ISSN 2449-8955 European Journal of Biological Research *Research Article*

DOI: http://dx.doi.org/10.5281/zenodo.3228890

Antifungal activity of the rhizome extracts of *Pulsatilla vulgaris* against *Candida glabrata*

Grażyna Łaska*, Aneta Sienkiewicz

Department of Agri-Food Engineering and Environmental Management, Białystok University of Technology, Wiejska 45A, 15-351 Białystok, Poland

* Corresponding author: Phone: +48 602499654; E-mail: g.laska@pb.edu.pl

Received: 30 March 2019; **Revised submission:** 17 May 2019; **Accepted:** 23 May 2019

ABSTRACT: *Pulsatilla vulgaris* Mill. ("Pasque flower", Ranunculaceae) is rare and a threatened plant species in Europe. It produces biologically active secondary metabolites. *P. vulgaris* is also known herbal drug used for centuries in traditional Chinese and Korean medicine. The rhizomes of *P. vulgaris* have been traditionally used for treatment of headaches, neuralgia, insomnia, hyperactivity, bacterial skin infections, septicemia, cough and bronchitis. In the present study, the extracts of leaves and rhizomes of *P. vulgaris* were evaluated for their antifungal, antimicrobial, antimalarial and cytotoxic activities. The results showed the antifungal activity of crude extracts of the rhizome of *P. vulgaris* against the yeast *Candida glabrata* with an IC_{50} of 11 µg/ml. These results indicate that the selected medicinal plant could be further investigated for identifying compounds that may be responsible for the observed activity and that may represent new leads in fungal drug discovery.

Keywords: *Pulsatilla vulgaris* subsp. *vulgaris*; Ranunculaceae; Leaves and rhizomes extracts; Biological activity; Microbiological assays.

1. INTRODUCTION

Phytochemical studies of *Pulsatilla* species revealed the presence of a high diversity of secondary metabolites [1]. Many bioactive compounds have been reported from the extract of *Pulsatilla* species such as anemonin [2] and protoanemonin [3, 4], hederagenin [5], oleanolic saponins and lupane-type saponins [6, 7] and antimicrobial cinnamic acid derivatives [8] or anti-acne activities of pulsaquinone, hydropulsaquinone and 1,4-quinone derivatives [9]. The triterpene saponins were isolated from *P. chinensis* (Bunge) Regel [10-12], *P. koreana* Nakai [2, 6, 13], *P. cernua* (Thunb.) Bercht. et Opiz. [14, 15], *P. dahurica* (Fisch. ex DC.) Spreng. [16], *P. turczaninovii* Kryl. et Serg. [17], *P. nigricans* Storck [18], *P. pratensis* (L.) Mill. [19] and *P. patens* subsp. *multifida* (G.A. Pritzel) Zämelis [20] (Table 1). 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 [21]. Chromatographic fractionation of the root extract from *P. patens* subsp. *patens* (L.) Mill. collected in Poland resulted in the isolation of three known oleanane-type glycosides identified as hederagenin 3-*O*-β-D-glucopyranoside, hederagenin 3-*O*-β-D-galactopyranosyl-(1→2)-β-D-glucopyranoside [22], and saponin D [23]. In the course of our studies on medicinal plants we evaluated the extracts of leaves and rhizomes of

Pulsatilla vulgaris Mill. for their antifungal, antimicrobial, antimalarial activities, and cytotoxicity to mammalian cell lines.

Table 1. Common *Pulsatilla* species in medicine.

Species	Action	Main components	References
Pulsatilla patens subsp. *multifida*	antifungal antimicrobial antitumor/cytotoxic molluscicidal antidiabetic antileishmanial	triterpene saponins	[20]
Pulsatilla koreana			[2, 6, 13]
Pulsatilla chinensis			[10-12]
Pulsatilla cernua			[14, 15]
Pulsatilla dahurica			[16]
Pulsatilla turczaninovii			[17]
Pulsatilla montana subsp. *balcana*	antioxidant antibacterial antifungal antiviral hepatoprotective anticancer anti-inflammatory	phenolics flavonoids anthocyanidins	[21]

The species of *P. vulgaris* was not extensively studied. Although the first report on pharmacodynamic properties, the distribution of saponins and tannins in this plant as well as pharmacology of isolated compounds came in 20th and 40th of the last century [24-26], they were followed only recently by few works on physiology [27, 28], genetic characteristics of the species [29, 30], ecology [31, 32] and various aspects of developmental biology [33]. In the fresh leaves and rhizomes of *P. vulgaris* the presence of the glycoside ranunculin was observed, which is converted to anemonine when the plant is dried [34, 35]. GC-MS analysis of the silylated methanolic extract of the leaves and rhizomes of *P. vulgaris* in our laboratories revealed the presence of carboxylic acids, such as benzoic, caffeic, malic, and succinic acids [5].

Relevant pharmacologic information regarding *P. vulgaris* is very scarce [9, 36-38]. However, the study done by Saify et al. [39] demonstrates the ability of *P. vulgaris* to reduce smooth muscle spasm. The extract from plant material appears to support the traditional use of this species as an antispasmodic [39]. This extract also can protect human cells against combined xenobiotic effects [40]. An important active constituent of *P. vulgaris* is protoanemonin [41-43]. Protoanemonin has been reported to have antibacterial, antimalarial and antifungal activity, however, it has been found to be cytotoxic as well. Plant extract from *P. vulgaris* showed antibacterial activity and inhibited the growth of *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* [44]. Due to the lack of current pharmacologic information about this species a study that examined the effect of protoanemonin which was extracted from *P. chinensis* was reviewed. Pharmacological study of secondary metabolites from *P. chinensis* showed that this compound possess anti-inflammatory affect on intestinal cells [45]. Additionally, analyses of biologically active metabolites from *P. koreana* showed that protoanemonin possesses antifungal activity and acts as an antibiotic [46]. Kanetoshi et al. [37] estimated that plant extract from *P. vulgaris* contains anticancer components active to three cell lines.

P. vulgaris is confined to dry grasslands, in sparsely wooded pine forests or meadows, often on a sunny sloping side with calcium-rich soil, where it grows at an altitude between 110-580 m. It grows well in fertile, humusy, gritty, and medium moisture well-drained soils in full sun to light shade. The best performance occurs in cool climates where plants are also more apt to tolerate drier conditions. There are three distinguishing subspecies of *P. vulgaris*, *P. vulgaris* subsp. *vulgaris*, subsp. *grandis* (Wender.) Zamels and subsp. *gotlandica* (Johanss.) Zamels & Paegle.

2. MATERIALS AND METHODS

2.1. Plant material

The leaves and rhizomes of *P. vulgaris* subsp. *vulgaris* were obtained from cultivation at the Herbarium "The Herbal Corner" located in Podlaskie Province, in North-Eastern Poland in May 2013 and identified by Prof. Grażyna Łaska from the Bialystok University of Technology, Faculty of Civil and Environmental Engineering, Poland.

2.2. General experimental procedures

The plant material in the form of crude rhizomes (56.1 g) and leaves (21.8 g) was extracted by accelerated solvent extraction (ASE) method (Buchi E-916) with 80% methanol and evaporated under reduced pressure. The crude extracts of rhizomes (0.8 g) and leaves (0.7 g) were resolved in 99.8% methanol and analyzed for their antimicrobial and antimalarial activities. The rhizome extract was also tested for cytotoxicity against cancer and healthy mammalian cell lines.

2.3. Antimicrobial assay

All organisms were obtained from the American Type Culture Collection (Manassas, VA) and include 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. Susceptibility testing was performed using a modified version of the CLSI (formerly NCCLS) methods [47-50]. *M. intracellulare* was tested using a modified method of Franzblau et al. [51]. Samples were serially-diluted in 20% DMSO/saline and transferred in duplicate to 96-well flat bottom microplates. Microbial inocula were prepared by correcting the OD_{630} of microbe suspensions in incubation broth to afford final target inocula. Drug controls [Ciprofloxacin (ICN Biomedicals, USA) for bacteria and Amphotericin B (ICN Biomedicals, Ohio) for fungi] were included in each assay. All organisms were read at either 530 nm using the Biotek Powerwave XS plate reader (Bio-Tek Instruments, Vermont) or 544 ex/590 em, (*M. intracellulare, A. fumigatus*) using the Polarstar Galaxy Plate Reader (BMG LabTechnologies, Germany) prior to and after incubation. Minimum fungicidal or bactericidal concentrations were determined by removing 5 µL from each clear well, transferring to agar and incubating. The MFC/MBC was defined as the lowest test concentration that kills the organism (allows no growth on agar).

2.4. Assay for antimalarial activity

The antimalarial activity was determined against chloroquine sensitive (D6) strain of *Plasmodium falciparum* by measuring plasmodial LDH activity according to the procedure of Makler and Hinrichs [52]. A suspension of red blood cells infected with *P. falciparum* (200 µL, with 2% parasitemia and 2% hematocrit in RPMI 1640 medium supplemented with 10% human serum and 60 µg/ml amikacin) was added to the wells of a 96-well plate containing 10 µl of diluted sample. The plate was incubated at 37^{o}C, for 72 h in a modular incubation chamber with 90% N_2, 5% O_2, and 5% CO_2. Parasitic LDH activity was determined by mixing 20 µl of the incubation mixture with 100 µl of the MalstatTM reagent (Flow Inc., Portland, OR) and incubating at room temperature for 30 min. Twenty microliters of a 1:1 mixture of NBT/PES (Sigma, St. Louis, MO) was then added and the plate was further incubated in the dark for 1 h. The reaction was then stopped by adding 100 µl of a 5% acetic acid solution and the absorbance was read at 650 nm. Artemisinin and chloroquine were included as the drug controls. IC_{50} values were computed from the dose response curves of growth inhibition using XLfit 4.2 (IDBS, USA).

2.5. Assay for cytotoxicity

The in vitro cytotoxicity of the rhizome extract was determined against a panel of cancer and non-cancer cell lines. The assay was performed in 96-well tissue culture-treated plates. The cells were seeded to the wells of 96-well plate at a density of 25,000 cells/well and grown for 24 h. Samples at different concentrations were added and cells were further incubated for 48 h. Cell viability was determined by Neutral Red method [53]. IC_{50} values were obtained from dose response curves. Doxorubicin was included as drug control.

3. RESULTS

Microbiological assays of the rhizome extracts of *P. vulgaris* showed activity against fungal pathogen *Candida glabrata* with an IC_{50} value of 11 µg/ml. The results of all antimicrobial activity tests are shown in Tables 2 and 3.

Table 2. The test results of the antimicrobial activity of rhizomes and leaves extracts of *Pulsatilla vulgaris* Mill. (primary screen).

Tested strain	Extracts from rhizomes (50 µg/ml)	Extracts from leaves (50 µg/ml)	Amphotericin B (5 µg/ml)	Ciprofloxacin (1 µg/ml)
C. albicans	24	11	100	ND
C. glabrata	98	14	100	ND
C. krusei	16	15	100	ND
A. fumigatus	11	21	99	ND
C. neoformans	40	0	82	ND
S. aureus	16	7	ND	89
MRSA	9	9	ND	94
E. coli	24	20	ND	96
P. aeruginosa	11	7	ND	100
M. intracellulare	0	0	ND	72

The results in %, ND – not determined.

Table 3. Dose response (IC_{50} in µg/ml) results of the rhizome extracts of *Pulsatilla vulgaris* Mill.

Test strain	Extracts from rhizomes	Amphotericin B	Ciprofloxacin
C. albicans	NA	0.19	ND
C. glabrata	11	0.37	ND
C. krusei	NA	0.67	ND
A. fumigatus	NA	sty.17	ND
C. neoformans	NA	0.18	ND
S. aureus	NA	ND	0.09
MRS	NA	ND	0.08
E. coli	NA	ND	0.01
P. aeruginosa	NA	ND	0.07
M. intracellulare	NA	ND	0.37

The results in IC_{50}, ND – not determined, NA – not active at 200 µg/ml.

The extracts from the rhizomes and leaves of *P. vulgaris* showed decreased ability to inhibit the growth of the other bacteria (*Staphylococcus aureus*, *MRSA*, *Escherichia coli*, *Pseudomonas aeruginosa*), and four different fungi (*Candida albicans*, *Candida krusei*, *Aspergillus fumigatus*, *Cryptococcus neoformans*)

pathogenic to humans. These extracts did not show any ability to inhibit the growth of the bacteria *Mycobacterium intracellulare* (Tables 2-3).

Antimalarial assays of the extracts from the rhizomes and leaves of *P. vulgaris* showed very low activity (1-8% of inhibition) against the protozoan, when the antimalarial drug chloroquine (positive control) showed 94-98% of inhibition.

The rhizome extract showed cytotoxicity to all the cell lines included in the assay. As shown in Table 4, the IC_{50} for cytotoxicity was in the range of 35-57 µg/ml for each cell line indicating a general cytotoxic activity throughout the panel of cancer and non-cancer cells.

Table 4. Cytotoxicity of *Pulsatilla vulgaris* Mill. rhizome extract towards a panel of mammalian cell lines.

Sample name	IC_{50} µg/ml					
	SK-MEL	**KB**	**BT-549**	**SK-OV-3**	**LLC-PK1**	**Vero**
Rhizome extract	44	42	57	35	42	39
Doxorubicin*	1.7	1.7	2.2	2.3	1.6	>5

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

4. DISCUSSION

The *Pulsatilla* species (Ranunculaceae) produces a high diversity of secondary metabolites with a biological activity. The triterpene saponins, flavonoids and anthocyanidins from various *Pulsatilla* subsp. have demonstrated multiple biological properties including antitumor [46, 54, 55], cognition-enhancing [56, 57], neuroactive [58], neuroprotective [59], immunomodulatory [60], antioxidant [61], antimicrobial [20] and cytotoxic [12] activities. Additionally, they have potential beneficial effects as a chemopreventive agent for critical health conditions including cancer. Treatment with *Pulsatilla* saponin D resulted in inhibition of cell growth/proliferation, angiogenesis and induction of apoptosis in cancer [62]. *Pulsatilla* saponin D isolated from the root of *Pulsatilla koreana* Nakai showed potent inhibition rate of tumor growth (IR, 82%) at the dose of 6.4 mg/kg on the BDF1 mice bearing LLC cells [6]. The extracts of the rhizomes or roots from other species of the *Pulsatilla* species have been used for amoebic, dysentery, malaria, epistaxis, and internal hemorrhoids [9].

The fact that *Pulsatilla* species produce the high content of a variety of secondary metabolites allows an intense search for new natural-product derived drugs, especially new antibiotics and antifungal agents. It is very important because currently used antifungal drugs are not effective in 15-20% of cases against *Candida glabrata* [63]. *Candida glabrata* (H.W. Anderson) S.A. Mey & Yarrow (1978) is a pathogenic ascomycete yeast, which is the second most frequent causative agent of human candidiasis [63]. *C. glabrata* is an opportunistic pathogen of the urogenital tract, and of the bloodstream. It is especially aggressive in HIV positive people, and the elderly [64]. There are two potential virulence factors that contribute to the pathogenicity of *C. glabrata*. The first is a series of adhesins coded by the EPA (epithelial adhesin) genes. These genes, located in the subtelomeric region, can respond to environmental cues that allow them to be expressed “en masse” so the organism can adhere to biotic and abiotic surfaces in microbial mats. This is also the suspected mechanism by which *C. glabrata* forms microbial “biofilms” on urinary catheters, and less commonly in-dwelling catheters. It also causes problems with dental devices, such as dentures [64].

Although *C. glabrata* is listed as the second most virulent yeast after *Candida albicans*, little information is available regarding its identification and treatment of infection. A major phenotype and potential virulence factor that *C. glabrata* possesses is low-level intrinsic resistance to the azole drugs, which are the most commonly prescribed antifungal medications. These drugs, like fluconazole and ketoconazole,

are not effective in 15-20% of cases against *C. glabrata* [63]. It is still highly vulnerable to polyene drugs such as amphotericin B and nystatin, along with variable vulnerability to flucytosine and caspofungin. Amphotericin B vaginal suppositories are used as an effective form of treatment in combination with boric acid capsules as they are not absorbed into the blood stream. Amphotericin B vaginal suppositories have also been used in case studies to treat chronic infections, both symptomatic and asymptomatic [63]. However high renal toxicity and other side effects of amphotericin B contained drugs make the use of such therapy the last resort approach. In the light of the limitation of existing antifungal therapy against *C. glabrata* the search for new safer drugs and natural products-derived agents or herbal preparations is highly desirable. The high antifungal activity (IC_{50} 11 µg/ml) of crude rhizome extracts of *P. vulgaris* against *C. glabrata* and the relatively high cytotoxicity (IC_{50} 35-57 µg/ml) towards a panel of mammalian cell lines prompts further research on isolation and identification of biologically active components from this species. The designated activity of rhizome extract of *P. vulgaris* below the threshold of observed toxicity qualify this species for further studies toward homeopathic therapy of candidiasis caused by pathogenic *C. glabrata*.

In 2015, the application for an invention patent titled "The use of *Pulsatilla vulgaris* Mill. in the treatment of fungal diseases" was submitted to the Polish Patent Office by the authors of this publication. Pharmaceutical application of extracts from *P. vulgaris* was patented by other authors [65-67]. The first invention provides an herbal extract pharmaceutical composition including *P. vulgaris* and its use in medicine. The application of this therapeutic extract increases the effectiveness in treating bloating. The second invention relates to an herbal composition comprising extract from *P. vulgaris* and use of this for preventing or treating skin diseases. The next application as antimicrobial composition includes an effective extract of plant of the Ranunculaceae family in that *P. vulgaris*.

The genus *Pulsatilla* comprises about 30 species, but *P. vulgaris* is an allotetraploid (2n=32) and may have occurred following hybridization between *P. patens* (2n=16) and *P. pratensis* (2n=16) [68]. *Pulsatilla vulgaris* Mill. is an early-flowering, long-lived, polycarpic hemicryptophyte herb of conservation concern and specialist species of calcareous grasslands across central Europe, ranging from France in the south to Sweden at its northern limit [33]. The current range this species is characterized by a high level of fragmentation, since numbers and sizes of populations have declined considerably during the last few decades, mainly as a consequence of land-use changes [29]. The reasons for the loss this species include mainly ploughing-up of calcareous grassland [31], cessation of traditional grazing practices [69], increased above-ground competition from coarse grasses and shrubs, what caused impossibility colonized restored habitats [32]. Consequently, small and fragmented populations showed signs of genetic depauperation due to genetic drift [29].

Numerous applications of *P. vulgaris* in traditional medicine are one of the reasons for reducing the abundance of this species. *P. vulgaris* is listed as "near threatened" by the International Union for Conservation of Nature [70]. Currently *P. vulgaris* is listed as vulnerable (VU category) in Ukraine, Slovakia [71], Sweden [72] and United Kingdom [73]. In Germany, it is classified as lower risk (LR category), however subsp. *grandis* (Wender.) Zamels listed as critically endangered (CR category) and subsp. *vulgaris* listed as endangered (EN category) [74]. This species is also listed as endangered (EN category) in Switzerland [75] and as critically endangered (CR category) in Austria [76]. In the "Red List of Vascular Plants in Poland" [77] and "Polish Red Data Book of Plants" [78] it is classified as extinct (EX category) species. In order to obtain larger amount of plant material for future study, a cooperation agreement was signed between the Bialystok University of Technology and Botanical Garden "Herbal Corner", from where cultivated plant species were transferred from the Botanical Garden to our laboratories.

P. vulgaris in Poland is mainly cultivated. In Denmark, Germany and Sweden it is still relatively widespread, but appears to have declined, especially in Sweden [72] and Germany, where it's populations are now small and highly fragmented [29]. In Austria, only around 2000 plants now survive in 23 sites [79] and Switzerland where it is very rare [33]. In Belgium it is confined to two small areas (Quentin Groom, pers. comm.). In Luxembourg it is declined from 28 to 5 localities [80]. In England *P. vulgaris* is a threatened herb

that declined from 130 to 33 sites between 1750 and the 1960s [31]. *P. vulgaris* appears to be extinct in Finland, where it has not been seen since the 1930s [81].

Acknowledgments: We thank Prof. Jordan K. Zjawiony, Dr Melissa Jacob and Dr Shabana Khan from University of Mississippi for determining biological activity of the extracts.
This study was supported by a grant nr S/WBiIŚ/5/16 from the Ministry of Science and Higher Education of Poland and by grants nr AI 27094 from NIH, NIAID, Division of AIDS and from USDA Agricultural Research Service Specific Cooperative Agreement no. 58-6408-1-603 of the United States.

Author Contributions: GŁ suggested the concept, writing the manuscript and approved the final version. AS did extensive literature search and writing the manuscript.

Conflict of Interest: The authors declare no conflict of interest.

REFERENCES

1. Fu Y, Chen H. Studies on the pharmacognostic identification, chemical composition and pharmacology of *Pulsatilla*. Acta Acad Med. CPAF 2006; 5: 483-487.
2. Bang SC, Kim Y, Lee JH, Ahn BZ. Triterpenoid saponins from the roots of *Pulsatilla koreana*. J Nat Prod. 2005; 68: 268-272.
3. Cheon SA, Choi BK, Jeong CS, Li DW, Lee EB. The anti-inflammatory and analgesic actions of the root of *Pulsatilla koreana*. Korean J Pharmacogn. 2000; 31: 174-184.
4. Chung SW, Chung CG, Lim SB, Kim JK, So EH. The antimicrobial effect of *Pulsatilla koreana* extracts to oral micro-organism. J Korean Acad Periodontol. 2000; 30: 661-674.
5. Łaska G, Sienkiewicz A. Secondary metabolites from *Pulsatilla* species and their biological activity. Planta Med. 2014; 80-PN4.
6. Kim Y, Bang SC, Lee JH, Ahn BZ. *Pulsatilla* saponin D: the antitumor principle from *Pulsatilla koreana*. Arch Pharm Res. 2004; 27: 915-918.
7. Łaska G, Sienkiewicz A, Piotrowska-Niczyporuk A. Study on HPLC fingerprint characteristic of *Pulsatilla patens* (L.) Mill. Planta Med. 2016; S1-S381.
8. Lee SH, Byeon MS, Kim MK. Selective growth inhibitor toward human intestinal bacteria derived from *Pulsatilla cernua* root. J Agr Food Chem. 2001; 49: 4656-4661.
9. Cho SC, Sultan MZ, Moon SS. Anti-acne activities of pulsaquinone, hydropulsaquinone, and structurally related 1,4-quinone derivatives. Arch Pharm Res. 2009; 32: 489-494.
10. Mimaki Y, Yokosuka A, Kuroda M, Hamanaka M, Sakuma C, Sashida Y. New bisdesmosidic triterpene saponins from the roots of *Pulsatilla chinensis*. J Nat Prod. 2001; 64: 1226-1229.
11. Shu Z, Chen Z, Liu YL, Zhu WF, Feng YL, Xu QM, et al. A new oleanane-type triterpenoidal saponin from *Pulsatilla chinensis*. Nat Prod Res. 2013; 27: 2196-2201.
12. Xu K, Shu Z, Xu QM, Liu YL, Li XR, Wang YL, et al. Cytotoxic activity of *Pulsatilla chinensis* saponins and their structure-activity relationship. J Asian Nat Prod Res. 2013; 15: 680-686.
13. Yang H, Cho YW, Kim SH, Kim YC, Sung SH. Triterpenoidal saponins of *Pulsatilla koreana* roots. Phytochemistry 2010; 71: 1892-1899.
14. Xu YL, Bai L, Liu YH, Liu Y, Xu TH, Xie SH, et al. A new triterpenoid saponin from *Pulsatilla cernua*. Molecules 2010; 15: 1891-1897.
15. Fan WH, Liu JY, Gong YX, Ma J, Zhou N, Xu YN. A new triterpenoid saponin from *Pulsatilla cernua*. Nat Prod Sci. 2013; 19: 150-154.

16. Sun H, Wang Y, Zhang XQ, Zhao SX, Ye WC. Chemical constituents of *Pulsatilla dahurica*. Chem Nat Comp. 2009; 45: 764-765.
17. Xu HJ, Shi XW, Ji X, Du YF, Zhu H, Zhang LT. A rapid method for simultaneous determination of triterpenoid saponins in *Pulsatilla turczaninovii* using microwave-assisted extraction and high performance liquid chromatography-tandem mass spectrometry. Food Chem. 2012; 135: 251-258.
18. Samadder A, Das J, Das S, Khuda-Bukhsh AR. Dihydroxy-isosteviol-methyl-ester, an active biological component of *Pulsatilla nigricans*, reduces arsenic induced cellular dysfunction in testis of male mice. Environ Toxicol Phar. 2012; 34: 743-752.
19. Rolski S, Przyborowski L. Dissert Pharm. 1961; 13: 349-355.
20. Ye WC, Ji NN, Zhao SX, Che CT. A New Cytotoxic Saponin from *Pulsatilla patens* var. *multifida*. Pharm Biol. 2001; 39: 7-10.
21. Danova K, Bertoli A, Pistelli L, Dimitrow D, Pistelli L. *In vitro* culture of Balkan endemic and rare *Pulsatilla* species for conservational purposes and secondary metabolites production. Bot Serb. 2009; 33: 157-162.
22. Sharma V, Łaska G, Radhakrishnan SVS, Jacob MR, Zjawiony JK. Phytochemical investigation and pharmacological evaluation of *Pulsatilla patens* var. *patens*. Planta Med. 2014; 80-PD3.
23. Łaska G, Sienkiewicz A, Piotrowska-Niczyporuk A. New Polish species with isolated *Pulsatilla* saponin D. Planta Med. 2016; 82: S1-S381.
24. Luft G. The distribution of saponins and tannins in the plant. Monatshefte fuer Chemie 1926; 47: 259-284.
25. Raymond-Hamet L. Pharmacodynamic properties of *Anemone pulsatilla*. Bull Sci Pharmacol. 1927; 34: 143-151.
26. Kroeber L. On the pharmacology and therapeutic use of the anemonine drugs from Ranunculaceous plants. Pharmazie. 1949; 4: 181-190.
27. Weryszko-Chmielewska E, Sulborska A. Staminodial nectary structure in two *Pulsatilla* (L.) species. Acta Biol Cracov Ser Bot. 2011; 53: 94-103.
28. Liu DD, Chao WM, Turgeon R. Transport of sucrose, not hexose, in the phloem. J Exp Bot. 2012; 63: 4315-4320.
29. Hensen I, Oberprieler C, Wesche K. Genetic structure, population size, and seed production of *Pulsatilla vulgaris* Mill. (Ranunculaceae) in Central Germany. Flora 2005; 200: 3-14.
30. DiLeo MF, Graf R, Holderegger R, Rico Y, Wagner HH. Highly polymorphic microsatellite markers in *Pulsatilla vulgaris* (Ranunculaceae) using next-generation sequencing. Appl Plant Sci. 2015; 3(7): apps.1500031.
31. Walker KJ, Pinches CE. Reduced grazing and the decline of *Pulsatilla vulgaris* Mill. (Ranunculaceae) in England. UK. Biol Conserv. 2011; 144: 3098-3105.
32. Piqueray J, Saad L, Bizoux JP, Mahy G. Why some species cannot colonise restored habitats? The effects of seed and microsite availability. J Nat Conserv. 2013; 21: 189-197.
33. Pfeifer E, Holderegger R, Matthies D, Rutishauser R. Investigation on the population biology of a flagship species of dry meadows: *Pulsatilla vulgaris* Mill. in north-eastern Switzerland. Bot Helv. 2002; 112: 153-172.
34. drugs.com, 2000-2014. Drugs.com know more be sure, Auckland 0632 New Zealand: Mission Statement "to empower patients with the knowledge to better manage their own healthcare and to improve

consumer safety by assisting in the reduction of medication errors". http://www.drugs.com/npp/pasque-flower.html.

35. Bundesministerium für Gesundheit, Pharmabund.net-Arzneimittelinforamtion für alle. http://www.pharmnet-bund.de/dynamic/de/index.html (accessed 25 February 2014).
36. Bone K. A clinical guide to blending liquid herbs: herbal formulations for the individual patient. Churchill Livingstone, St Louis, Missouri, 2003.
37. Kanetoshi A, Inoue S, Anetai M, Fujimoto T, Aoyagi M, Sato M. An in vitro screening test for anti-cancer components of wild plants in Hokkaido. Report-Hokkaido Institute of Public Health. 2005; 55: 49-53.
38. Łaska G, Sienkiewicz A, Stocki M, Sharma V, Zjawiony JK, Jacob M. Secondary metabolites from *Pulsatilla patens* and *Pulsatilla vulgaris* and their biological activity. Planta Med. 2015; 81-PM_122.
39. Saify ZS, Noor F, Mushtaq N, Dar A. Assessment of *Anemone pulsatilla* for some biological activities. Pak J Pharm Sci. 1998; 11: 47-53.
40. Gasnier C, Laurant C, Decroix-Laporte C, Mesnage R, Clair E, Travert C, et al. Defined plant extracts can protect human cells against combined xenobiotic effects. J Occup Med Toxicol. 2011; 6: 3.
41. Jürgens A, Dötterl S. Chemical composition of pollen volatiles in Ranunculaceae: Genera-specific profiles in *Anemone*, *Aquilegia*, *Caltha*, *Pulsatilla*, *Ranunculus*, and *Trollius* species. Am J Bot. 2004; 91: 1969-1980.
42. Mills S, Bone K. The essential guide to herbal safety. Elsevier, St Louis, 2005.
43. Foster S, Johnson R. National geographic desk reference to natural medicine. National Geographic, Washington, 2006.
44. Baer H, Holden M, Seegal BC. The nature of the antibacterial agent from *Anemone pulsatilla*. J Biol Chem. 1946; 162: 65-68.
45. Duan H, Zhang Y, Xu J, Qiao J, Suo Z, Hu G, et al. Effect of anemonin on NO, ET-1 and ICAM-1 production in rat intestinal microvascular endothelial cells. J Ethnopharmacol. 2006; 104: 362-366.
46. Bang SC, Lee JH, Song G, Kim DH, Yoon MT, Ahn BZ. Antitumor activity of *Pulsatilla koreana* saponins and their structure-activity relationship. Chem Pharm Bull. 2005; 53: 1451-1454.
47. NCCLS: National Committee on Clinical Laboratory Standards, 2002; 22: 15.
48. NCCLS: National Committee on Clinical Laboratory Standards, 2002; 22: 16.
49. NCCLS: National Committee on Clinical Laboratory Standards, 2003; 23: 18.
50. NCCLS: National Committee on Clinical Laboratory Standards, 2006; 26: 2.
51. Franzblau SG, Witzig RS, McLaughlin JC, Torres P, Madico G, Hernandez A, et al. Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar blue assay. J Clin Microbiol. 1998; 36: 362-366.
52. Makler MT, Hinrichs DJ. Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia. Am J Trop Med Hyg. 1993; 48: 205-210.
53. Borenfreund E, Babich H, Martin-Alguacil N. Rapid chemosensitivity assay with human normal and tumor cells in vitro. In Vitro Cell Dev Biol. 1990; 26: 1030-1034.
54. Xu QM, Shu Z, He WJ, Chen LY, Yang SL, Yang G, et al. Antitumor activity of *Pulsatilla chinensis* (Bunge) Regel saponins in human liver tumor 7402 cells in vitro and in vivo. Phytomedicine 2012; 19: 293-300.
55. Hong SW, Jung KH, Lee H, Son MK, Yan HH, Kang NS, et al. SB365, *Pulsatilla* saponin D, targets c-Met and exerts antiangiogenic and antitumor activities. Carcinogenesis 2013; 34: 2156-2169.

56. Han CK, Park YH, Jin DQ, Hwang YK, Oh KB, Hand JS. SK-PC-B70M from *Pulsatilla koreana* improves scopolamine-induced impairments of memory consolidation and spatial working memory. Brain Res. 2007; 1184: 254-259.
57. Seo JS, Kim TK, Leem YH, Lee KW, Park SK, Baek IS, et al. SK-PC-B70M confers anti-oxidant activity and reduces β levels in the brain of Tg2576 mice. Brain Res. 2009; 1261: 100-108.
58. Yoo HH, Lee SK, Lim SY, Kim Y, Kang MJ, Kim EJ, et al. LC-MS/MS method for determination of hederacolchiside E, a neuroactive saponin from *Pulsatilla koreana* extract in rat plasma for pharmacokinetic study. J Pharm Biomed Anal. 2008; 48: 1425-1429.
59. Liu JY, Guan YL, Zou LB, Gong YX, Hua HM, Xu YN, et al. Saponins with neuroprotective effects from the roots of *Pulsatilla cernua*. Molecules 2012; 17: 5520-5531.
60. Dai L, Wang H, Chen Y. The immune enhancing effect of PcG2A-a glycoprotein isolated from dried root of *Pulsatilla chinensis* (Bunge) Regel. Chin J Biochem Pharm. 2000; 21: 230-231.
61. Li HB, Wong CC, Cheng KW, Chen F. Antioxidant properties in vitro and total phenolic contents in methanol extracts from medicinal plants. Food Sci Tech. 2008; 41: 385-390.
62. Son MK, Jung KH, Lee HS, Lee H, Kim SJ, Yan HH, et al. SB365, *Pulsatilla* saponin D suppresses proliferation and induces apoptosis of pancreatic cancer cells. Oncol Rep. 2013; 30: 801-808.
63. Fidel F, Vazquez J, Sobel J. *Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. Clin Microbiol Rev. 1999; 12: 80-96.
64. Bethea EK, Carver BJ, Montedonico AE, Reynolds TB. The inositol regulon controls viability in *Candida glabrata*. Microbiology 2009; 156: 452-462.
65. http://worldwide.espacenet.com/publication Details/ (accessed 11 October 2012).
66. http://worldwide.espacenet.com/publication Details/ (accessed 12 June 2008).
67. http://worldwide.espacenet.com/publication Details/ (accessed 4 October 2005).
68. Böcher TW. Beiträge zur Zytologie der Gattung Anemone. Botanisk Tidsskrift 1934; 42: 183-206.
69. Butaye J, Adriaens D, Honnay O. Conservation and restoration of calcareous grasslands: A concise review of the effects of fragmentation and management on plant species. Biotechnol Agro Soc Environ. 2005: 9: 111-118.
70. IUCN, The IUCN Red List of threatened species. Version 2014.1. www.iucnredlist.org. (accessed 12 June 2014).
71. Witkowski ZJ, Król W, Solarz W, eds. Carpathian list of endangered species. WWF and Institute of Nature Conservation, Polish Academy of Sciences, Vienna-Krakow, 2003.
72. Gärdenfors U. The 2010 Red List of Swedish species. ArtDatabanken, SLU, Uppsala, 2010.
73. Cheffings C, Farrell L, eds. The vascular plant red data list for Great Britain. Joint Nature Conservation Committee, Peterborough, 2005.
74. Ludwig G, Schnittler M. Red List of threatened plants in Germany. Bundesamt für Naturschutz, Bonn, 1996.
75. Moser D, Gygax A, Bäumler B, Wyler N, Palese R. Red List of the threatened ferns and flowering plants of Switzerland. Bundesamt für Umwelt, Wald und Landschaft, Zentrum des Datenverbundnetzes der Schweizer Flora, Conservatoire et Jardin botaniques de la Ville de Genève, Bern, Chambésy, 2002.
76. Niklfeld H, Schratt-Ehrendorfer L, eds. Red List of threatened plants of Austria. Grüne Reihe, Bundesministerium für Umwelt, Jugend und Familie, Vienna, 1999.
77. Zarzycki K, Szeląg Z. 2006. Red list of vascular plants in Poland. In: Mirek Z, Zarzycki K, Wojewoda W, Szeląg Z, eds. PAN, Inst. Bot., Kraków, 9-20.

78. Kaźmierczakowa R, Zarzycki K, Mirek Z. Polish Red Data Book of Plants. PAN, Inst. Bot., Kraków, 2014.
79. Franz E. Population development, habitat preference and causes of endangerment of the Pasque Flower (*Pulsatilla vulgaris* Mill.) in Austria between 1991 and 2005. Linzer Biologische Beitraege 2005; 37: 1145-1176.
80. Colling G. Red list of the vascular plants of Luxembourg. Ferrantia 2005; 42: 1-77.
81. Rassi P, Alanen A, Kanerva T, Mannerkoski I, eds. The 2000 Red List of Finnish species. Ympäristöministeriö & Suomen ympäristökeskus, Helsinki, 2001.