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EXPERIMENTAL PAPER

Accumulation of phenolic compounds in the purple betony herb (*Stachys officinalis* L.) originated from cultivation

KATARZYNA BĄCZEK*, OLGA KOSAKOWSKA, JAROSŁAW L. PRZYBYŁ, ZENON WĘGLARZ

Laboratory of New Herbal Products
Department of Vegetable and Medicinal Plants
Warsaw University of Life Sciences – SGGW
Nowoursynowska 166,
02-787 Warsaw, Poland

*corresponding author: phone: +4822 5932258; e-mail: katarzyna_baczek@sggw.pl

Summary

Introduction: Purple betony (*Stachys officinalis* L., *Lamiaceae*) is a perennial of versatile medicinal usage. Nowadays, in Poland betony herb is collected exclusively from wild growing plants. Decreasing number of its natural sites results in lack of the herb supply and thus, in its limited usage. **Objective**: The aim of the study was to determine the effects of the age of plant and term of raw material harvest on its yield and quality in cultivation conditions. **Methods**: The observations were carried out on 2- and 3-year-old plants. During vegetation the herb was collected for four times. The raw material was subjected to chemical analysis. Tannins (as pyrogallol equivalent) were determined according to Polish Pharmacopoeia, phenolic acids and flavonoids – by HPLC. **Results**: The mass of herb, both in the second and third year, had increased from the beginning of vegetation up to seed setting. The highest content of tannins was found in the herb collected at the vegetative stage of plant development (2.05% in the second and 2.91% in the third year). Four phenolic acids (chlorogenic, ferulic, caffeic and rosmarinic acids) and five flavonoid compounds (orientin, luteolin-7-glucoside, apigenin-7-glucoside, apigenin-3-glucoside, apigenin) were identified in the obtained raw materials. In these groups, the dominant compounds were caffeic acid and apigenin. The highest content of caffeic acid was found at the beginning of plant vegetation, whereas apigenin – at the stage of full blooming and seed setting. **Conclusion**: In cultivation conditions, purple betony produces high mass of herb which may be used as a valuable raw material in herbal industry.

Key words*: accumulation of biomass, tannins, phenolic acids, flavonoids*

INTRODUCTION

Purple betony (*Stachys officinalis* L., syn. *Betonica officinalis* (L.) Trev.) known also as wood betony, common betony, woundwort or Bishop's wort is a perennial from the *Lamiaceae* family occurring in Europe, North Africa and Asia [1-4]. The underground organs of the plant consists of a short rhizome with roots. It produces basal leaves and stems which terminate in pink or red-purple inflorescence. It grows in moist, partly shaded sites, at the edges of forests, meadows and pasturelands [3, 5]. Wild growing plants are the source of betony herb (*Betonicae herba*) – a medicinal raw material. The herb is rich in polyphenols, especially tannins and flavonoids. It also contains some bitter substances, iridoids, diterpenic lactons and small amount of essential oil [1, 2, 5–8]. Extracts from these raw material reveal astringent, anti-inflammatory, antihemmorhagic and antirheumatic activity [8–10]. The cytotoxic and antifungal actions of betony herb were confirmed in many studies. In traditional medicine, it is used against diarrhoea, internal bleeding, mouth or throat inflammations, in the upper respiratory tract disorders, cough and to treat liver disorders, migraine, convulsions and infected wounds [5, 6, 10–12]. In spite of rich chemical profile of the plant and its versatile medicinal usage in the past, nowadays it is not included into any European pharmacopoeia. Such situation results probably from decreasing number of natural sites where it grows, and thus from narrow stock for harvest [2, 4 ,13]. In Poland, problems with supply of this raw material from the natural sites have been noted [14]. Some other wild growing medicinal plants are also no longer in use, or are used only incidentally, due to supply problems, e.g. *Filipendula vulgaris* L., *Comarum palustre* L. Cultivation of such species may solve the problem. It gives also an opportunity to produce standardised raw material with expected quality parameters.

Until now, only preliminary investigation on purple betony cultivation and its reproduction potential have been done [13, 15, 16]. Those studies indicate the possibility of introduction of the species into cultivation. However, given the external factors affecting the wild growing plants, it seems that the development of plants in cultivation conditions will be different than in the wild. Therefore, this paper attempts to determine the effects of the age of plant and term of raw material harvest on the yield and quality of purple betony herb, with special respect to the accumulation of phenolics.

MATERIAL AND METHODS

The field experiment was established at the experimental field of Department of Vegetable and Medicinal Plants, Warsaw University of Life Sciences – SGGW. The object of the study were two- and three-year-old plants of purple betony (*Stachys officinalis* L.). The seeds collected from the natural site in the Eastern Poland (population of Siemiatycze, seeds deposited in National Centre for Plant Genetic

Resources, Polish GeneBank, accession No. 503105) were sown in the greenhouse at the end of May, 2012. The seedlings were planted out in the field, in the randomized block design (3 replications), at the beginning of September. Plant density was 70 x 40 cm. The herb (5 plants within one replication) was collected in the second and third year (2013 and 2014): at the beginning of vegetation (rosette of basal leaves; May), at the beginning of blooming (late June), at the stage of full blooming (early July) and at the beginning of seed setting (late July). It was dried at 35°C. The weight of herb per plant was determined and the raw materials were subjected to chemical analysis.

Determination of the total content of tannins

The total content of tannins (expressed as pyrogallol equivalent, %) was determined spectrophotometrically, according to Polish Pharmacopoeia VIII [17]. The presented results are mean values from 3 replications.

Determination of the content of phenolic compounds using HPLC

Validation

Commercially available standards (ChromaDex®) were separately dissolved in 10 ml volumetric flask with MeOH (methanol) according to the ChromaDex's Tech Tip 0003: Reference Standard Recovery and Dilution and used as standard stock solutions. Further calibration levels were prepared by diluting these solutions with methanol in 10 ml volumetric flasks (injected volume ranges: 10, 50, 100, 200, 500 and 1000 μl) and mix all stock solutions together. The working solutions were injected (1 μl) on a column in six replicates (n=6) using SIL-20A to generate a seven-point calibration curve, using CLASS VP™ 7.3 chromatography software. The peak table and spectra library (190–450 nm) of individual compounds were created. Standard curve parameters were calculated with statistical service e-stat (http://www.chem.uw.edu.pl/stat/e-stat/). Signal-to-noise ratio approach were used to determined LOD (S/N of 3:1) and LOQ (S/N of 10:1).

Sample preparation

Air-dry, finely powdered and homogenized raw material (1.000 g of herb) was extracted with 100 ml of methanol in Büchi Labortechnik AG Extraction System B-811. Soxhlet hot extraction with twenty-five extraction cycles, flushing and drying was used. After the evaporation of solvent, the residue was dissolved in 10 ml

of methanol. The obtained extracts were filtered with Supelco Iso-Disc™ Syringe Tip Filter Unit, PTFE membrane, diameter 25 mm, pore size 0.20 μm and subjected to HPLC.

Parameters of separations

The analyses were performed using a Shimadzu chromatograph equipped with auto sampler SIL-20A, photodiode array detector SPD-M10A VP PDA and CLASS VP™ 7.3 chromatography software. A modern C-18 reversed-phase column with core-shell technology (Phenomenex Kinetex® 2.6 μm, C18, 100 Å, 100 · 4.60 mm i.d.) was used as solid phase. Binary gradient of mobile phase A (deionised water adjusted to pH 3 with phosphoric acid) and B (ACN /acetonitrile/ adjusted to pH 3 with phosphoric acid) was used as follows: 0.01 min – 18% B; 0.50 min – 18% B; 5.50 min – 30% B; 5.54 min – 95% B; 6.50 min – 95% B; 6.51 min – 18% B; 11.00 min – STOP. The following conditions were applied: flow rate 1.2 ml · min^{-1}, oven temperature 35°C, total time of analysis 11 min, injection volume: 1 μl.

Parameters of integration

UV-spectra were recorded between 190 and 450 nm. Peak identification was conducted by comparison of retention time and UV-spectra recorded between 190 and 800 nm of standards. Detection wave applied: 300 nm (caffeic acid), 330 nm (chlorogenic acid, rosmarinic acid, ferulic acid), 336 nm (apigenin, apigenin-7-glucoside, apigenin-3-glucoside), 347 nm (orientin /luteolin-8-glucoside/, luteolin-7-glucoside). The content of determined compounds was calculated in mg · 100 g^{-1} of dry matter. The results presented are mean values from three replications.

Statistical analysis

The results were analysed with one-way ANOVA and Tukey's HSD test at $\alpha=0.05$ significance level using Statgraphics Plus for Windows v. 4.1 software. They were expressed as mean ± standard deviation (SD).

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS AND DISCUSSION

Purple betony herb has been collected for medicinal purpose exclusively from the wild growing plants [2, 4, 12]. There is little information on its cultivation. Basic data on ornamental *Stachys* species cultivation are available on web pages of

botanical gardens and horticultural companies [18]. Field experiments on this plant have been carried out in the Czech Republic and in Poland. They concern mainly the intraspecific variability of the species [2, 4, 13] and evaluation of accessions deposited in botanical gardens [15, 16]. It was found that wild growing populations differ significantly in the morphological traits, i.e. in the height and width of plants, in the length of inflorescences, in the shape of leaves, leaf area as well as in seed parameters [2, 4, 13]. Some investigation on the chemical profile of betony herb and its activity has been done as well. It was confirmed that this raw material contains only small amount of essential oil (up to 0.02%), but is rich in phenolic compounds such as phenolic acids (hydroxycinnamic acid derivatives) and flavonoids (luteolin and apigenin derivatives) [1, 7, 16, 19-21]. The information on the concentration of these substances in different plant organs is also available [22]. However, the accumulation of secondary metabolites during plant ontogenesis is unknown. There are also no data concerning the factors influencing the purple betony development.

In present study, the accumulation of biomass and phenolics in the herb of cultivated purple betony in two years of plant vegetation has been determined. In the second and third year of plant vegetation, the weight of air-dry herb had increased from the beginning of vegetation, when plants were in vegetative phase (84.5 g in the second and 137.5 g per plant in the third year of vegetation), until the stage of seed setting (165.0 g per plant in the second and 175.2 g per plant in the third year of vegetation). It correlated with increment of shoots number and their length (tab. 1). According to Dušek *et al*. [2, 4], the height of betony plants may vary from 45.4 to 96.8 cm. In our research the stem elongation was observed particularly from the beginning of blooming until the seed setting. In the second year during this period the shoots reached 79.7 cm and in third year 93.2 cm (tab. 1).

Table 1.

The morphological traits, herb weight and the total content of tannins in *Stachys officinalis*

Age of plants	Stage of development	Number of shoots per plant	Length of shoots [cm]	Air-dry weight of herb [g · plant^{-1}]	Total content of tannins [%]
2nd year of plant vegetation	Beginning of vegetation	–	–	84.5d±7.2	2.05c±0.17
	Beginning of blooming	36.6c±3.7	64.5b±9.0	105.2c±8.6	1.72d±0.09
	Full blooming	48.5b±4.3	78.5b±8.7	150.0b±14.8	1.86d±0.14
	Seed setting	52.1ab±4.8	79.7b±7.9	165.0ab±14.9	1.77d±0.10
3rd year of plant vegetation	Beginning of vegetation	–	–	137.5bc±12.4	2.91a±0.20
	Beginning of blooming	47.4b±5.1	71.2b±6.6	158.0ab±14.2	2.54b±0.15
	Full blooming	53.8ab±5.3	92.1a±10.8	172.1a±16.6	2.69b±0.18
	Seed setting	56.8a±5.5	93.2a±12.5	175.2a±17.1	1.82d±0.13

Values in columns marked with the same letter do not differ significantly at $\alpha = 0.05$

According to Buchwald and Dedio [15], the total content of tannins in herb of purple betony varies from 2.0 to 3.9 mg · g^{-1}. Vundać *et al.* [10] report that the herb collected at the stage of full blooming characterize with the total content of tannins on the level of 1.36%. Another literature data state a content of up to 15% [8]. In our results, total content of tannins in betony herb depended on the age of plant and term of harvest. Their content was higher in the third year of vegetation in comparison to the second year. The highest content of those metabolites was observed in the herb collected in the vegetative phase of plant development. The concentration of tannins in herb collected from plants in generative phase (beginning or full blooming as well as seed setting stage) was lower (tab. 1).

In the raw material nine phenolic compounds were identified, including four phenolic acids (chlorogenic, ferulic, caffeic and rosmarinic acids) and five flavonoid compounds (orientin, luteolin-7-glucoside, apigenin-7-glucoside, apigenin-3-glucoside, apigenin) (fig. 1). Among phenolic acids, ferulic and rosmarinic acids were present in the raw material only in small amounts. There was no clear relationship between their content and the developmental stage of plants. The content of chlorogenic acid was the highest at the full blooming and seed setting stage, whereas the content of caffeic acid was detected in the highest concentration in herb collected at the beginning of vegetation. This consistency was observed both in the second and third year of plant vegetation (tab. 2). The identified phenolic acids have been previously reported as constituents of betony herb along with other acids e.g. sinapic, protocatechuic, neochlorogenic, isochlorogenic and *p*-coumaric [16, 21, 22].

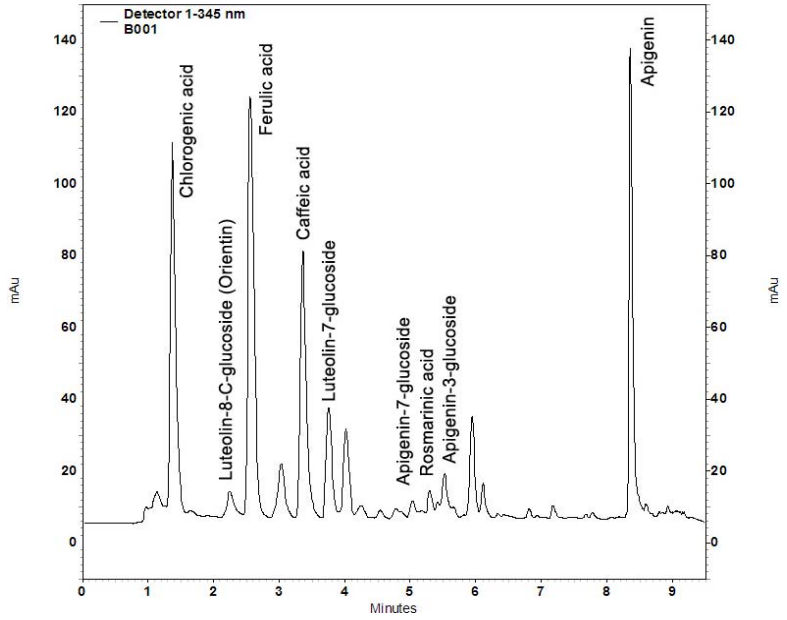

Figure 1.

An example of HPLC chromatogram of purple betony herb extract

Irrespective of the plant age and their developmental stage, the dominant flavonoid compounds were luteolin-7-glucoside and apigenin. The content of both constituents had increased from the beginning of vegetation until the full blooming stage and, in the case of apigenin, it had increased even up to seed setting phase. Orientin was detected in the highest amounts at the beginning of vegetation whereas the content of apigenin-7-glucoside and apigenin-3-glucoside was the highest at the beginning and full blooming (tab. 3). The above-mentioned flavonoid constituents also has been earlier identified in betony aboveground organs by other authors. However, most of them show only the chemical composition of the herb without reports on the content of particular flavonoids [1, 7, 20]. Some other articles present information on the total content of flavonoid compounds [2, 5, 8,10, 13, 15].

CONCLUSION

In summary, the analysis of changes in the accumulation of identified phenolics in betony herb in the second and third year of vegetation indicate that the raw material is the richest in tannins at the beginning of vegetation. The content of caffeic acid, i.e. the dominant one, was the highest in the herb collected at the beginning of vegetation, whereas the dominant flavonoid compound, i.e. apigenin – at the stage of full blooming and seed setting. The most intensive increment of mass of betony herb was observed between the beginning of flowering and seed setting, in the third year of plant vegetation.

Taking into consideration the results of our study it seems that purple betony, as a perennial, may be exploit in cultivation for at least 3 years and give high and good quality yield of herb.

Table 2.

The content of identified phenolic acids in the herb *Stachys officinalis* (mg · 100 g^{-1})

Age of plants	Stage of development	Chlorogenic acid	Ferulic acid	Caffeic acid	Rosmarinic acid
2nd year of plant vegetation	Beginning of vegetation	97.51d±6.76	16.89b±1.11	215.08b±16.94	4.10c±0.29
	Beginning of blooming	113.27cd±7.44	16.45b±1.03	160.82d±14.58	2.82e±0.13
	Full blooming	124.77c±8.13	12.89c±0.56	137.47e±11.01	3.43d±0.22
	Seed setting	123.57c±8.08	14.63bc±0.98	116.16f±9.26	6.91a±0.38
3rd year of plant vegetation	Beginning of vegetation	105.30d ±6.59	15.19b±1.01	257.43a ±22.34	6.25a±0.32
	Beginning of blooming	104.10d±6.93	16.12b±1.08	208.20bc±17.18	4.55c±0.25
	Full blooming	141.92b±9.76	14.01bc±0.93	191.37c ±15.49	5.10b±0.28
	Seed setting	175.28a±11.12	23.10a±1.56	162.31d±14.00	4.41c±0.25

Values in columns marked with the same letter do not differ significantly at $\alpha = 0.05$

Table 3.

The content of identified flavonoid compounds in the herb *Stachys officinalis* (mg · 100 g^{-1})

Age of plants	Stage of development	Orientin	Luteolin-7-glucoside	Apigenin-7-glucoside	Apigenin-3-glucoside	Apigenin
2nd year of plant vegetation	Beginning of vegetation	49.05b±3.43	99.77d±8.03	8.02e±0.50	14.26e±0.82	149.98d ±12.56
	Beginning of blooming	18.91d±1.55	87.00e±6.56	10.41e±0.73	43.14c±3.34	179.00c±13.08
	Full blooming	17.62d±1.49	132.34b±9.14	17.78c±0.98	46.86c±3.65	185.40c±14.39
	Seed setting	5.33f±0.26	116.16c±8.02	9.83e±0.79	26.88d±1.23	201.64b±17.47
3rd year of plant vegetation	Beginning of vegetation	108.86a±6.94	119.02c ±8.13	14.89d±0.80	27.70d±1.27	123.06e±8.99
	Beginning of blooming	22.36c±1.69	176.96a±12.28	25.48b±1.12	102.29a±8.18	130.40e±9.86
	Full blooming	27.68c±1.88	184.00a±13.33	37.49a±1.73	74.41b±4.65	187.19c±15.21
	Seed setting	12.77e±0.74	126.13b±9.45	9.93e±0.71	26.88d±1.12	273.92a±22.16

Values in columns marked with the same letter do not differ significantly at $\alpha=0.05$

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Conflict of interest: Authors declare no conflict of interest.

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AKUMULACJA ZWIĄZKÓW FENOLOWYCH W ZIELU BUKWICY LEKARSKIEJ (*STACHYS OFFICINALIS L.*) POCHODZĄCEJ Z UPRAWY

KATARZYNA BĄCZEK*, OLGA KOSAKOWSKA, JAROSŁAW L. PRZYBYŁ, ZENON WĘGLARZ

Laboratorium Nowych Technologii Wytwarzania Produktów Zielarskich
i Oceny ich Jakości
Katedra Roślin Warzywnych i Leczniczych
Szkoła Główna Gospodarstwa Wiejskiego w Warszawie
ul. Nowoursynowska 166
02-787 Warszawa

*autor, do którego należy kierować korespondencję: tel: +4822 5932258;
e-mail: katarzyna_baczek@sggw.pl

Streszczenie

Wstęp: Bukwica lekarska (*Stachys officinalis* L., *Lamiaceae*) to bylina wszechstronnie wykorzystywana jako roślina lecznicza. Obecnie w Polsce ziele bukwicy zbiera się wyłącznie z roślin dziko rosnących. Malejąca liczba naturalnych stanowisk tej rośliny skutkuje brakiem surowca na rynku, a w konsekwencji jego ograniczonym stosowaniem. **Cel**: Celem pracy było określenie, w warunkach uprawy, wpływu wieku rośliny i terminu zbioru surowca na jego plon i jakość. **Metody**: Obserwacje przeprowadzono na roślinach dwu- i trzyletnich. W sezonie wegetacyjnym ziele zbierano w czterech terminach. Surowiec ten poddano analizie chemicznej. Zawartość garbników (w przeliczeniu na pirogalol) oceniono zgodnie z Farmakopeą Polską, a kwasów fenolowych i flawonoidów – przy użyciu HPLC. **Wyniki**: Masa ziela, zarówno w drugim, jak i trzecim roku uprawy, wzrastała od początku wegetacji do początku zawiązywania nasion. Najwyższą zawartością garbników charakteryzowało się ziele zebrane w okresie wegetatywnego rozwoju roślin (2,05% w drugim i 2,91% w trzecim roku). W pozyskanym surowcu zidentyfikowano cztery kwasy fenolowe (chlorogenowy, ferulowy, kawowy i rozmarynowy) oraz pięć związków flawonoidowych (orientynę, 7-glukozyd luteoliny, 7-glukozyd apigeniny, 3-glukozyd apigeniny, apigeninę). W tych grupach chemicznych dominowały kwas kawowy i apigenina. Najwyższą zawartość kwasu kawowego stwierdzono na początku wegetacji roślin, a apigeniny – w fazie pełni kwitnienia i na początku zawiązywania nasion. **Wnioski**: W warunkach uprawy bukwica lekarska wytwarza dużą masę ziela, które możne być użyte jako cenny surowiec w przemyśle zielarskim.

Słowa kluczowe: *przyrost masy, garbniki, kwasy fenolowe, flawonoidy*