en-28-oic]} \) acid. Pentacyclic ketone, 28-norolean-17-en-3-one, was identified tentatively from the mass spectral data that were not confirmed by the \( I^T \) value.

Natural triterpenoids, especially lupane series, have recently attracted the attention of experts in the field of pharmacology due to their anti-inflammatory, antitumor and chemopreventive properties [24,25]. Betulinic acid was screened in vitro against a panel of human cancer cell lines and showed a strong inhibition against several human melanoma lines; at the same time it displayed

| Compound | CAS | Analytical parameters \( I^T \text{exp} \), \( I^T \text{obs} \) | Target ions \( m/z \) | \( M^+ \) | VOC | Essential oil |
|----------|-----|---------------------------------------------------|------------------|---------|-----|----------------|
| 14-Hydroxy-4,5-dihydro-β-β-caryophyllene | - | 1684 | 1685 | 95,82,79,93,67 | 222 | 0.8 | 36.8 |
| 14-Hydroxy-4,5-dihydro-β-isocaryophyllene | - | 1704 | 1704 | 95,82,79,93,67 | 222 | 0.1 | - |
| 14-Hydroxy-β-isocaryophyllene | - | 1721 | 1722 | 73,155,91,131,105 | - | trace | 0.6 |
| 14-Hydroxy-α-humulene | 75678-90-9 | 1745 | 1746 | 119,79,121,187 | 220 | 0.2 | 3.3 |
| 14-Hydroxy-β-caryophyllene acetate | - | 1787 | 1787 | 91,43,41,69,79 | 262 | 0.2 | 0.2 |
| \( n \)-Tricosane | - | - | 2300 | - | - | - |
| \( n \)-Pentacosane | - | - | 2500 | - | - | 0.4 |
| 14-Acetoxy-α-humulene acetate | - | - | - | - | - | 0.1 |
| 14-Acetoxy-4,5-dihydro-β-caryophyllene | - | - | - | - | - | 0.4 |
| 14-Hydroxy-4,5-epoxy-β-caryophyllene | - | - | - | - | - | 0.9 |

**Group composition**

- Monoterpene and monoterpenoids: 2.2%
- Sesquiterpenes: 77.2%
- Sesquiterpenoids: 13.3%
- Other: 7.3%

\( ^a \) According to [9].

\( ^b \) trace – < 0.01 of TIC.

\( ^c \) identified tentatively.
low cytotoxicity on healthy cells [24]. Besides, betulinic and oleanolic acids were found to exhibit strong anti-HIV activity.

The methanol extract from *B. litwinowii* buds contained predominantly carbohydrates and 79% in the total ion current of the chromatogram was formed by the peaks of α- and β-anomers of fructose and glucose (Table 2). Biologically active components of this extract were presented by free amino acids and small amounts of vitamins, myo-inositol and α-tocopherol.

It would be interesting to compare the chemical composition of the buds exudates from *B. litwinowii* and from other representatives of *Betula* genera. It seems that the exudate composition of the species under investigation is similar to the composition of the extract from *Betula pubescens* (downy birch) buds which is also very rich in sesquiterpenols, sesquiterpene phenylpropenoids and flavonoids [26]. However, *B. pubescens* exudates are deprived of the isomers of 14-hydroxy-4,5-dihydrocaryophyllenes and their esters with p-coumaric and ferulic acids (the main components of *B. litwinowii* buds exudate). From the other side, only *B. pubescens* buds contain small amounts (0.27–0.97% of TIC) of nor-sesquiterpenoids, birkenal and birkenol.

![Figure 2: Mass spectra of the TMS derivative of 14-hydroxy-4,5-dihydro-β-caryophyllene ferulate (a) and 14-hydroxy-β-caryophyllene caffeate (b).](image)
| Compound | Analytical parameters | Extract |
|----------|----------------------|---------|
| Lactic acid, TMS | 1070, 1074 | 147,73,117,190,191 | - | - | 0.2 |
| Alanine, TMS | 1111, 1114 | 116,73,147,190,218 | - | - | 0.1 |
| Valine, TMS | 1227, 1225 | 144,73,218,145,147 | - | - | 0.06 |
| Leucine, TMS | 1285, 1284 | 158,73,147,132,218 | - | - | 0.1 |
| H₂PO₄⁻, TMS | 1288, 1289 | 299,300,73,314,133 | 314 | 0.05 | 0.07 |
| Glycerol, TMS | 1292, 1294 | 147,73,205,218,103 | - | 0.03 | 1.5 |
| Proline, TMS | 1304, 1303 | 142,73,216,147,143 | 259 | - | 0.8 |
| Isoleucine, TMS | 1306, 1309 | 158,73,218,147,159 | - | - | 0.04 |
| Succinic acid, TMS | 1324, 1324 | 147,73,247,172,129 | 262 | trace* | 0.2 |
| (E)-β-Caryophyllene | 1418, 1418 | 93,133,91,41,69 | 204 | 0.4 | - |
| α-Humulene | 1453 | 93,80,121,41,147 | 204 | 0.9 | - |
| Isoeugenol, TMS | 1477, 1478 | 206,236,205,221,179 | 236 | 0.05 | - |
| β-Selinene | 1485, 1485 | 204,105,93,107,97 | 204 | 0.02 | - |
| α-Selinene | 1495, 1494 | 189,204,93,81,107 | 204 | 0.03 | - |
| α-Bulnesene | 1504, 1505 | 93,67,79,119,81 | 204 | 0.03 | - |
| Malic acid, TMS | 1511, 1510 | 73,147,233,265,335 | - | - | 0.1 |
| 5-Oxoproline, TMS | 1530, 1527 | 156,73,147,157,75 | - | - | 0.1 |
| Erythritol, TMS | 1539, 1535 | 73,217,147,205,103 | - | - | 0.1 |
| Des-methyl-Caryophylla-8(14)-en-5-one | 1539, 1538 | 79,82,41,55,124 | 206 | 0.5 | - |
| β-Aminobutyric acid, TMS | 1542, 1546 | 174,304,147,175,176 | - | - | 0.05 |
| 4-Hydroxyphenyl ethanol, di-TMS | 1579, 1579 | 179,73,282,267,193 | 282 | 0.05 | - |
| Caryophyllene oxide | 1582, 1581 | 79,43,61,93,95 | 220 | 0.3 | - |
| Humulene epoxide II | 1607, 1606 | 43,67,61,138,109 | 220 | 0.4 | - |
| Sesquiterpenoid C₁₅H₂₅O | 1625 | - | 82,79,67,95 | 220 | 0.1 | - |
| 4-Hydroxybenzoic acid, di-TMS | 1636, 1636 | 267,223,193,73,282 | 282 | 0.1 | - |
| 4,5-Dihydro-β-caryophyllen-14-al | 1640, 1638 | 82,79,67,41,95 | 220 | 0.4 | - |
| Glutamic acid, TMS | 1641, 1646 | 246,73,147,230,218 | - | - | 0.06 |
| β-Arabinopyranose, TMS | 1649, 1646 | 204,217,191,73,147 | - | - | 0.5 |
| β-Betulinal | 1650, 1650 | 91,69,41,79,105 | 218 | 0.09 | - |
| 6-Hydroxy-β-caryophyllene, TMS | 1682, 1682 | 73,156,169,131,189 | 292 | 0.02 | - |
| 14-Hydroxy-β-isocaryophyllene, TMS | 1702, 1707 | 73,144,133,95,91 | 292 | 0.6 | - |
| 4-(p-Hydroxyphenyl)-butanol-2, TMS | 1723, 1721 | 295,310,73,137 | 310 | trace | - |
| 14-Hydroxy-β-caryophyllene, TMS | 1729, 1725 | 73,155,131,91,187 | 292 | 8.0 | - |
| 14-Hydroxy-4,5-dihydroisocaryophyllene, TMS | 1734, 1734 | 73,75,82,93,357 | 294 | 1.0 | - |
| α-Xylopyranose, TMS | 1739, 1740 | 204,217,73,191,147 | - | - | 1.6 |
| Arabinitol, TMS | 1760, 1759 | 217,317,73,103,307 | - | - | 0.5 |
| 14-Hydroxy-4,5-dihydrocaryophyllene, TMS | 1760, 1762 | 73,75,82,119,91 | 294 | 4.0 | - |
| Vanillic acid, TMS | 1777, 1776 | 297,312,267,233,282 | 312 | trace | 0.04 |
| β-Xylopyranose, TMS | 1795, 1790 | 204,217,73,191,147 | - | - | 1.9 |
| Sesquiterpenoid C₁₅H₂₅O₃, mono-TMS? | 1815 | - | 119,73,147,91,291 | 306 | 0.1 | - |
| α-Fructofuranose, TMS | 1846, 1847 | 217,73,437,147,218 | - | - | 3.3 |
| β-Fructofuranose, TMS | 1854 | 217,73,437,204,147 | - | - | 23.1 |
| α-Galactopyranose, TMS | 1900, 1899 | 204,191,73,217,147 | - | - | 3.1 |
| α-Glucopyranose, TMS | 1932, 1931 | 204,191,73,217,147 | - | - | 25.0 |
| Sesquiterpenoid C₁₅H₂₅O₃, di-TMS | 1935 | - | 189,73,147,279,133 | 382 | 0.8 | - |
| Sesquiterpenoid C₁₅H₂₅O₃, di-TMS | 1937 | - | 189,73,147,279,133 | 382 | 0.8 | - |
| Sesquiterpenoid C₁₅H₂₅O₃, di-TMS | 1945 | - | 189,73,147,279,133 | 382 | 1.1 | - |
Table 2: Averaged chemical composition (% of TIC, n = 3) of extractive substances from B. litwinowii buds.

| Compound                                      | Analytical parameters | Extract          |
|-----------------------------------------------|-----------------------|------------------|
|                                               | $I_{Exp}^*$            | $I_{DB}^*$       | Target ions (m/z) | M⁺ ether | methanol |
| β-Galactopyranose, TMS                        | 1945                  | 1945             | 204,191,73,147,217 | -        | 4.6      |
| p-Coumaric acid, TMS                          | 1948                  | 1947             | 219,293,73,308,249 | 308.04   | -        |
| Mannitol, TMS                                 | 1972                  | 1972             | 319,73,205,147,217 | -        | 0.6      |
| Glucitol, TMS                                 | 1980                  | 1981             | 319,73,205,147,217 | -        | 0.6      |
| β-Glucopyranose, TMS                          | 2030                  | 2031             | 204,191,73,147,217 | -        | 27.7     |
| n-Heneicosane, TMS                            | 2100                  | 2100             | 57,71,85,43,99    | 296.08   | -        |
| Ferulic acid, TMS                             | 2103                  | 2104             | 338,323,73,308,249 | 338.08   | -        |
| myo-Inositol, TMS                             | 2128                  | 2128             | 305,217,318,73,147 | -        | 1.1      |
| Caffeic acid, TMS                             | 2155                  | 2155             | 396,219,73,381,307 | 396.01   | -        |
| Linoleic acid, TMS                            | 2216                  | 2216             | 73,75,337,81,129  | 352.01   | -        |
| α-Linolenic acid, TMS                         | 2220                  | 2218             | 73,75,79,95,129   | 350.02   | -        |
| Octadecanoic acid, TMS                        | 2250                  | 2250             | 341,117,73,75,129 | -        | Trace    |
| n-Tricosane                                   | 2300                  | 2300             | 57,71,85,43,99    | 342.03   | -        |
| 1-Eicosanol, TMS                              | 2360                  | 2360             | 355,356,73,103,43 | - 0.3    | -        |
| Eicosanoic acid, TMS                          | 2448                  | 2447             | 369,117,73,132,129 | 384.02   | -        |
| Uridine, TMS                                  | 2471                  | 2469             | 217,73,147,169,299 | 460.0    | 0.1      |
| n-Pentacosane                                 | 2500                  | 2500             | 57,71,85,43,99    | 352.04   | -        |
| 1-Docosanol, TMS                              | 2554                  | 2557             | 383,384,75,57,103 | 398.08   | -        |
| 5-Hydroxy-4',7-dimethoxyflavone               | 2681                  | 2680             | 300,134,121,193,166 | 300.01   | -        |
| n-Heptacosane                                 | 2700                  | 2700             | 57,71,85,43,99    | 380.09   | -        |
| Sucrose, TMS                                  | 2711                  | 2713             | 361,73,217,147,437 | -        | 0.05     |
| α-Maltose, TMS                                | 2739                  | 2744             | 204,191,217,73,361 | -        | 0.2      |
| α-Cellobiose, TMS                             | 2758                  | 1758             | 204,191,217,217,361 | -        | 0.3      |
| 5-Hydroxy-4',7-dimethoxyflavanone, TMS        | 2775                  | 2773             | 357,238,178,371,73 | 372.04   | -        |
| Flavonoid?                                    | 2777                  |                  | 179,358,192,357,177 | -        | 0.2      |
| Isosakuranetin, TMS                           | 2818                  | 2818             | 415,296,416,73,429 | 430.05   | -        |
| Tetracosanoic acid, TMS                       | 2845                  | 2845             | 425,117,73,132,135 | 440.05   | -        |
| Sakuranetin chalcone, TMS                     | 2868                  | 2865             | 487,488,73,238,311 | 502.07   | -        |
| Sakuranetin, TMS                              | 2878                  | 2877             | 415,238,200,179,192 | 430.07   | -        |
| Naringenin, TMS                               | 2895                  | 2895             | 473,73,296,474,179 | 488.01   | -        |
| n-Nonacosane                                  | 2900                  | 2900             | 57,71,85,43,99    | 408.01   | -        |
| epi-Catechin, TMS                             | 2907                  | 2910             | 368,73,355,267,297 | -        | Trace    |
| 5-Hydroxy-4',7-dimethoxyflavone               | 2927                  | 2927             | 298,269,297,255,135 | 298.01   | -        |
| Catechin, TMS                                 | 2935                  | 2935             | 368,355,73,179,267 | 650.07   | 0.9      |
| Gentibiose, TMS                               | 2984                  | 2989             | 204,73,217,361,103 | -        | 0.2      |
| 3,5-Dihydroxy-4',7-dimethoxyflavone, TMS      | 3035                  | 3035             | 443,444,73,400,429 | 458.14   | -        |
| Hexadecanoic acid, TMS                        | 3046                  | 3044             | 453,117,132,145,73 | 468.07   | -        |
| Homoeriodictyol, TMS                          | 3051                  | 3050             | 501,502,503,485,429 | 516.16   | -        |
| Pectolinaringenin, TMS                         | 3060                  | 3063             | 443,444,445,427,413 | 458.29   | -        |
| 5,7-Dihydroxy-4'-methoxyflavone (acacetin), TMS| 3064                  | 3067             | 413,414,170,370,73 | 428.01   | -        |
| 3,5,7-Trihydroxy-4'-methoxyflavone (kaempferide), TMS | 3100                  | 3100             | 501,502,503,373,429 | 516.16   | 0.3      |
| 3,4',5,7-Tetrahydroxy flavone (kaempferol), TMS| 3112                  | 3114             | 559,560,561,73,272 | -        | Trace    |
| 5,7-Dihydroxy-3',4',5-trimethoxyflavone?      | 3136                  |                  | 473,474,443,73,487 | 488.13   | 0.1      |
| α-Tocopherol, TMS                             | 3150                  | 3147             | 502,237,236,73,277 | 502.01   | 0.1      |
| 5,7,4'-Trihydroxy-4'-methoxyflavone, TMS      | 3140                  | 3139             | 501,502,73,503,515 | 516.05   | -        |
| 5,7,4'-Trihydroxy-3'-methoxyflavone, TMS      | 3148                  | 3143             | 501,502,73,503,516 | 516.12   | -        |
| 14-Hydroxy-β-caryophyllene p-coumarate, TMS   | 3170                  | 3168             | 219,236,73,220,202 | 438.121  | -        |
3.4 Antimicrobial activity

In the present study, the antimicrobial activities of the buds extracts were tested against three groups of microorganisms: Gram-positive bacteria *Staphylococcus aureus* and *Bacillus cereus*, Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*, as well as pathogenic fungi *Candida albicans*. As can be seen, bud extracts inhibited the tested Gram-positive bacteria and *C. albicans*, however their inhibitory activity against two Gram-negative bacteria was rather occasional (Table 3). The extracts did not inhibit *E. coli*, but did inhibit *P. aeruginosa*. Therefore, birch buds extracts may be used in therapy against Gram-positive pathogens and *P. aeruginosa*. According to our (unpublished) data, the exudate from *B. pubescens* buds inhibited the latter species with nearly same MIC value (0.04 mg mL⁻¹). Most likely, it is explained by the above mentioned similarity in the chemical composition of bud exudates from these birch species.

The inhibitory spectra of *B. litwinowii* buds extracts were compared with the antimicrobial activity of some types of European propolis, known as “natural antibiotics”. Surprisingly, in many cases the buds extract demonstrates the lower MIC values than was registered by the authors [27,28] in their experiments with 70% ethanol extracts of propolis. It may be explained by the fact that ethanol-water extracts of propolis contain rather high (up to 30%) amount of “ballast” substances like sugars, alcohols and sugar acids [29] which do not possess antimicrobial activity.

Although definitive studies of the biological activity of *B. litwinowii* buds extracts do not appear in the literature, the biological action of many of its constituents is well...
documented. For example, Pepeljnjak [30] correlated the flavonoid content with activity of propolis against *Bacillus subtilis*. Hydroxycinnamic acids (*p*-coumaric, ferulic and caffeic) and their esters are also well known for their antibacterial and antifungal activities [31]. Hence, the above mentioned action of buds extracts against Gram-positive pathogens can be linked with the high content of phenolics: hydroxycinnamic acid derivatives and flavonoids. Flavonoids have been demonstrated to exhibit the broad spectrum of biological activities including antimicrobial action and anti-inflammatory properties resulting from their radical-scavenging action [21].

4 Conclusions

1. It seems to be promising to apply the described approach of sample preparation (successive extraction) for screening of extractable compounds of buds of other birch species with a view to search for novel pharmacologically promising natural substances.

2. The obtained experimental material demonstrates that *B. litwinowii* buds contained a wide range of chemical compounds with well-documented or tentatively assigned biological activities. In spite of the relatively small occurrence of some of these components (for instance, betulinol or betulinic acid), their medicinal effects can be pronounced due to synergistic interaction with other chemicals.

3. The above mentioned exudate covering birch buds (it has a viscous consistency in summer) is a plant precursor of another remedy, propolis. It is known that composition of propolis depends mainly on the local flora. For this reason, it will be interesting to investigate the composition and biological activity of propolis collected by honeybees in the regions of natural spreading of *B. litwinowii* growing mainly in East-Northern Turkey, North Iran, Caucasus and South Ural [1].

Acknowledgements: The authors would like to thank Kinga Nowak, MSc from the Dendrology Institute of the Polish Academy of Sciences for providing bud samples and identification of the plant material. The project was partly (I.Świecicka) supported by the funds in the frame of “Specific scientific equipment–2012”, of the Ministry of Science and Higher Education of Poland.

References

[1] Browicz K., In: Davis P.H. (Ed.), Flora of Turkey and the East Aegean Islands, University Press, Edinburg, 1972, Vol. 7, 688
[2] Folkard C. (Ed.), Encyclopedia of Herbs and Their Uses, Dorling Kindersley Pub. Inc., New York, 1995
[3] Penoel D., Aromatherapy for Health Professionals, Churchill Livingstone, Edinburgh, 1995
[4] Klika K.D., Demirci B., Salminen J.-P., Ovcharenko V.V., Vuorela S., Can Başer K.H., Pihlaja K., Eur. J. Org. Chem., 2004, 2627
[5] European Pharmacopeia, 7th Edition, Council of Europe, Strasbourg, 2010
[6] Isidorov V.A., Krajewska U., Bal K., Jaroszynska J., Niesluchowska A., Vetchinnikova L., Fuksman I., Chem. Anal., 2000, 45, 513
[7] Demirci B., Can Başer K.H., Demirci F., Hamann M.T., J. Nat. Prod., 2000, 63, 902
[8] Isidorov V.A., Krajewska U., Vinogorova V.T., Vetchinnikova L.V., Fuksman I.L., Bal K., Biochem. System. Ecol., 2004, 32, 1
[9] Can Başer K.H., Demirci B., Arkivos, 2007, 7, 335
[10] Orav A., Arak E., Boikova T., Raal A., Biochem. System. Ecol., 2011, 39, 744
[11] Keinänen M., Julkunen-Tiitto R., J. Chromatogr. A, 1998, 793, 370
[12] Vedernikov D.N., Galashkina N.G., Roshchin V.I., Rastit. Res., 2007, 43, 84 (in Russian)
[13] Isidorov V.A., Vinogorova V.T., J. Natuforsch., 2003, 58c, 355
[14] Isidorov V.A., Szczepaniak L., J. Chromatogr. A, 2009, 1216, 8998
[15] Isidorov V.A., Szczepaniak L., Bakier S., Food Chem., 2014, 142, 101
[16] Szczepaniak L., Isidorov V.A., J. Chromatogr. A, 2011, 1218, 7061
[17] Szczepaniak L., Walejko P., Isidorov V.A., Anal. Sci., 2013, 29, 643
[18] NIST Chemistry WebBook, National Institute of Standards and Technology, Gaithersburg, 2013, MD 20899: http://webbook.nist.gov.chemistry
[19] Greenaway W., Whatley F.R., J. Chromatogr., 1990, 519, 145
[20] Greenaway W., Whatley F.R., Phytochemistry, 1991, 30, 1887
[21] Burdock G.A., Food Chem. Toxicol., 1998, 36, 347
[22] Uwai K., Osanai T., Kanno S., Takeshita M., Ishikawa M., Bioorg. Med. Chem., 2008, 16, 7795
[23] Pan M.-H., Lai C.-S., Ho C.-T., Food Funct., 2010, 1, 15
[24] Pisha E., Chai H., Lee I., Chagwedera T.E., Farnsworth N.R., Cordell G.A., Beecher C.W.W., et al., Nat. Med., 1995, 1, 1046
[25] Gallo M.B.C., Sarachine M.J., Int. J. Biomed. Pharm. Sci., 2009, 3(1), 46
[26] Isidorov V.A., Szczepaniak L., Wróblewska A., Pirożnikow E., Biochem. System. Ecol., 2014, 52, 41
[27] Mohammadzadeh S., Shariatpanahi M., Hamedi M., Ahmadkhaniha R., Samadi N., Ostad S.N., Food Chem., 2007, 103, 1097
[28] Popova M., Trusheva B., Khismatullin R., Gavrilova N., Legotkina G., Lapunov J., Bankova V., Nat. Prod. Commun., 2012, 7, 617
[29] Greenaway W., May J., Scaysbrook T., Whatley F.R., Z. Naturforsch., 1991, 46c, 111
[30] Pepeljnjak S., Jalseniak I., Maysinger D., Pharmazie, 1985, 40, 122
[31] Binutu O.A., Adesogan K.E., Okugun J.I., Planta Med., 1996, 62, 352