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GŁ: designed the study and codirected laboratory work; AS: participated in laboratory work; MS: GC-MS analysis; JKZ, VS: conducted chromatographic fractionation; AB, APN: HPLC analysis; MJ, SK: determination of biological activity; GŁ, AS: drafted the manuscript

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ORIGINAL RESEARCH PAPER

Phytochemical screening of *Pulsatilla* species and investigation of their biological activities

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Abstract
The present study aimed to identify biologically active secondary metabolites from the rare plant species, *Pulsatilla patens* subsp. *patens* and the cultivated *P. vulgaris* subsp. *vulgaris*. Chromatographic fractionation of the ethanolic extract of the roots of *P. patens* subsp. *patens* resulted in the isolation of two oleanane-type glycosides identified as hederagenin 3-*O*-β-D-glucopyranoside (2.7 mg) and hederagenin 3-*O*-β-D-galactopyranosyl-(1→2)-β-D-glucopyranoside (3.3 mg, patensin). HPLC analysis of the methanolic extract of the crude root of *P. patens* subsp. *patens* and *P. vulgaris* subsp. *vulgaris* revealed the presence of *Pulsatilla* saponin D (hederagenin 3-*O*-α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→4)]-α-L-arabinopyranoside). Chromatographic analysis using GC-MS of the silylated methanolic extracts from the leaves and roots of these species identified the presence of carboxylic acids, such as benzoic, caffeic, malic, and succinic acids. The extracts from *Pulsatilla* species were tested for their antifungal, antimicrobial, and antimalarial activities, and cytotoxicity to mammalian cell lines. Both *P. patens* subsp. *patens* and *P. vulgaris* subsp. *vulgaris* were active against the fungus *Candida glabrata* with the half-maximal inhibitory concentration (IC_{50}) values of 9.37 μg/mL and 11 μg/mL, respectively. The IC_{50} values for cytotoxicity evaluation were in the range of 32–38 μg/mL for *P. patens* subsp. *patens* and 35–57 μg/mL for *P. vulgaris* subsp. *vulgaris* for each cell line, indicating general cytotoxic activity throughout the panel of evaluated cancer and noncancer cells.

Keywords
pasque flower subspecies; oleanane-type triterpenoid glycosides; carboxylic acids; antifungal and antimicrobial activities; cytotoxicity; medicinal applications

Introduction

Plants are known to produce different biologically active substances, including secondary metabolites. It had been reported that plants from the *Pulsatilla* genus contain ranunculin, anemonin, protoanemonin, triterpenes, and saponins (9%) of mainly the oleanane and lupane-type [1,2]. These metabolites were also subjected to analyses in order to determine the contents of polyphenol compounds, flavonoids, and anthocyanidins

[3,4]. The triterpene saponins were isolated from *P. chinensis* (Bunge) Regel [5], *P. koreana* Nakai [6], *P. cernua* (Thunb.) Bercht. et Opiz. [7], *P. dahurica* (Fisch. ex DC.) Spreng. [8], *P. turczaninovii* Kryl. et Serg. [9], *P. nigricans* Storck [10], *P. pratensis* (L.) Mill. [11], and *P. patens* subsp. *multifida* (G. A. Pritzel) Zämelis [12]. Polyphenolic compounds such as flavonoids and anthocyanidins are produced by *P. montana* subsp. *balcana* (Velen.) Zämelis & Paegle, *P. halleri* subsp. *rhodopaea* (Stoj. et Stef.) K. Krause, and *P. slaviankae* (Zimmer.) Jordanov & Kožuharov [13]. The saponins produced by *Pulsatilla* species show high biological activity across a wide range as an anticancer [14], neuroactive [15], neuroprotective [16], immunomodulating [17], cognitive function enhancing [18], antioxidant [19], antimicrobial [20], and cytotoxic agents [21].

Pulsatilla species are rarely of toxicological importance, as while they are moderately hazardous, they are mutagenic and contain cellular poison [22]. The active ingredient in fresh plant material, a terpenoid glucoside known as ranunculin, is broken down to protoanemonin [23]. Protoanemonin is a reactive compound with an exocyclic methylene group and is mutagenic, because it can bind to the SH-group of proteins and DNA. Protoanemonin is an irritant lactone that causes allergic dermatitis in human skin [24], and internally, paralysis of the central nervous system and gastrointestinal disturbance [25]. When the plant is dried, the protoanemonin dimerises to form the less toxic anemonin, if it has not already alkylated proteins [23].

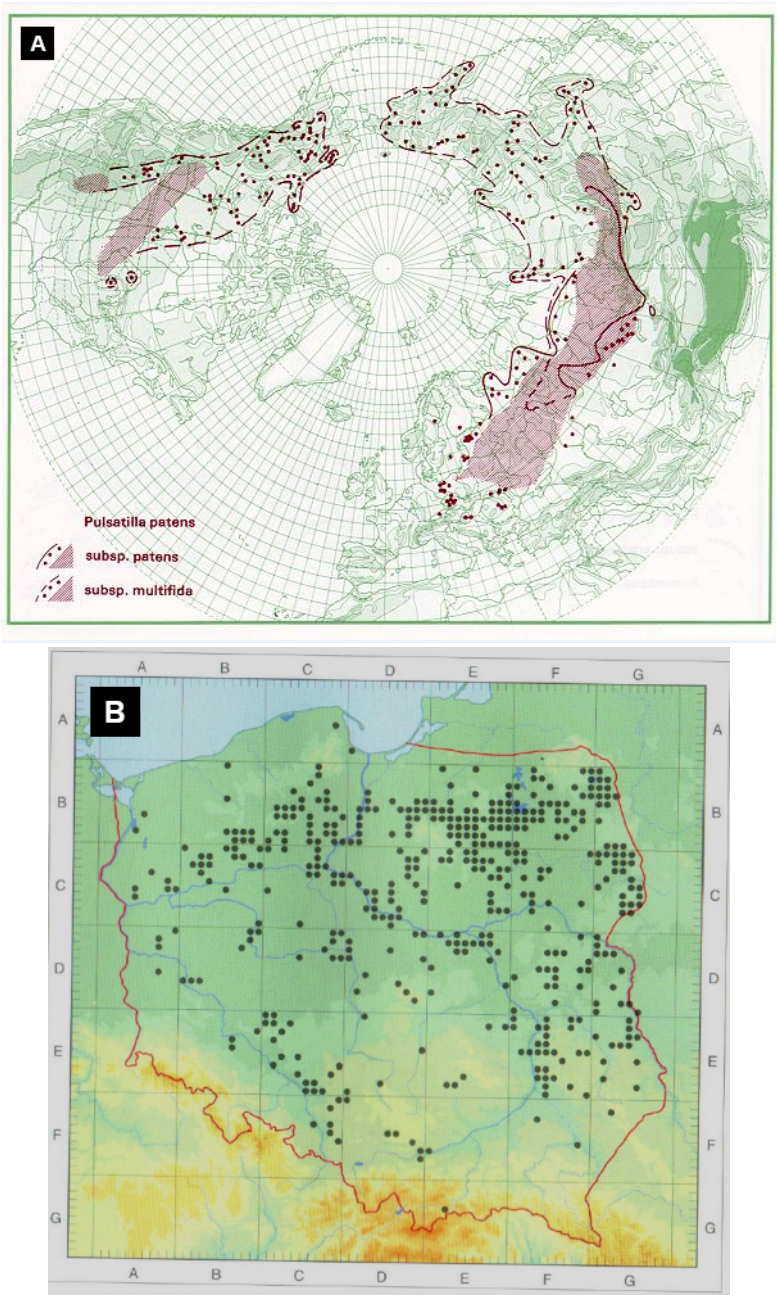


Fig. 1 Distribution of *Pulsatilla patens* subsp. *patens* (**A**) in Europe, Asia, and North America [31], and (**B**) in Poland [40].

Despite the potential toxicity of *Pulsatilla* species, their natural products have been used for centuries in traditional Chinese and Korean medicine for the treatment of many diseases and ailments. They are known as herbal drugs used for the homeopathic treatment of eye ailments, earache, stress, anxiety, tension, skin eruptions, rheumatism, headaches, neuralgia, insomnia, hyperactivity, bacterial skin infections, septicemia, bronchitis, coughs, and asthma [26].

The *Pulsatilla* genus is composed of 38 species in the Northern Hemisphere [23]. The species of *Pulsatilla patens* subsp. *patens* and *P. vulgaris* subsp. *vulgaris* have not been extensively studied. The existing research focuses primarily on genetic characteristics of the species [27], ecology [28], and various aspects of developmental biology [29]. *Pulsatilla patens* subsp. *patens* is a lowland species of Boreo-meridional-continental distribution [30] and is native to Europe, Russia, Mongolia, China, Canada, and the United States [31] (Fig. 1A).

Pulsatilla patens subsp. *patens* is an endangered plant and is considered critically threatened in the Czech Republic [32], included in the red data books of Germany as an endangered plant [33], vulnerable in Sweden [34], relatively restored in Lithuania, and in decreasing number in the St. Petersburg and Kaliningrad [35] regions of Russia, Latvia [36], and Slovakia [37]. In Finland and Estonia, the populations of *P. patens* subsp. *patens* are considered to be relics [38]. In Poland, *P. patens* subsp. *patens* has been strictly protected since 1958 and requires

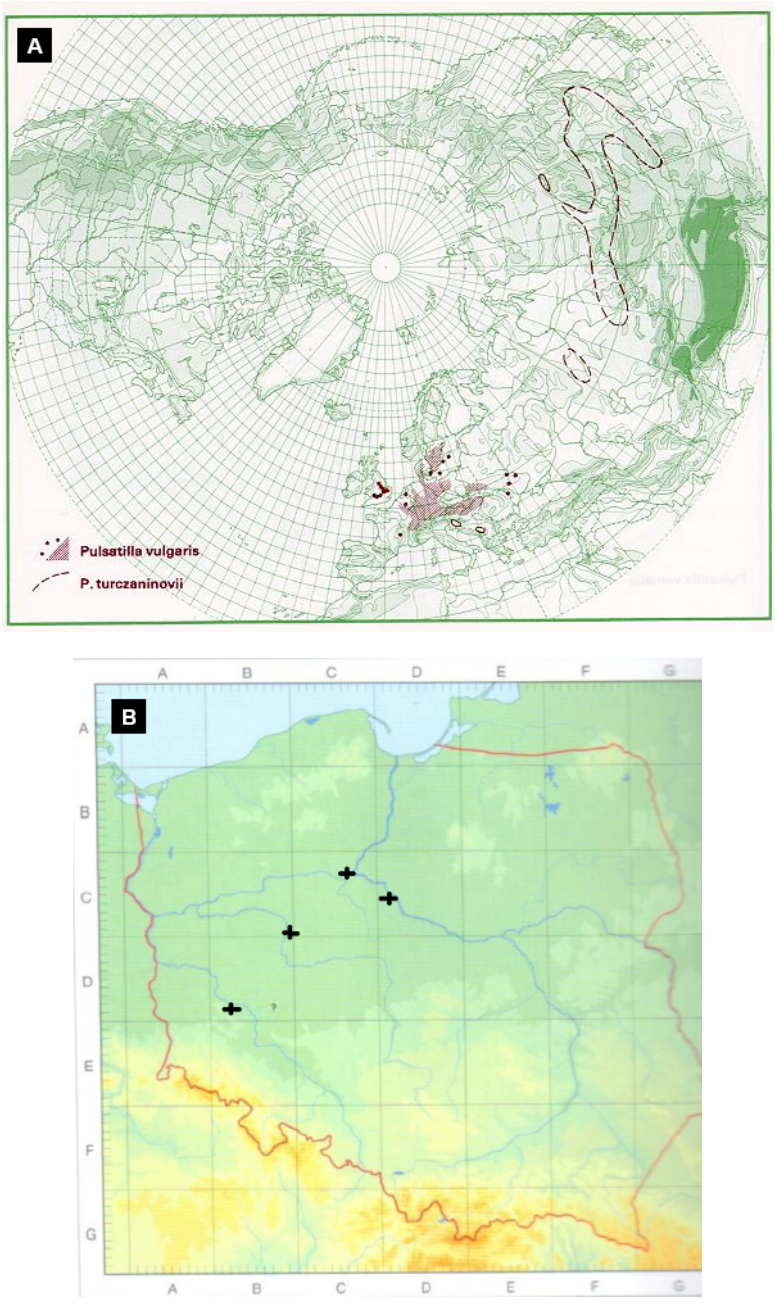


Fig. 2 Distribution of *Pulsatilla vulgaris* subsp. *vulgaris* (**A**) in Central Europe [31] and (**B**) in Poland [40].

active protection [39]. On the territory of Poland, *P. patens* subsp. *patens* reaches the western border of its range and the number of its sites clearly decreases towards the west and south [40] (Fig. 1B).

Pulsatilla vulgaris subsp. *vulgaris* is a species occurring across Central Europe, ranging from France in the south to Sweden at its northern limit [31] (Fig. 2A) and is listed as "near threatened" by the International Union for Conservation of Nature [41]. Currently, *P. vulgaris* subsp. *vulgaris* is listed as vulnerable (VU category) in Ukraine, Slovakia [42], Sweden [43], and the United Kingdom [44]. This species is also classified as endangered (EN category) in Germany [45] and Switzerland [46] and as critically endangered (CR category) in Austria [47]. In Poland, it is listed as an extinct (RE category) species [48] and mainly cultivated. *Pulsatilla vulgaris* subsp. *vulgaris* is currently extinct in Poland on natural sites [49]. It was only reported at four sites before 1930 [40] (Fig. 2B) on the habitats of the pine forests of the All. *Dicrano-Pinion*.

The aim of this study was the identification of biologically active secondary metabolites from the rare plant species *Pulsatilla patens* subsp. *patens* and the cultivated *P. vulgaris* subsp. *vulgaris*. In the course of our study, we evaluated the extracts of leaves and roots of these plant species for their antifungal, antimicrobial, and antimalarial activities, and cytotoxicity to mammalian cell lines.

Material and methods

Material collection

The leaves and roots of *P. patens* subsp. *patens* were collected in May 2013–2015 at the Knyszynska Forest near Bialystok City, NE Poland, while *P. vulgaris* subsp. *vulgaris* were obtained from cultivation at the Herbarium "The Herbal Corner" in May 2015, Podlaskie Province, NE Poland. Plant material was identified by Prof. Grażyna Łaska from the Bialystok University of Technology, Faculty of Civil and Environmental Engineering, Poland.

Extraction and chromatographical analysis

TLC/CC analysis. For thin layer chromatography (TLC) and column chromatography (CC), ethanolic extracts were prepared from roots of *P. patens* subsp. *patens* (K_2). Dried roots of *P. patens* subsp. *patens* (10 g) were extracted with 1 L of 50% aqueous EtOH at room temperature for 12 h and then filtered. The extraction was repeated three times. The combined filtrates were concentrated in a vacuum evaporator to obtain a syrupy brown residue (0.95 g). Then 5 mg of ethanolic extract from roots of *P. patens* subsp. *patens* (K_2) was transferred to normal and reverse phase (RP) TLC on silica gel with

fluorescence (stationary phase). The following solvent system was used for normal phase TLC: hexanes–ethyl acetate (C_6–H_{14}–$C_4H_8O_2$; 60:40), chloroform–methanol ($CHCl_3$–MeOH; 90:10) (Fig. 3) and for reverse phase TLC: water–methanol (H_2O–MeOH; 30:70), water–methanol (H_2O–MeOH; 70:30). The solvent system H_2O–MeOH (30:70) was found to be best for RP-TLC. A solution of H_2SO_4–MeOH (5:95) and UV 254 and 366 nm were used as a detecting reagent (Fig. 3). In the next step, an ethanolic extract of the roots of *P. patens* subsp. *patens* was fractionated by CC (0.9 g) using $CHCl_3$–MeOH gradient (90:10). Compounds were eluted by reverse-phase C_{18} column using the H_2O–MeOH (30:70) solvent system (Fig. 4). The structures of the isolated compounds were determined by ^1H and ^{13}C-NMR spectroscopy.

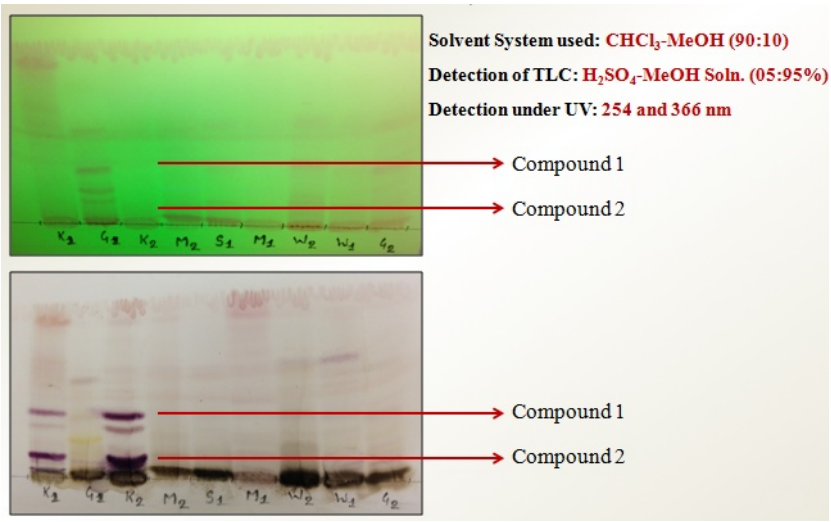


Fig. 3 TLC image of the ethanolic extract of roots (K_2) of *Pulsatilla patens* subsp. *patens* and identification of Compound 1 and 2.

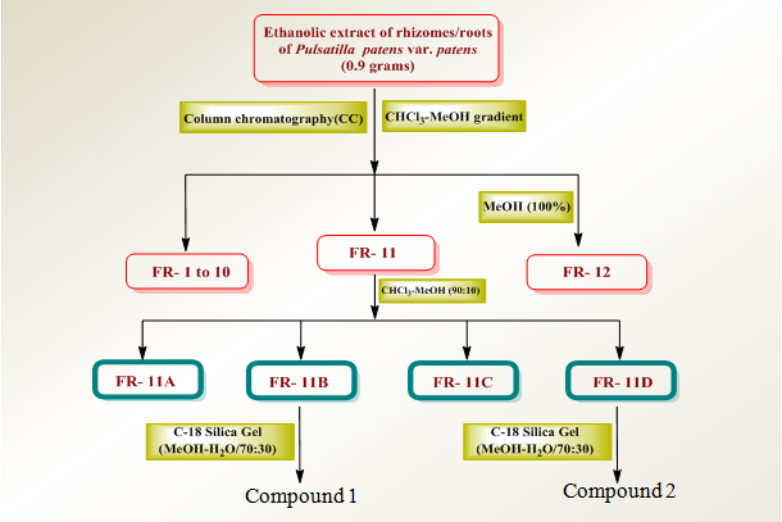


Fig. 4 Column chromatography of the ethanolic extract of *Pulsatilla patens* subsp. *patens* roots and isolation of Compound 1 and 2.

HPLC analysis. The fraction which exhibited the most potent activity was again chromatographed by high performance liquid chromatography (HPLC) using Agilent Technologies 1260 Infinity system. The HPLC profiles were determined at 35°C on a COSMOSIL Packed Column 5C-18-MS-II (4.6 µm × 250 mm) (Nacalai, USA) eluted with methanol (A) and water (B) as mobile phases in a linear gradient elution with the flow rate of 1 mL/min. The elution program was as follows: 0–8 min, 90% A to 77%

A, 8–25 min, changed to 71% A, 25–40 min, to 60% A, 40–50 min, to 50% A, 50–75 min, to 10% A, 75–80 min, to 0% A [50]. The volume of sample injected was 35 µL. Fluorescence was monitored by an Agilent 1260 Infinity Fluorescence Light Detector (Agilent Technologies, USA; 210 nm excitation and 460 nm emission wavelength). The analytical data were integrated using the Agilent OpenLAB software. The fraction was purified by HPLC to give *Pulsatilla* saponin D. The peak of *Pulsatilla* saponin D was identified based on retention time with respect to the reference standard (purchased from Toronto Research Chemicals) previously run in an analytical HPLC.

GC-MS analysis. The pressurized solvent extractions were performed using a Büchi E-916 Speed Extractor. The raw materials were extracted with methanol (leaves 32.3 g, and roots, 23.6 g of *P. patens* subsp. *patens*; leaves, 21.8 g and roots, 56.1 g of *P. vulgaris* subsp. *vulgaris*). The extraction time was 30 min, temperature 100°C and pressure 1.2 × 10^7 Pa. Three extracts were prepared from each raw material. Extracts were filtered through paper filters. The methanol was dried using a rotor evaporator.

Each dry residue (10 mg) was redissolved with 1 mL of pyridine and 200 µL BSTFA [*N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide] added. Next, the mixture was heated at 60°C for 30 min. The solution was analyzed by gas chromatography / mass spectrometry (GC-MS) using an Agilent 7890A gas chromatograph equipped with an Agilent 5975C mass selective detector. The injection of a 1-µL sample was performed using an Agilent 7693A autosampler. The separation was performed on an HP-5MS (30 m × 0.25 mm × 0.25 µm film thickness) fused silica column at a helium flow rate of 1 mL/min. The injector worked in split mode (1:5), and the injector temperature was 280°C. The initial column temperature was 50°C, rising to 310°C at 3°C/min and the final temperature was held for 10 min. The ion source and quadrupole temperatures were 230°C and 150°C, respectively. The ionization energy (EIMS) was 70 eV. The detection was performed in full scan mode from 41 to 600 amu.

After integration, the percentage of each component in the total ion current (TIC) was calculated. Mass spectral data and calculated retention indices were used to identify the compounds. The mass spectrometric identification was carried out with an automated system of GC-MS data processing supplied by the National Institute of Standards and Technology (NIST). Retention indices of the analytes were determined, taking into account C_{10}–C_{40}*n*-alkanes retention times and comparing them with the NIST database.

Antimicrobial assay

For the antimicrobial assay, we used the fungi *Candida albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 204305 and the bacteria *Staphylococcus aureus* ATCC 29213, methicillin-resistant *S. aureus* ATCC 33591 (MRS), *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068 from the American Type Culture Collection (Manassas, VA). The testing was performed using a modified version of the CLSI method (formerly NCCLS) [51–54] except for *M. intracellulare*, where a modified method by Franzblau et al. [55] was used. All samples were diluted in 20% DMSO/saline and then transferred in duplicate to microplates. In order to obtain final target inocula, microbial inocula were prepared by correcting the optical density at 630 nm (OD_{630}) of the microbe suspensions in the incubation broth. In each assay, drug controls were used, with ciprofloxacin (ICN Biomedicals, USA) for the bacteria and amphotericin B (ICN Biomedicals, Ohio) for the fungi. The readings were made for all organisms before and after incubation at either 530 nm using the Biotek Powerwave XS plate reader (Bio-Tek Instruments, USA) or 544 excitation / 590 emission, (*M. intracellulare*, *A. fumigatus*) using the Polarstar Galaxy Plate Reader (BMG LabTechnologies, Germany). To determine the minimum fungicidal or bactericidal concentrations required, 5 µL was removed from each clear well, transferred to agar, and incubated. The MFC/MBC (the lowest test concentration that kills the organism) was also established.

Antimalarial assay

In the antimalarial assay, the chloroquine sensitive (D6) strain of *Plasmodium falciparum* was used and plasmodial LDH activity was measured using the procedure devised by Makler and Hinrichs [56]. To the wells of a 96-well plate containing 10 µL of diluted sample, a suspension of red blood cells infected with *P. falciparum* of 200 µL was introduced, together with 2% parasitemia and 2% hematocrit in RPMI 1640 medium supplemented with 10% human serum and 60 µg/mL Amikacin. Then, the plate was incubated at 37°C for 72 h with 90% N_2, 5% O_2, and 5% CO_2 using a modular incubation chamber. In order to determine the parasitic LDH activity, 20 µL of the incubation mixture was mixed with 100 µL of the Malstat reagent (Flow Inc., USA) and incubated at room temperature for 30 min. Then, 20 µL of a 1:1 mixture of NBT/PES (Sigma, USA) were added and the plate was incubated in the dark for 1 h. To stop the reaction, 100 µL of a 5% acetic acid solution was added and the absorbance was read at 650 nm. The drug controls were artemisinin and chloroquine. The half-maximal inhibitory concentration (IC_{50}) values were calculated on the basis of the dose response curves of growth inhibition using XLfit 4.2 (IDBS, USA).

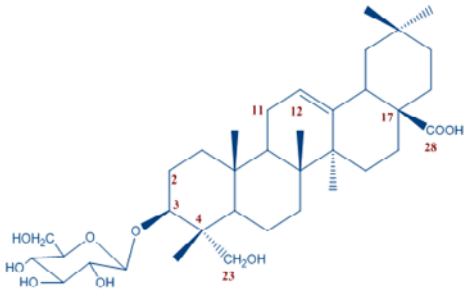
Cytotoxicity assay

In the cytotoxicity assay, the root extract was tested against a panel of cancer and non-cancer cell lines. In this assay, we used 96-well tissue culture-treated plates. The cells were seeded at a density of 25,000 cells/well and grown for 24 h. Next, samples of the extract at different concentrations were added and cells were incubated for 48 h. The neutral red method [57] was used to determine the cell viability. The IC_{50} values were obtained on the basis of dose response curves. Doxorubicin was used as the control drug.

Results

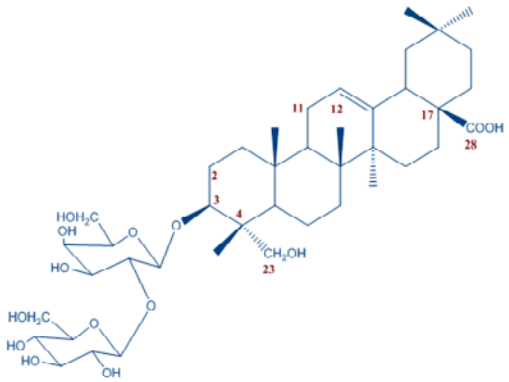
Chromatographic fractionation of the ethanolic extract (0.9 g) of the roots of *P. patens* subsp. *patens* resulted in the isolation of two oleanane-type glycosides identified as hederagenin 3-*O*-β-D-glucopyranoside (2.7 mg) and hederagenin 3-*O*-β-D-galactopyranosyl-(1→2)-β-D-glucopyranoside (3.3 mg, patensin) (Fig. 5).

Compound 1



Hederagenin 3-*O*-β-D-glucopyranoside

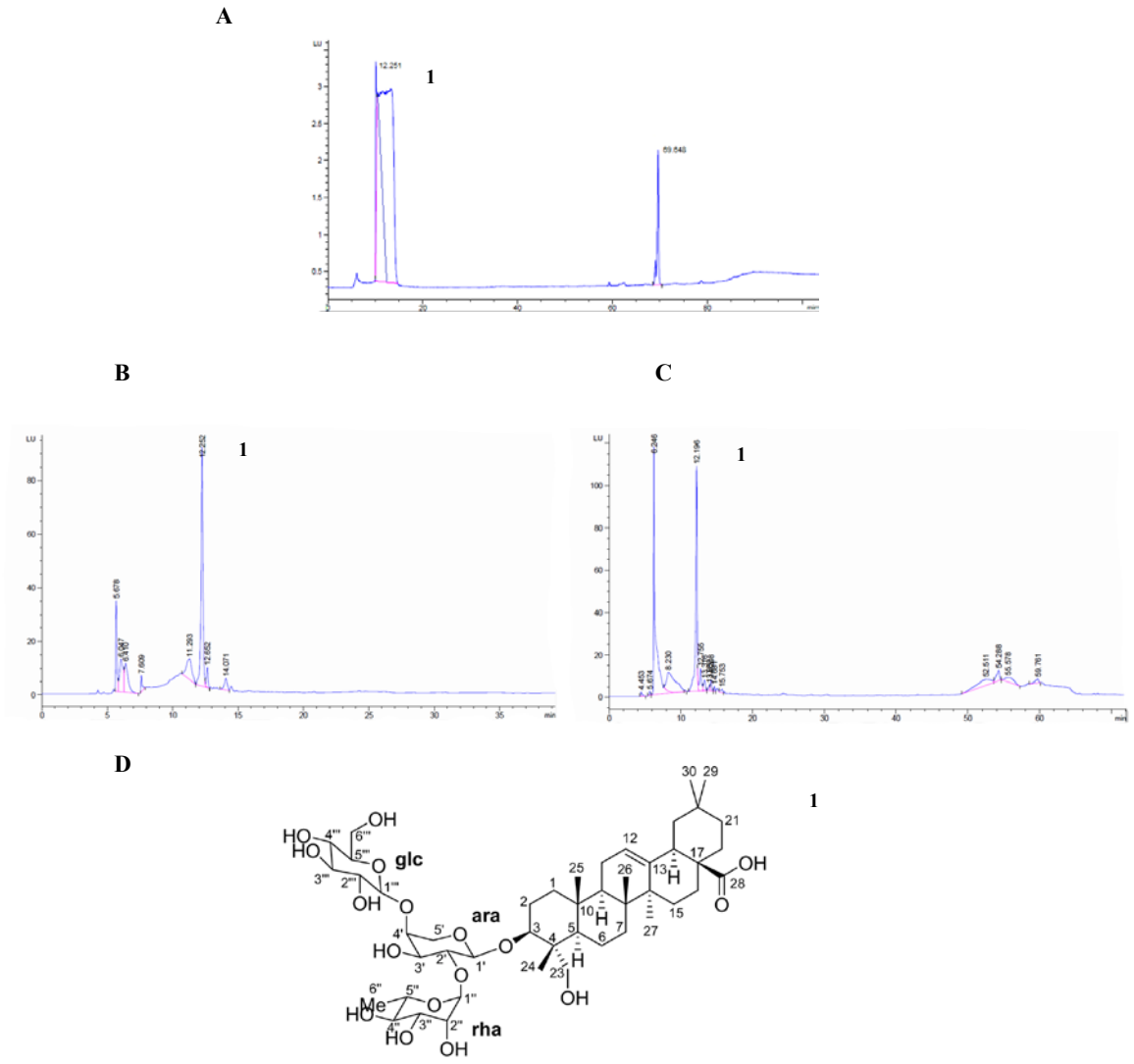
Compound 2



Hederagenin 3-*O*-β-D-galactopyranosyl-(1→2)-β-D-glucopyranoside

Fig. 5 Structures of two oleanane-type glycosides isolated from *Pulsatilla patens* subsp. *patens*.

HPLC analysis of the silylated methanolic extract of the crude root of *P. patens* subsp. *patens* and *P. vulgaris* subsp. *vulgaris* revealed the presence of *Pulsatilla* saponin D – hederagenin 3-*O*-α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→4)]-α-L-arabinopyranoside (Fig. 6). Analysis of the chromatographic spectra of the extracts from *P. patens* subsp. *patens* and *P. vulgaris* subsp. *vulgaris* showed similar spectra to the standard solution chromatogram of *Pulsatilla* saponin D. The other chromatographic peaks for the extracts of *P. patens* subsp. *patens* and *P. vulgaris* subsp. *vulgaris* also showed the presence of triterpenoid saponins, which will need to be investigated further. The *Pulsatilla* saponin D has not been isolated in *Pulsatilla* medicinal plants in Poland until now, with this type of study being conducted here for the first time. *Pulsatilla* saponin D, $C_{47}H_{76}O_{17}$, has multiple demonstrated biological properties including antifungal, antimicrobial, and cytotoxic activities.



Hederagenin 3-*O*-α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→4)]-α-L-arabinopyranoside

***Pulsatilla* saponin D.** Synonyms: 3β-[(*O*-β-D-glucopyranosyl-(1→4)-*O*-[α-L-rhamnopyranosyl-(1→2)]-α-L-arabinopyranosyl)oxy]-23-hydroxyolean-12-en-28-oic acid; hederacolchiside A; hederagenin 3-*O*-[β-D-glucopyranosyl(1→4)-[α-L-rhamnopyranosyl(1→2)]]-α-larabinopyranoside; hederagenin 3-*O*-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-larabinopyranoside; SB 365.

Fig. 6 HPLC chromatogram of standards *Pulsatilla* saponin D – 1 (**A**); HPLC spectrum of the triterpenoid saponins (*Pulsatilla saponin* D – 1) in *Pulsatilla patens* subsp. *patens* (**B**); HPLC spectrum of the triterpenoid saponins (*Pulsatilla saponin* D – 1) in *Pulsatilla vulgaris* subsp. *vulgaris* (**C**) and the structure of *Pulsatilla* saponin D (1) isolated from *Pulsatilla patens* subsp. *patens* and *Pulsatilla vulgaris* subsp. *vulgaris* (**D**).

Carbohydrates were the main components of the extracts from *P. patens* subsp. *patens* and *P. vulgaris* subsp. *vulgaris*. The content of carbohydrates in each extract from *P. patens* subsp. *patens* was over 60% while those from *P. vulgaris* subsp. *vulgaris* were over 90% carbohydrates. The analyzed extracts that were the most abundant were saccharose, glucopyranose and fructofuranose. The extracts from the leaves of these species revealed the presence of carboxylic acids, such as benzoic, caffeic, malic, and succinic acids. Furthermore, glyceric, quinic, and protocatechuic acids were identified in the leaf extracts of *P. patens* subsp. *patens*, while the roots extracts from *P. vulgaris* subsp. *vulgaris* contained the smallest amount of carboxylic acids (Tab. 1).

Microbiological assays of the root extracts showed activity against the fungal pathogen *Candida glabrata*, indicated by the IC_{50} value of 9.37 μg/mL for *P. patens* subsp. *patens* and 11 μg/mL for *P. vulgaris* subsp. *vulgaris* (Tab. 2, Tab. 3). This result is particularly important as with the increasing use of immunosuppressants, the number of cases of inflammation of mucous membranes and particular systems by *C. glabrata* have significantly increased, especially in people with human immunodeficiency virus. *Candida glabrata* has become the second most important agent responsible for surface (oral cavity, esophagus, vaginal, or urinary duct) or systemic inflammation. The main problem with the inflammation caused by *C. glabrata* is their innate resistance to azole antibiotics that are very effective against other *Candida* species. Treatment of *C. glabrata*-caused inflammation often require the use of amphotericin B that is toxic for humans and can trigger undesired side effects [58]. It has been experimentally shown that the root extracts form *Pulsatilla* species can contribute to new, safer drugs in antifungal therapy against *C. glabrata*.

Tab. 1 Relative chemical composition (% of TIC) of *Pulsatilla patens* subsp. *patens* and *P. vulgaris* subsp. *vulgaris* extracts.

Group of compounds	$t_{ret.}$ (min)	Fresh leaves of *P. patens*	Fresh roots of *P. patens*	$t_{ret.}$ (min)	Fresh leaves of *P. vulgaris*	Fresh roots of *P. vulgaris*
Carbohydrates such as:		69.77 ±3.49	88.17 ±4.41		94.14 ±4.71	96.46 ±4.82
α-Fructofuranose	32,494	2.12 ±0.11	3.37 ±0.17	42,998	4.28 ±0.21	1.02 ±0.05
β-Fructofuranose	42,678	2.53 ±0.13	7.68 ±0.38	43,286	4.39 ±0.22	4.19 ±0.21
α-D-Glucopyranose	45,290	13.44 ±0.67	10.19 ±0.51	45,928	16.80 ±0.84	16.89 ±0.84
β-D-Glucopyranose	48,449	17.44 ±0.87	9.42 ±0.47	49,055	19.53 ±0.98	16.81 ±0.84
Saccharose	66,784	14.98 ±0.75	25.74 ±1.29	67,461	23.52 ±1.18	53.65 ±2.28
Carboxylic acids such as:		21.83 ±1.09	3.33 ±0.17		1.13 ±0.06	0.07 ±0.01
Laevulic acid	-	-	-	15,035	0.05 ±0.01	-
Benzoic acid	19,169	0.03 ±0.01	0.06 ±0.01	19,765	0.04 ±0.01	-
4-Hydroxyvaleric acid	-	-	-	20,763	0.16 ±0.01	-
Succinic acid	22,526	0.34 ±0.02	0.17 ±0.01	23,108	0.07 ±0.01	-
Malic acid	30,260	1.04 ±0.05	1.62 ±0.08	30,861	0.73 ±0.04	0.07 ±0.01
2-Hydroxyglutaric acid	-	-	-	32,448	0.04 ±0.01	-
(E)-Ferulic acid	50,432	0.01 ±0.01	0.02 ±0.01	-	-	-
(E)-Caffeic acid	51,967	1.49 ±0.08	-	52,588	0.06 ±0.01	-
Glyceric acid	23,728	8.46 ±0.42	0.05 ±0.01	-	-	-
3-Phenyllactic acid	33,465	-	0.28 ±0.01	-	-	-
Protocatechuic acid	41,965	2.12 ±0.11	-	-	-	-
Shikimic acid	42,394	-	0.22 ±0.01	-	-	-
Quinic acid	44,256	7.58 ±0.38	0.58 ±0.03	-	-	-
Amino acids		2.24 ±0.11	0.58 ±0.03		-	-
Other compounds		5.90 ±0.30	3.86 ±0.19		0.57 ±0.03	1.39 ±0.07
Unidentified compounds		0.17 ±0.01	4.06 ±0.20		4.16 ±0.21	2.08 ±0.10

Tab. 2 The test results of the antimicrobial activity of leaf and root extracts of *Pulsatilla patens* subsp. *patens* and *Pulsatilla vulgaris* subsp. *vulgaris* (primary screen).

Tested strain	Extracts from leaves of *P. p.* (50 µg/mL)	Extracts from roots of *P. p.* (50 µg/mL)	Extracts from leaves of *P. v.* (50 µg/mL)	Extracts from roots of *P. v.* (50 µg/mL)	Amphotericin B (5 µg/mL)	Ciprofloxacin (1 µg/mL)
C. albicans	2*	27	11	24	100	ND
C. glabrata	0	100	14	98	100	ND
C. krusei	0	17	15	16	100	ND
A. fumigatus	7	3	21	11	99	ND
C. neoformans	0	22	0	40	82	ND
S. aureus	0	8	7	16	ND	89
MRSA	0	10	9	9	ND	94
E. coli	5	11	20	24	ND	96
P. aeruginosa	4	4	7	11	ND	100
M. intracellulare	0	0	0	0	ND	72

* The results in %. ND – not determined; *P. p.* – *Pulsatilla patens*; *P. v.* – *Pulsatilla vulgaris*.

Tab. 3 Dose response (IC_{50} in µg/mL) results of the roots extracts of *Pulsatilla patens* subsp. *patens* and *P. vulgaris* subsp. *vulgaris*.

Test strain	Extracts from roots of *P. patens*	Extracts from roots of *P. vulgaris*	Amphotericin B	Ciprofloxacin
C. albicans	NA	NA	0.19	ND
C. glabrata	9.37*	11	0.37	ND
C. krusei	NA	NA	0.67	ND
A. fumigatus	NA	NA	1.17	ND
C. neoformans	42.27	NA	0.18	ND
S. aureus	NA	NA	ND	0.09
MRS	NA	NA	ND	0.08
E. coli	NA	NA	ND	0.01
P. aeruginosa	NA	NA	ND	0.07
M. intracellulare	NA	NA	ND	0.37

* The results in IC_{50}. ND – not determined; NA – not active at 200 µg/mL

The extracts from the leaves and roots of this plant species showed lower ability to inhibit the growth of the four different fungi (*Candida albicans*, *C. krusei*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*) and other bacteria (*Staphylococcus aureus*, MRSA, *Escherichia coli*, and *Pseudomonas aeruginosa*) pathogenic to humans (Tab. 2, Tab. 3). As far as *C. albicans*, *A. fumigatus*, *E. coli*, and *P. aeruginosa* are concerned, the leaf and root extracts have been found to show biological activity. Although their activity is rather low, the extracts are still highly valuable as *C. albicans* is a frequent fungus pathogen in humans. This species is responsible for the painful inflammation of mucous membranes, e.g., vagina inflammation in women or oral-pharyngeal thrush in AIDS patients. In certain groups of sensitive patients, this species is responsible for serious and life threatening bloodstream inflammation and subsequent inflammation of internal organs [59]. The extracts from *Pulsatilla* species are also attractive in fighting *A. fumigatus*, which is one of the most common saprophytic fungi in the air. With an increasing number of patients treated with immunosuppressants, the incidence of serious and usually lethal invasive aspergillosis has also considerably increased worldwide [60]. Extracts from *Pulsatilla* species are also promising agents for the treatment of *E. coli* caused inflammation. This bacteria strain is the dominant facultative anaerobic

Tab. 4 The test results of the antimalarial activity of leaf and root extracts of *Pulsatilla patens* subsp. *patens* and *P. vulgaris* subsp. *vulgaris*.

Tested strain	*Plasmodium falciparum* (D6 % inhibition)
Chloroquine	97
Chloroquine	94
Chloroquine	98
Extracts from leaves of *P. patens*	0
Extracts from roots of *P. patens*	27
Extracts from leaves of *P. vulgaris*	8
Extracts from roots of *P. vulgaris*	1

component of the flora of the human large intestine. It can cause inflammation of the urinary tract, sepsis, meningitis, intestinal diseases, and diarrhea, and is difficult to treat as it is resistant to antimicrobial agents [61]. The *Pulsatilla* extracts should also be considered in fighting *P. aeruginosa*, the opportunistic pathogen responsible for hospital infections in patients with immunodeficiency. This pathogen causes inflammation of the lungs related to the use of a respirator, inflammation of the urinary tract, wounds in patients with serious burns, as well as sepsis [62]. Unfortunately, the extracts considered are inactive against *M. intracellulare*.

In our antimalarial assays, the extracts from the leaves and roots showed low activity against the protozoan (27% of inhibition for *P. patens* subsp. *patens* and 1–8% for *P. vulgaris* subsp. *vulgaris*), while the antimalarial drug chloroquine (positive control) showed 94–98% inhibition (Tab. 4).

The root extracts showed cytotoxicity to all the cell lines included in the assay. As shown in Tab. 5, the IC_{50} for cytotoxicity of *P. patens* subsp. *patens* was in the range of 32–38 μg/mL and for *P. vulgaris* subsp. *vulgaris*, 35–57 μg/mL for each cell line, indicating general cytotoxic activity throughout the panel of cancer and noncancer cells.

Tab. 5 Cytotoxicity of *Pulsatilla patens* subsp. *patens* and *P. vulgaris* subsp. *vulgaris* roots extracts towards a panel of mammalian cell lines.

	IC_{50} μg/mL					
Sample name	SK-MEL	KB	BT-549	SK-OV-3	LLC-PK1	Vero
Root extract of *P. patens*	34.0	38.0	32.0	32.0	36.0	32.0
Root extract of *P. vulgaris*	44.0	42.0	57.0	35.0	42.0	39.0
Doxorubicin*	1.7	1.7	2.2	2.3	1.6	>5.0

Cell lines: SK-MEL – skin melanoma; KB – epidermal carcinoma; BT-549 – breast cancer; SK-OV-3 – ovarian cancer; LLC-PK1 – kidney epithelial; Vero – kidney fibroblast. * Positive control drug.

Discussion

The extract of the roots of *P. patens* subsp. *patens* revealed the presence of two oleanane-type glycosides identified as hederagenin 3-*O*-β-D-glucopyranoside and hederagenin 3-*O*-β-D-galactopyranosyl-(1→2)-β-D-glucopyranoside (patensin). Structures of both compounds were established via comparing ^{1}H and ^{13}C-NMR spectral data on compounds reported earlier by other authors [63,64]. Patensin, $C_{42}H_{68}O_{14}$, a triterpenoid glycoside, was also isolated from the ethanolic extract of roots of *P. patens* subsp. *multifida* [63] and five oleanane-type glycosides and two triterpene saponins were also isolated from the roots of *P. patens* subsp. *multifida* [64]. Phytochemical studies of new secondary metabolites from *P. patens* subsp. *multifida* have revealed the presence of a hitherto unknown saponin [12]. This saponin isolated from the methanolic extract of this plant has been found to inhibit the growth of skin cancer. Roots from *P. patens* subsp. *multifida* have been used historically in traditional Chinese medicine [26] as the agent shows anticancer, antimalarial, and antibacterial properties. At present, it is used as a homeopathic drug for the treatment of the ear conditions, exanthema, rheumatism, bronchitis, coughing, asthma, as well as an agent for alleviating stress and anxiety.

The triterpene acid and triterpene glycosides (saponins *Pulsatilla* A and B) were isolated from the plant material of *P. chinensis* [65]. The triterpene acid shows high cytotoxic activity against the malignant cells of lung cancer. The triterpene saponins are used for the treatment of inflammatory conditions [66] and reveal high antiprotozoal

[67], antibacterial [68], antiparasitic [69], antifungal [70], and molluscicidic [71] activities. These saponins have also been used for the treatment of indigestion, premenstrual tension syndrome, and psychosomatic disturbances [72].

Six new saponins, five lupanes, and one oleanane [hederagenin 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside], along with 11 known saponins, were also isolated from the roots of *P. koreana* [2]. The extracts from *P. koreana* were also determined to contain ranunculin, anemonin, protoanemonins, triterpenes, and saponins (9%). As evidenced in studies on the pharmacological use of components isolated from the extract, its protoanemonins show antifungal properties and antibiotic activity. Moreover, the biologically active compounds isolated from the root extract of *P. koreana* have been found to show anticancer [73], anti-inflammatory [74], antiparasitic [75], and antibacterial [76] activities.

HPLC analysis of the root extract of *P. patens* subsp. *patens* and *P. vulgaris* subsp. *vulgaris* revealed the presence of *Pulsatilla* saponin D – hederagenin 3-*O*-α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→4)]-α-L-arabinopyranoside. Results of numerous phytochemical studies have confirmed high content of triterpenoid saponins of lupane or oleanane-type in the extract of the root of *P. koreana* and *P. chinensis* [2,77] and their chromatographic spectra [50] are similar to those obtained for *P. patens* subsp. *patens* and *P. vulgaris* subsp. *vulgaris* (Fig. 7A). All structures of triterpenoid saponins (pulsatilloside, *Pulsatilla* saponin, anemoside, hederacolchiside, and cussosaponin) isolated from *P. koreana* roots were elucidated by comparative analysis of spectral data [78].

Analysis of HPLC fingerprints of different species of *Pulsatilla* (*P. koreana*, *P. chinensis*, *P. ambigua*, *P. dahurica*, *P. turczaninovii*) showed significant differences between their HPLC fingerprints, but with 10 common peaks (Fig. 7B). These common peaks not only can be used to classify and authenticate *Pulsatilla* species, but also provide important

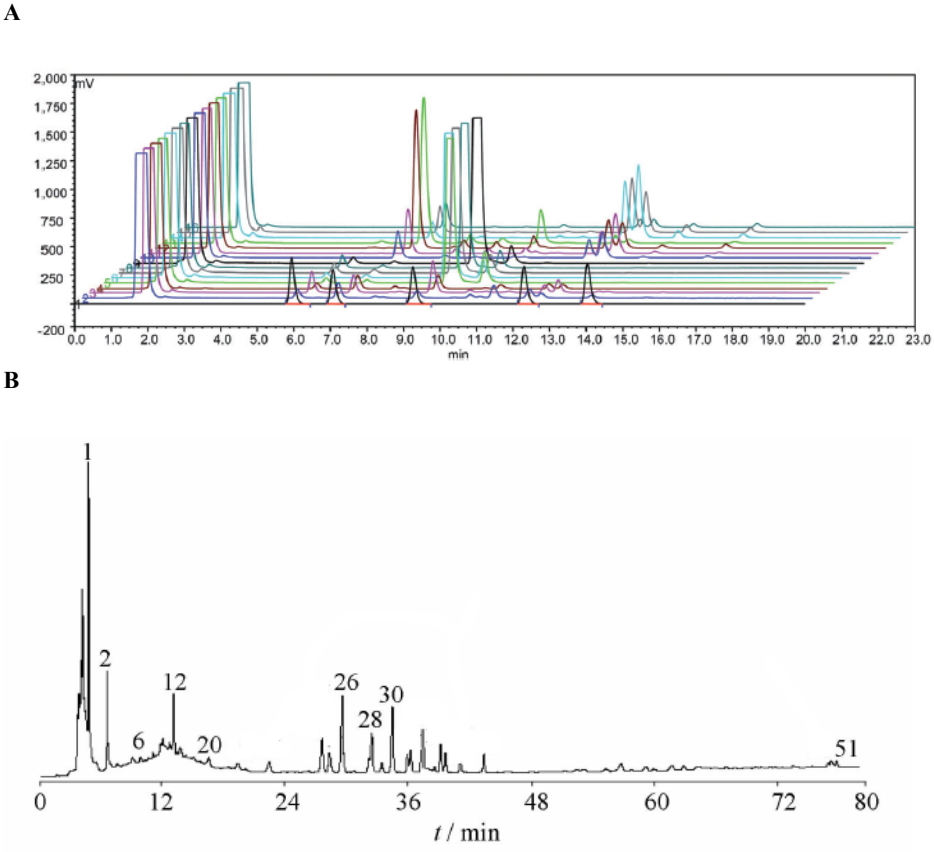


Fig. 7 HPLC-ELSD chromatogram of mixed standards and 15 samples extract of *Pulsatilla koreana* Nakai with spectrum of the triterpenoid saponins (pulsatilloside E, *Pulsatilla* saponin H, anemoside B4, hederacolchiside E, and cussosaponin C-5) (**A**) [78], and 10 common peaks of different *Pulsatilla* species on HPLC fingerprint study (**B**) [50].

references for HPLC fingerprints and quality control of *Pulsatilla* medicinal plants [50]. These studies confirmed chromatographic spectra can show differences depending on the *Pulsatilla* species. Significant differences between *P. koreana* and *P. dahurica*, and between *P. turczaninovii* and *P. ambigua* were observed. Slight differences in the chromatographic spectra were also found for *P. patens* subsp. *patens* and *P. vulgaris* subsp. *vulgaris*; however, the presence of the triterpenoid saponins and the biological activity of their plant extracts were demonstrated for the first time in this work. This is very important because the triterpenoid saponins, such as *Pulsatilla* saponin D ($C_{47}H_{76}O_{17}$), have demonstrated multiple biological properties including antifungal, antimicrobial, and cytotoxic activities. These compounds are also produced from the root of several *Pulsatilla* species, including *P. koreana* [1], *P. chinensis* [79], and *P. cernua* [80]. In particular, *Pulsatilla* saponin D shows high cytotoxic activity against cancer cells. *Pulsatilla* saponin D isolated from the root of *P. koreana* showed potent inhibition rate of tumor growth (IR, 82%) at the dose of 6.4 mg/kg on BDF1 mice bearing Lewis lung carcinoma (LLC) cells [1]. This compound is also proving to be promising for the treatment of Alzheimer's disease [15].

It has been reported that the extracts from other species containing triterpenoid saponins, with hederagenin being the active ingredient, showed marked inhibition of serotonin (5-HT), norepinephrine (NE), and dopamine (DA) transporters [81]. Monoamine transporter inhibition studies on pure isolated compounds have not been reported yet, hence the importance of performing CNS studies on isolated compounds from *Pulsatilla* species. In future, we will focus on pure isolated compounds from *P. patens* subsp. *patens* and *P. vulgaris* subsp. *vulgaris* and submit them to the Psychoactive Drug Screening Program (PDSP), Chapel Hill, NC for monoamine transporter inhibition studies for extracellular monoamines including serotonin (5-HT), norepinephrine (NE), and dopamine (DA) transporters. The two oleanane-type glycosides have been sent to the National Center for the Natural Product Research (NCNPR) at the University of Mississippi to evaluate their antimicrobial activity.

Currently, this preparation, which is called SB31, is undergoing phase II clinical studies in Korea. It was previously reported that SB31 exhibited cytotoxic activity against some human cancer cell lines and potent antitumor activity on a mouse tumor model. An active antitumor constituent from the roots of *P. koreana* was isolated and the main component was found to be deoxypodophyllotoxin (DPT), which exhibited antiangiogenic activity and good antitumor activity against mice bearing LLC cells. However, the DPT content of Pulsatillae Radix, which is an active constituent of SB31, is too low for discernible antitumor activity. This suggests that DPT is not the major constituent responsible for the antitumor activity of *P. koreana* roots.

The extracts from *P. cernua* have been found to contain anthocyanidins, as well as triterpene saponins and phytosterols [82]. The natural dyes isolated from *P. cernua* show antiallergic, antifungal, anticancer, and antiulcer activity. Moreover, they improve blood flow in coronary arteries and reduce blood pressure so are attractive substances for the treatment of atherosclerosis. The anthocyanidins isolated from the extracts have been used in medicine as perspiration-promoting agents in the treatment of colds and in the treatment of inflammation of the stomach and intestines.

The flavonoids and tannins isolated from the methanolic extract from *P. nigricans* show considerable antianxiety effect [83]. *Pulsatilla nigricans* is one of the species used in traditional medicine for the treatment of nervous exhaustion and anxiety. Therefore, their high activity permits their application for the treatment of depression. The confirmation of our results shows the extracts from *P. nigricans* can also be used as antifungal agents and to alleviate the weakness of an organism.

Phytochemical studies of the extracts from *P. montana* subsp. *balcana*, *P. halleri* subsp. *rhodopaea*, and *P. slaviankae* have shown polyphenolic compounds, flavonoids, and anthocyanidins [13]. The highest contents of these substances were established in the extracts from *P. montana* subsp. *balcana* and *P. halleri* subsp. *rhodopaea*. The antioxidative properties of *P. slaviankae* were a consequence of the presence of astragalin, isoquercetin, quercetin, kaempferol, caffeic acid, and isorhamnetin in the aboveground shoots of this species [84]. The natural biologically active substances isolated from this species may play an important role in the protection of humans against breast cancer, colon cancer, prostate cancer, and leukemia. Phytochemical studies of plant material from the species *P. montana* subsp. *balcana* have also revealed the presence of caffeic

acid, chlorogenic acid, gallic acid, methylgallic acid, ferulic acid, and ellagic acid [85]. Our results also identified caffeic acid, and so are in some accordance with the observation of these previous studies. The therapeutic effects related to the presence of these polyphenolic compounds include diastole of blood vessels, and assistance in stabilizing the blood pressure of patients with heart disease and hypertension.

The chemical composition of extracts from the leaves and roots of *P. patens* subsp. *patens* and *P. vulgaris* subsp. *vulgaris* were significantly different. The leaf extracts, in contrast to the root extracts, contained up to 22% carboxylic acid for *P. patens* subsp. *patens* and 2% for those of *P. vulgaris* subsp. *vulgaris*, whereas the leaf extracts showed lower biological activity. Chemical composition of extracts from dry and raw materials from this species were slightly different (Łaska and Sienkiewicz, unpublished data), while the extracts from fresh material were the most rich with compounds. Upon drying, some compounds were lost through evaporation or destruction.

However, phytochemical analysis of *P. patens* subsp. *patens* and *P. vulgaris* subsp. *vulgaris* showed nearly similar results due to the presence of phytochemical constituents. A previous study on the chemotaxonomic characteristics of *Pulsatilla* species revealed that *P. patens* subsp. *patens*, *P. vulgaris* subsp. *vulgaris*, and *P. grandis* Wenderoth contain only delphinidin glycosides, whereas the remaining species [*P. halleri* subsp. rhodopaea (Stoj. & Stef.) K. Krause, *P. montana* subsp. *balcana* (Vel.) Zamels, *P. pratensis* (L.) Mill., *P. rubra* (Lamk.) Delarbre, and *P. vulgaris* subsp. *vulgaris* var. *touranginiana* E. G. Camus] contain pelargonidin and delphinidin glycosides [86]. Our studies revealed the presence of the triterpenoid saponins and the biological activity of the extracts from *P. patens* subsp. *patens* and *P. vulgaris* subsp. *vulgaris*. Therefore, isolated saponins have chemotaxonomic features that may be recognized as specific to *Pulsatilla* species.

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