

EKSPERIMENTINIAI TYRIMAI

Variability of phenolic compounds in flowers of *Achillea millefolium* wild populations in Lithuania

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Key words: *Achillea millefolium*; phenolic compounds; flavonoids; flowers.

Summary. *Achillea millefolium* L. sensu lato (yarrow) is the best-known species of the genus *Achillea* due to numerous medicinal applications both in folk and conventional medicine. Phenolic compounds such as flavonoids and phenol carbonic acids are present in yarrow and constitute one of the most important groups of pharmacologically active substances. In the present study, yarrow flowers gathered from native populations in different locations of Lithuania were analyzed for phenolic compound composition. High-performance liquid chromatography (HPLC) was used for chemical analyses. Eight phenolic compounds – chlorogenic acid and flavonoids, namely vicenin-2, luteolin-3',7-di-O-glucoside, luteolin-7-O-glucoside, rutin, apigenin-7-O-glucoside, luteolin, and apigenin – were identified in the extracts from yarrow flowers. Considerable variation in accumulation of phenolic compounds among the flowers from different locations was observed. The samples were divided into two main groups based on chemical composition: the first group was characterized by lower than the mean total amount of the identified phenolics; the second was formed from samples accumulating higher concentrations of investigated secondary metabolites. The total amount of the identified phenolics in yarrow flowers from different populations varied from 13.290 to 27.947 mg/g.

Introduction

There are about 100 species of the genus *Achillea* L. (Asteraceae) widely spread over the Northern hemisphere (1). The plants of this genus have been used as healing agents due to their numerous medicinal properties by many cultures for hundreds of years (1, 2). The raw material of yarrow constitutes one of the oldest and most important medicines widely used both in folk and conventional Lithuanian medicine (3). However, additional scientific research is still required to clarify its phytochemicals and their potential healing qualities, as well as to expand the knowledge on its use and safety (4). The phenolic compounds such as flavonoids and phenol carbonic acids are considered as one of the most important groups of pharmacologically active compounds present in *Achillea* species. Spasmolytic activity on the isolated rabbit intestine (5) as well as on isolated terminal guinea-pig ilea (6) has been documented for a flavonoid fraction. It has been reported that antiphlogistic activity of *Achillea millefolium* L. sensu lato (s.l.) (yarrow) might be

mediated by the flavonoids (2). However, recent investigations concerning the inhibition of the serine protease human neutrophil elastase (HNE) and the matrix metalloproteinases MMP-2 and -9 by dicaffeoylquinic acids and the flavonoid fractions demonstrated that these fractions, at least partially, might additionally contribute to the antiphlogistic activity of yarrow (7). The flavones, isolated from the aerial parts of *Achillea atrata* L. subsp. *multifida*, were found to possess *in vitro* antimicrobial activity (8). Furthermore, a study of the methanol extract from *Achillea ageratum* L. in several experimental models showed the analgesic and antipyretic properties, whereas phytochemical investigation of the extract revealed the presence of flavonic compounds, thus suggesting that flavonoids might induce the respective effects (9). Recent attention has been directed towards the dicaffeoylquinic acids present in yarrow, since these phenolic acids were shown to cause the choleretic effects (10). Two flavonoids casticin (11) and centaureidin (12), respectively, derived from *A. millefolium* and *A. clavennae* L., were

observed to exert cytotoxic activity. The results from *in vitro* assay, based on recombinant MCF-7 cells, indicated that flavonoids from *A. millefolium* exhibit estrogenic activity (13). Moreover, recent experiments have confirmed that phenolic substances are completely extracted into the common application forms of yarrow; thereby, these compounds may exert their polyvalent pharmacological effects (6, 7). Thus, in order to assess comprehensively the quality of herbal drug preparations, it is of crucial importance to determine the composition of the flavonoid and phenol carbonic acid complex in yarrow herb material.

The raw material of yarrow (*Millefolii herba*) is described in various pharmacopoeias (2, 5, 14) and consists of upper part of whole plant. Moreover, in some pharmacopoeias only yarrow flowers (*Millefolii flos*) are officially regarded as the raw material (2). The present study was conducted to determine the qualitative and quantitative composition of phenolic compounds in yarrow flowers, as well as their distribution and variability among plants from different populations.

Materials and methods

Plant material

The plant material represents aerial parts of *A. millefolium* s.l. plants randomly gathered from 22 wild populations located in different parts of Lithuania in 2006 (Table 1). All samples were collected at full flowering stage. The capitula, which further were referred as flowers, were separated from corymbs before drying. The material was dried at room temperature (20–25°C), in the ventilated lodge, avoiding direct sunlight for two weeks. Dry material was packed into multi-layer paper bags and stored in the dark room at an ambient temperature.

Loss on drying was carried out for powdered sample by drying it in an oven at 100–105°C as described in the European Pharmacopoeia (14). All obtained results were recalculated for absolutely dried material.

Sample extraction

To obtain a homogeneous plant matrix for the solvent extraction, air-dried plant material was milled

Table 1. Characteristics of *Achillea millefolium* wild populations in Lithuania

No.	Site	District	Location	Habitat
1	Vytėnai	Kaunas	Central Lithuania	Roadside
2	Bitvanas	Kaunas	Central Lithuania	Meadow
3	Vandžiogala	Kaunas	Central Lithuania	Abandoned field
4	Jonava	Jonava	Central Lithuania	Roadside
5	Užumiškiai	Kaunas	Central Lithuania	Meadow
6	Labūnava	Kėdainiai	Central Lithuania	Abandoned field
7	Liogailiškiai	Kėdainiai	Central Lithuania	Abandoned field
8	Tiskūnai	Kėdainiai	Central Lithuania	Meadow
9	Gudiniškės	Alytus	Southern Lithuania	Abandoned field
10	Seimeniškiai	Alytus	Southern Lithuania	Meadow
11	Obelija	Alytus	Southern Lithuania	Meadow
12	Milastonys	Alytus	Southern Lithuania	Meadow
13	Taurapilis mound	Utena	North-eastern Lithuania	Meadow
14	Šakarva	Ignalina	North-eastern Lithuania	Meadow
15	Tumelinos forest	Ignalina	North-eastern Lithuania	Forest edge
16	Zarasai	Zarasai	North-eastern Lithuania	Abandoned field
17	Stelmužė	Zarasai	North-eastern Lithuania	Forest edge
18	Tauragė	Tauragė	South-western Lithuania	Abandoned field
19	Gargždai	Klaipėda	Western Lithuania	Abandoned field
20	Laugaliai	Klaipėda	Western Lithuania	Roadside
21	Grišlaukė	Kretinga	Western Lithuania	Meadow
22	Biržuvėnai	Telšiai	North-western Lithuania	Forest edge

The numbers, showing the location of population, are used throughout this paper.

at room temperature and sieved using the sieve with 355- μ m openings. An ultrasonic bath BioSonic UC100 (Coltène/Whaledent, Mahwah, NJ, USA) was used for sonication extractions. Approximately 0.25 g (accurate weight, weighed with 0.0001 g precision) of powdered plant material were sonicated with 25 mL of the 70% (v/v) aqueous ethanol for 30 min. After centrifugation at 12 000 rpm for 10 min, the supernatant was adjusted to 25 mL in a measuring flask. To perform the high-performance liquid chromatography (HPLC) analysis of all obtained extracts, they were filtered through the membrane filter with a pore size of 0.22 μ m (Carl Roth GmbH, Karlsruhe, Germany).

Reagents

Authentic standards of reference compounds – apigenin, apigenin-7-*O*-glucoside, luteolin, luteolin-7-*O*-glucoside, luteolin-3',7-di-*O*-glucoside, rutin, and chlorogenic acid – were purchased from Fluka (Buchs, Switzerland), Roth (Karlsruhe, Germany), and ChromaDex (Santa Ana, CA, USA). The analyte standards were of HPLC grade. Vicenin-2 was previously isolated in this laboratory. Trifluoroacetic acid (TFA) was obtained from Sigma-Aldrich (Seelze, Germany). The solvent acetonitrile, labeled as HPLC gradient grade, was supplied by Sigma-Aldrich (Buchs, Switzerland). Ethanol of 96% (v/v) was provided by Stumbras AB (Kaunas, Lithuania). Ultra pure water generated by a Simplicity™ Water Purification System (Millipore, Bedford, USA) was used throughout the HPLC experiment and for preparation of extraction solvents.

HPLC analysis

HPLC analysis of chlorogenic acid and flavonoids was performed using a liquid chromatographic Waters 2690 alliance HPLC system (Waters Corporation, Milford, MA, USA) equipped with Waters 2487 dual λ absorbance detector (UV/Vis) and Waters 996 photodiode array (PDA) detector. Separations were carried out using a 5- μ m Ascentis™ RP-Amide analytical column (150 $\times$ 4.6 mm) guarded with a guard column 5- μ m Supelguard™ Ascentis™ RP-Amide (20 $\times$ 4.00 mm) (SUPELCO, Bellefonte, PA, USA). The chromatographic separation was carried out using 0.1% trifluoroacetic acid solution in water as solvent A and 0.1% trifluoroacetic acid solution in acetonitrile as solvent B with the following gradient elution program: 0–25.5 min, 90–76% A, 10–24% B; 25.5–27 min, 76–72% A, 24–28% B; 27–45 min, 72–45% A, 28–55% B; 45–48 min, 45% A, 55% B; 48–52 min, 45–90% A, 55–10% B; 52–55 min, 90% A, 10%

B. The elution was monitored at 360 nm with a UV/Vis detector. This liquid chromatography method was used at a flow rate of 1.5 mL/min at an ambient temperature. The sample injection volume was 10 μ L. The confirmation of the identity of chromatographic peaks was achieved by comparison of retention times of samples with those of standard compounds and spectral characteristics of the eluting peaks, scanned with a diode-array detector (λ =200–400 nm), with those of authentic standards.

The quantification of the bioactive compounds was achieved by the external standard approach using a calibration curve established with five dilutions of each standard, and it gave correlation coefficients between 0.9940 and 0.9997. Standard solutions were prepared at the approximate concentration of constituent levels in the extracts of the samples. Each sample was analyzed twice, and the mean value was used for calculation.

Statistical analysis of data

The results were statistically analyzed using the program SPSS version 16 for Windows (SPSS Chicago, IL, USA). The hierarchical cluster analysis was applied to determine the relationships among plants from different populations according to their content of bioactive substances. Agglomerative approach, which uses Euclidean distance as a unit for measuring and furthest neighbor clustering algorithm, was employed. Graphical presentation of results was performed using the software SigmaPlot for Windows, version 10.0.

Results and discussion

Evidence of the beneficial therapeutic effects of the medicinal herbs and herbal medicines can be seen in their continued use. However, recent progress in modern therapeutics has stimulated the increasing use of natural products throughout the world, thus confirming that a scientific approach for the investigation of the pharmacologically active principles of phytopharmaceuticals represents a crucial importance in rational phytotherapy (15, 16). There is also considerable concern regarding the increased requirements on quality, safety, and efficiency of medications from botanical sources and plant raw material (17).

A. millefolium s.l. raw material is used in the preparation of a wide range of application forms, which include tinctures, liquid extracts, industrial tea mixtures as well as some multicomponent phytomedicines (2, 5, 18). Defined by heterogeneous nature of botanical matrix of yarrow raw material, the phytochemical

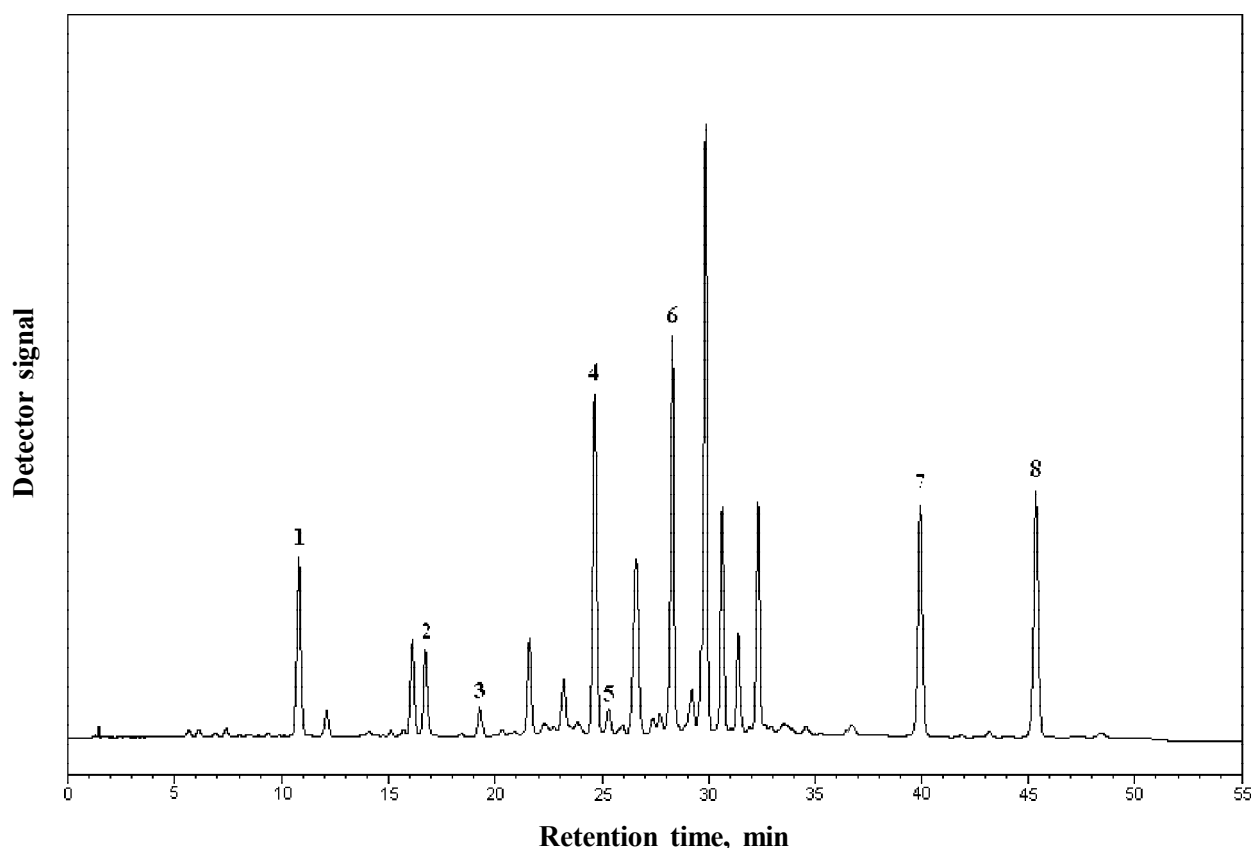


Fig. 1. Characteristic HPLC chromatogram of a 70% (v/v) ethanolic extract from *Achillea millefolium* flowers

Peaks identified: 1 – chlorogenic acid, 2 – vicenin-2, 3 – luteolin-3',7-di-*O*-glucoside, 4 – luteolin-7-*O*-glucoside, 5 – rutin, 6 – apigenin-7-*O*-glucoside, 7 – luteolin, 8 – apigenin.

composition of the crude drug is complex and comprises of phytochemical profiles of flowers, leaves, and stems.

Eight phenolic compounds – chlorogenic acid and seven flavonoids, namely vicenin-2, luteolin-3',7-di-*O*-glucoside, luteolin-7-*O*-glucoside, rutin, apigenin-7-*O*-glucoside, luteolin, and apigenin – were identified

in the extracts of yarrow flower. Typical HPLC chromatogram of the extract from flowers is shown in Fig. 1. As it can be seen, the peaks of apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside, luteolin, and apigenin were dominant among the identified analytes in the chromatographic profile of the extract.

The results of the phytochemical analysis indicate

Table 2. Phenolic compounds and their amounts in flowers of *Achillea millefolium* from different wild populations in Lithuania (n=22)

Compound	Quantity, mg/g			Std. error
	Min	Max	Mean	
Chlorogenic acid	2.837	12.679	8.030	0.625
Vicenin-2	0.739	3.047	1.593	0.123
Luteolin-3',7-di- <i>O</i> -glucoside	0.056	0.338	0.196	0.014
Luteolin-7- <i>O</i> -glucoside	2.107	5.885	3.871	0.244
Rutin	0.185	0.467	0.293	0.017
Apigenin-7- <i>O</i> -glucoside	3.334	7.546	5.284	0.226
Luteolin	0.561	1.253	0.802	0.036
Apigenin	0.448	2.562	1.210	0.146

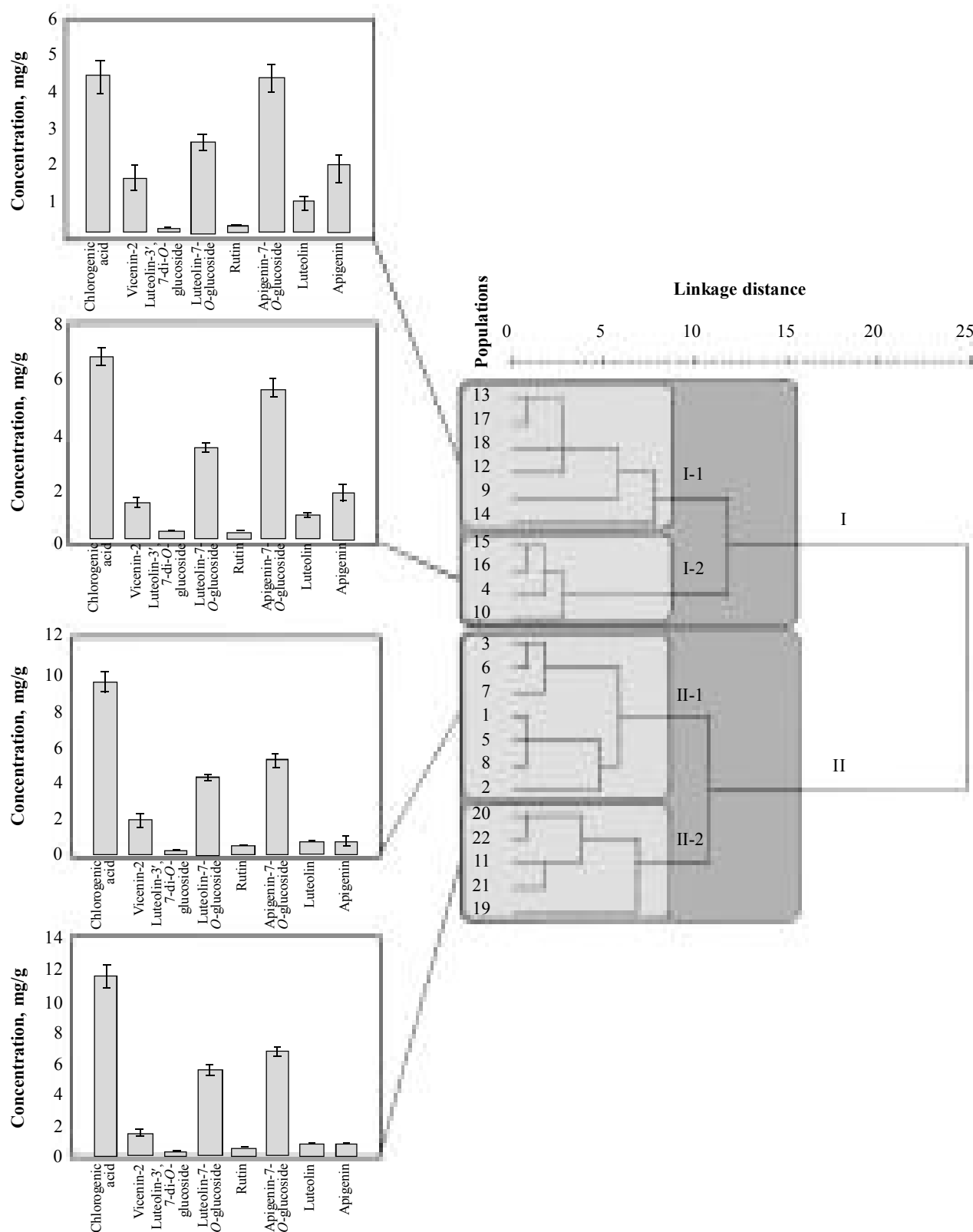


Fig. 2. The dendrogram of hierarchical cluster analysis of *Achillea millefolium* populations according to the content of phenolic compounds and profiles of the identified phenolics, expressed as mean values in mg/g

The error bars represent the standard error. Refer to Table 1 for details of sampling locations.

that total content of the identified phenolics ranged from 13.290 to 27.947 mg/g among wild populations of yarrow. The accumulation trends of chlorogenic acid and flavonoids highly varied and showed considerable quantitative heterogeneity among flower extracts of *A. millefolium* (Table 2). As it can be seen, chlorogenic acid (2.837–12.679 mg/g) predominated in the mixture of identified secondary metabolites. Regarding the composition of the flavonoid complex, the patterns of distribution in the analyzed samples of yarrow flowers are characterized by the dominance of apigenin-7-*O*-glucoside (3.334–7.546 mg/g) and luteolin-7-*O*-glucoside (2.107–5.885 mg/g), as well as their corresponding free aglycones – apigenin (0.448–2.562 mg/g) and luteolin (0.561–1.253 mg/g). Rather high amounts of apigenin and luteolin are of high importance in the pharmacological profile of yarrow, particularly in spasmolytic and estrogenic effects (6, 13). Concerning the pattern of flavonoid distribution, the results in present study are in good agreement with previously published data on *A. millefolium* (19, 20). According to the literature data, no study has been carried out on the intraspecies variation of individual phenolics in flowers of *A. millefolium*.

The results from phytochemical assay have revealed that there is a variation in accumulation of secondary metabolites among *A. millefolium* populations from different locations. Consequently, hierarchical cluster analysis was applied to determine the relationship among populations based on phenolic compounds accumulation. The dendrogram obtained by hierarchical cluster analysis on the matrix of 22 populations is shown in Fig. 2. All populations ranged into two high level clusters. The first cluster (I) bringing together 10 populations (No. 13, 17, 18, 12, 9, 14, 15, 16, 4, and 10) is characterized by lower than the mean total content of the identified phenolics (13.290–20.846 mg/g). Cluster I united two lower level clusters (I-1 and I-2). Cluster I-1 formed of No. 13, 17, 18, 12, 9, and 14 populations is characterized by the lowest contents of chlorogenic acid, luteolin-7-*O*-glucoside, rutin, apigenin-7-*O*-glucoside and the highest levels of luteolin and apigenin. Cluster I-2 consists of four populations No. 15, 16, 4, and 10, containing a lower than the mean amount of chlorogenic acid, vicenin-2, luteolin-7-*O*-glucoside, rutin and higher than the mean amount of luteolin and apigenin. The second cluster (II), formed by populations No. 3, 6, 7, 1, 5, 8, 2, 20, 22, 11, 21, and 19, was characterized by higher (with the exception of populations No. 2 and 6) mean total

amount (22.083–27.947 mg/g) of the identified phenolics. The second higher level cluster united two lower level clusters (II-1 and II-2). The seven populations (No. 3, 6, 7, 1, 5, 8, and 2) of cluster II-1 accumulated higher than the mean amounts of chlorogenic acid, vicenin-2, luteolin-3',7-di-*O*-glucoside, luteolin-7-*O*-glucoside, rutin, and the lowest levels of luteolin and apigenin. Cluster II-2 consisted of populations No. 20, 22, 11, 21, and 19 that produced the highest amounts of chlorogenic acid, luteolin-7-*O*-glucoside, rutin, apigenin-7-*O*-glucoside and lower than mean amounts of vicenin-2, luteolin-3',7-di-*O*-glucoside, and apigenin.

Considering the phytochemical variability in the amount of the evaluated constituents among populations, the observed diversity could have a genetic basis, as well as it may be attributed to the environmental differences, since the examined populations of *A. millefolium* were located in different regions of Lithuania and growing habitats differed from each other by multifactorial nature of distinctive locations (microclimate, soil, UV radiation, etc.). The raw material of yarrow is usually sourced from wild populations; thereby, chemical heterogeneity of this plant may cause quality irregularities of the crude drug. This should be taken into consideration in order to achieve consistent pharmaceutical quality drug.

Conclusions

The phytochemical investigations on *A. millefolium* flowers from wild populations have revealed a considerable variation in accordance with phenolic compounds that seems likely to arise from the influence of different growing habitats. The total content of the identified phenolics in wild populations of yarrow varied from 13.290 to 27.947 mg/g. Regarding the composition of the flavonoid complex, the patterns of distribution in the samples of yarrow flowers are characterized by the predominant formation of flavone-*O*-glycosides, namely apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside, and their corresponding free aglycones apigenin and luteolin. In view of an increasing demand for consistent raw material, careful monitoring of processing methodology of wild-harvested plant material has decisive impact to guarantee the homogeneity of quality on raw material.

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Lietuvoje natūraliose populiacijose surinktų paprastosios kraujažolės žiedų fenolinių junginių sudėties įvairovės tyrimas

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Raktažodžiai: *Achillea millefolium*, fenoliniai junginiai, flavonoidai, žiedai.

Santrauka. Paprastosios kraujažolės (*Achillea millefolium* L. *sensu lato*) augalinė žaliava bei jos preparatai plačiai vartojami medicinoje. Kraujažolės žaliavoje randami fenoliniai junginiai (flavonoidai ir fenilkarboninės rūgštys) yra vieni svarbiausių biologiškai aktyvių junginių, lemiančių įvairialypį jos farmakologinį poveikį. Efektyviosios skysčių chromatografijos metodu įvertintas fenolinių junginių kokybinės ir kiekinės sudėties rodiklių įvairavimas kraujažolės žieduose, surinktuose natūraliose populiacijose, esančiose skirtinguose Lietuvos regionuose, pagal sukauptus chlorogeno rūgšties bei flavonoidų: vicenino-2, liuteolin-3',7-di-O-gliukozido, liuteolin-7-O-gliukozido, rutino, apigenin-7-O-gliukozido, liuteolino ir apigenino kiekius. Tyrimų duomenimis, žiedams, surinktiems skirtingose populiacijose, būdingas ryškus fenolinių junginių kiekinės sudėties įvairavimas. Atlikus klasterinę analizę, tirti pavyzdžiai pagal jų fitocheminę sudėtį pasiskirstė į dvi pagrindines grupes. Pirmosios grupės fitocheminei sudėčiai būdingas mažesnis už vidutinį suminis identifikuotų fenolinių junginių kiekis, o antrąją grupę sudaro kraujažolės žiedų ėminiai, sukaupiantys didesnius tirtų antrinių metabolitų kiekius. Suminis identifikuotų veikliųjų junginių kiekis skirtingų populiacijų kraujažolės žieduose kito nuo 13,290 iki 27,947 mg/g.

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