KAUNAS UNIVERSITY
OF MEDICINE

RAIMONDAS BENETIS

EVALUATION OF PHENOLIC COMPOUNDS COMPOSITION AND BIOLOGICAL ACTIVITY OF PLANTS OF THE GENUS YARROW (*ACHILLEA* L.)

SUMMARY OF DOCTORAL DISSERTATION
BIOMEDICAL SCIENCES, PHARMACY (09 B)

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The doctoral dissertation was prepared at Kaunas University of Medicine during the years of 2004–2008.

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KAUNO MEDICINOS UNIVERSITETAS

Raimondas Benetis

KRAUJAŽOLĖS (*ACHILLEA* L.) GENTIES AUGALŲ FENOLINIŲ JUNGINIŲ SUDĖTIES IR BIOLOGINIO POVEIKIO ĮVERTINIMAS

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INTRODUCTION

Evidence of beneficial therapeutic effects of 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. The world market for such medicines was estimated to be US $ 60 billion, with annual growth rates of between 5 % and 15 % [Kartal, 2007]. Thereby confirming that a scientific approach for the investigation of the pharmacologically active principles of phytopharmaceuticals, as well as the determination of their mode of action represents a crucial importance in an evidence-based rational phytotherapy.

The raw material of yarrow (*Achillea millefolium* L.) constitutes one of the oldest and most important medicines widely used both in folk and conventional Lithuanian medicine [Radušienė, Gudaitytė, 2005]. The genus yarrow (*Achillea* L., *Asteraceae* Dumort.) has very broad ecological amplitude, thus the plants of this genus are widely distributed throughout the world. Three native species of the genus occur in Lithuania: *A. millefolium*, *A. cartilaginea* Ledeb. ex Rchb. and *A. ptarmica* L. Two latter indigenous species – *A. cartilaginea* and *A. ptarmica* – occur rarely and usually only in their typical habitats, whereas *A. millefolium* is widespread and frequent in the whole Republic [Galinis, 1980].

The main parameter in the quality assessment of the herbal raw material is the qualitative and quantitative composition of biologically active compounds in the crude drug. The raw material of yarrow contains a complex mixture of structurally diverse natural compounds, namely terpenes, alkaloids and bases, tannins, coumarins, saponins, sterols, vitamins, amino and fatty acids. However, additional scientific investigations are still required to clarify its phytochemicals and their potential healing qualities. Phenolic compounds such as flavonoids and phenolcarbonic acids are present in yarrow and constitute one of the most important groups of pharmacologically active substances. It is believed that spasmolytic [Barnes et al., 2002; Lemmens-Gruber et al., 2002], anti-inflammatory [Blumenthal et al., 2000; Benedek et al., 2007], antimicrobial [Aljancic et al., 1999], analgesic, antipy-

retic [Garcia et al., 1997], choleretic [Benedek et al., 2006], cytotoxic [Haidara et al., 2006; Trifunovic et al., 2006] and estrogenic [Innocenti et al., 2007] activities of *Achillea* plants are mainly attributed to the flavonoid and phenolcarbonic acid complex. Scientific literature lacks comprehensive information about the qualitative and quantitative composition of phenolic compounds in the raw material of yarrow. Moreover, up to now no phytochemical data have been reported about the morphologic, phenologic and population variability of phenolic compounds in yarrow. Thus, there is a considerable research interest towards the assay of the flavonoid and phenolcarbonic acid complex composition of various *Achillea* species, as well as towards the expanding of knowledge on its variation trends.

The assessment of variation in phytochemical composition of herbal raw materials requires knowledge of accumulation tendencies of biologically active compounds among different plant parts and tissues. The detection of morphologic variation in phenolic compounds' composition would enable a more precise evaluation of using possibilities of different organs of the plant for medical purposes and would allow an investigation of the nature of bioactive substances variability, as well as their role in metabolic processes.

Secondary metabolite composition of *A. millefolium* varied substantially within the developmental stages of the plant. Therefore, investigations are necessary for evaluation of accumulation patterns of phenolic compounds and their variation dynamics through phenological cycle, because it has a decisive impact to guarantee homogeneity of quality on raw material.

The raw material of yarrow is usually sourced from wild populations and only rarely derives from cultivated plants. Therefore, the assessment of *A. millefolium* phenolic compounds' composition and its variability among wild populations is of crucial importance for the evaluation of quality of local raw material of yarrow as well as for its purposive selection and conservation of resources from overexploitation.

In order to comprehensively assess the antioxidant potential of the extracts from the plants of the genus *Achillea* as well as their phenolic compounds it is of high importance to evaluate their free radical

scavenging capacity and their effects on the oxidative phosphorylation in isolated mitochondria. Mitochondria are an important intracellular source of reactive oxygen species (ROS) and, on the other hand, are also a critical target of its damaging effects. Oxidative stress-mediated impairments in mitochondrial function are considered to be implicated in a large variety of diseases and patho-physiological processes, such as cardiovascular, neurodegenerative diseases, ageing, cancer and diabetes. Assessment of antioxidant properties as well as influence on mitochondrial respiration and energy production of extracts from yarrow raw materials rich in flavonoids and phenolcarbonic acids would yield a more precise evaluation of the possibility of modulating mitochondrial function thus counteracting detrimental effects of ROS.

For identification and quality evaluation of herbal medicine it is necessary to use analytical approaches enabling the systemic characterization of compositions of samples with focus on quantitative and qualitative determination of bioactive secondary metabolites. Moreover, to ensure the reliability and repeatability of phytochemical research the developed analytical methods should be validated. In order to optimize the conditions for sample preparation of the botanical matrix it is essential to elucidate the influence of critical factors determinant selective isolation of phenolic compounds: solvent polarity and extraction method.

The determination of the flavonoids and phenolcarbonic acids variation patterns in plants and deepening the knowledge on their biological properties represent crucial issues in comprehensive evaluation of quality parameters of the plant raw materials, as well as in increasing the possibilities of their practical applicability.

The aim of the study was to comprehensively assess qualitative and quantitative composition and variability of flavonoids and phenolcarbonic acids on herbal raw material of spontaneous and introduced species of yarrow (*Achillea* L.), as well as to evaluate the biological activity of these plants.

The objectives of the study:

1. To develop an RP-HPLC method for analysis of the flavonoid and phenolcarbonic acid complex in the different taxa of yarrow;
2. To determine the qualitative and quantitative composition of

phenolic compounds in herb of *A. millefolium*, as well as their distribution and variability among different wild populations;

3. To evaluate the variation on qualitative and quantitative composition of phenolic compounds of *A. millefolium* different plant parts through its phenological cycle;
4. To analyze the composition of flavonoid and phenolcarbonic acid patterns in the accessions of *A. ptarmica*, *A. odorata*, *A. sibirica* and *A. filipendulina*, as well as to determine phenological changes in the phytochemical parameters;
5. To assess the antiradical activity of extracts obtained from herbal raw materials of different *Achillea* species;
6. To investigate the effect of the extract of *A. millefolium* herb on the oxidative phosphorylation in rat heart mitochondria.

Scientific novelty of the work. Evaluation of qualitative and quantitative composition of the flavonoid and phenolcarbonic acid complex on herb raw material of yarrow collected in Lithuania was carried out for the first time. This work for the first time provides results of determination of variability of phenolic compounds' composition on *A. millefolium* wild populations and assessment of phenolic compounds composition, as well as its variation among plant parts and phenological stages. The study provides information about the distribution patterns and variation trends in the composition of flavonoids and phenolcarbonic acids in raw materials of other species of the genus yarrow. The RP-HPLC method for separation of the flavonoid and phenolcarbonic acid complex in the different taxa of the genus *Achillea* was developed using a polymeric amide-bonded phase column. The method proved to be suitable for determination of the qualitative and quantitative phenolic composition in the ethanolic extracts of yarrow. The investigations of contribution of individual constituents to antioxidant activity of yarrow extracts have revealed the main components of the flavonoid and phenolcarbonic acid complex that possessed radical scavenging properties and their antiradical activity.

Collaborating with the researchers from the Laboratory of Biochemistry, Institute for Biomedical Research, the influence of extract of *A. millefolium* herb on rat heart mitochondrial function was tested. These investigations are new, since up to now no experimental data

have been reported about the effects of yarrow extract on mitochondrial energy metabolism.

Significance of the study. The RP-HPLC method was developed which allowed the qualitative and quantitative determination of phenolic compounds in the raw materials of *Achillea* species. It could be useful for the evaluation of pharmaceutical quality of yarrow raw materials by determining the content of phenolic compounds in the drug as additional parameters in the comprehensive quality control of the drug.

The presented research data regarding the biological activity (effect on cellular bioenergetics and their ability to scavenge free radicals) of extracts from the raw materials of *A. millefolium*, as well as phenolic compounds from them, are important for the development of new preparations with antiphlogistic, antimicrobial, antioxidant and antidiabetic activities.

Having completed the phytochemical analysis of other native and introduced yarrow species in Lithuania, the quantitative and qualitative parameters of raw materials of different yarrow taxa have been assessed, in order to evaluate the possibilities of using them for medicinal purposes. The obtained data from assessment of natural populations of *A. millefolium* according to the qualitative and quantitative composition of bioactive substances are important for the evaluation and conservation of indigenous plant genetic resources, as well as for more comprehensive understanding of dynamics of the evolutionary processes in this genus.

The data of variation in accumulation of phenolic compounds through phenological cycle may be useful in understanding of an *in situ* biosynthesis and transportation peculiarities of secondary metabolites during plant growth and development. The evaluation of other *Achillea* species revealed differences in quantitative and qualitative composition of phenolic compounds, thus confirming that distribution pattern of flavonoids is of chemotaxonomic relevance.

Antioxidant activity studies of the extracts from the raw materials of *Achillea* species revealed that their ability to scavenge free radicals depends on qualitative and quantitative composition of the flavonoid and phenolcarbonic acid complex. Presented results will be used for

developing preparations, dietary supplements with antioxidant properties and integrating them into medical practices.

To conclude, the presented results provide a comprehensive survey of the phytochemical distribution of phenolic compounds in spontaneous and introduced species of *Achillea* genus as well as further insights into their biological activity.

Approbation of the research results. The obtained research data were published in 3 scientific papers in reviewed scientific journals. The investigation results were presented in the following conferences: Usage of Baltic Sea Region States' Genofond Plants for Breeding and Interchange (Kaunas, October 4-6, 2006), Human and Nature Safety (Akademija, May 16-19, 2007), Youth Seeks Progress (Akademija, April 13-14, 2007), 4th Nordic Separation Science Society (NoSSS) International Conference (Kaunas, August 26-29, 2007).

Structure of the dissertation. The doctoral thesis (in Lithuanian) consists of the following parts: introduction, review of literature, research material and methods, results and discussion, conclusions, list of references consisting of 335 sources, list of publications related to the topic of thesis and appendices. The volume of dissertation is 130 pages; the results are presented in 22 tables and 34 figures.

RESEARCH OBJECTS, MATERIALS AND METHODS

The objects of the research were spontaneous (*A. millefolium* s.l., *A. cartilaginea* and *A. ptarmica*) and introduced (*A. odorata*, *A. sibirica* and *A. filipendulina*) yarrow species from wild populations and field collection of Medicinal and Aromatic Plants at the Institute of Botany (Vilnius). The plant material was randomly gathered from 114 wild populations located in different parts of Lithuania in 2004–2006. The raw material of *A. ptarmica*, *A. odorata*, *A. sibirica* and *A. filipendulina* was gathered from field collection, whereas the accessions of *A. cartilaginea* were collected from 4 wild populations in Great Loops of Nemunas Regional Park in 2007.

Chemical analysis of the soil was done at the Sector of Chemical Analysis of the Institute of Botany.

Materials. The reagents pure for analysis, HPLC gradient grade solvents and analyte standards of HPLC-grade were obtained from: Sigma-Aldrich Chemie (Switzerland), Carl Roth Gmbh (Germany), Fluka Chemie (Switzerland), ChromaDex (USA), Scharlau Chemie (Spain), Riedel-de Haën (Germany) and Acros organics (USA).

Instrumentation. HPLC analysis was performed using a liquid chromatographic Waters 2690 Alliance HPLC system (Waters Corporation, USA) equipped with Waters 2487 Dual λ Absorbance Detector (UV/Vis) and a Waters 996 Photodiode Array (PDA) Detector. Separations were carried out using a 5μm Ascentis™ RP-Amide analytical column (150×4.6 mm) guarded with a guard column 5μm Supelguard™ Ascentis™ RP-Amide (20×4.00 mm) (SUPELCO, USA). HPLC-DAD-ESI/MS experiments were performed with the following setup: Waters 1525 binary HPLC eluent pump (Waters Chromatography, USA), Waters 996 Photodiode Array Detector and Waters Micromass ZQ-2000 mass detector. Separations were carried out using a Ascentis™ RP-Amide column. An on-line HPLC-DPPH system was used for a rapid screening and identification of radical scavenging compounds. The binary pump system Beckman solvent module 126 (USA) was used for the elution gradient formation. Rheodyne injector with 20 μl injection loop was used for injection of samples. Analytes were separated on the Ascentis™ RP-Amide column. The eluting compounds were identified with UV detector Beckman System Gold 166 at 360 nm. From the detector the mobile phase with analytes is delivered by mixing tee to the reaction coil, where DPPH reagent solution is supplied. HPLC pump Gilson pump 305 (USA) was used to supply DPPH solution. The reaction coil was made of PEEK tubing of the following sizes: 15 m × 0.25 mm i.d. Detection of DPPH quenching was carried out at 520 nm. A Gilson UV/VIS detector 118 connected to a PC was used to monitor DPPH conversion.

Mitochondria were prepared by the method of differential centrifugation using a Centrifuge 5810R (Germany). For determination of mitochondrial protein concentration Hitachi 557 (Japan) spectrophotometer was used. Mitochondrial oxygen uptake rates were recorded

at 37 ºC by means of the Clark-type electrode system (Denmark) and polarographic system BioMed.

HPLC analysis. 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 LC method was used at a flow rate of 1.5 mL/min at an ambient temperature. The sample injection volume was 10 µL. 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 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.

HPLC-DAD-ESI/MS analysis. The stationary phase and the elution gradient were the same as those in the HPLC analysis. Mass spectra of phenolic compounds were scanned with ion trap MS after electrospray ionization (ESI) in negative and positive mode. ESI conditions for ionization were as follows: capillary voltage 3 kV, sample cone voltage +/- 25 V, extractor voltage 3 V. In both modes, the source temperature was maintained at 120 °C and the mass spectrometer was scanned from 100 to 800 *m/z*.

Investigation of radical scavenging activity of *Achillea* extracts by the on-line HPLC-DPPH method. The stationary phase and the elution gradient were the same as those in the HPLC analysis. The DPPH reagent was prepared in acetonitrile at the beginning of each day of analysis at a concentration of 0.02 mg/mL and kept protected from light. This solution was mixed with citrate buffer at a ratio of 1:1. The buffer, pH 7.6, contained 0.05 M citric acid and 0.1 M sodium citrate solutions. The flow of DPPH reagent was 1.5 mL/min. Reference antioxidant Trolox was used for quantitative assessment of antioxidant compounds, which possessed radical scavenging proper-

ties, activity in plant extracts. Direct dependence between the amount of Trolox (μg) and the depth of the peak for bleaching of DPPH (negative peak area) was confirmed by high correlation factor (R^2=0,998). Trolox content equivalent (E_T) was used for quantitative evaluation of activity of antiradical compounds present in extracts. Antiradical activity equivalent (A_T) was used to evaluate activity of radical scavengers in plant raw materials.

Preparation of rat heart mitochondria. Mitochondria were prepared by the method of differential centrifugation. All procedures were carried out in a cold room at 0–4 °C temperature. Hearts of male Wistar rats were excised and rinsed in ice-cold 0.9 % KCl solution. Subsequently the tissue was cut into small pieces and homogenized in a buffer (10 mL/g tissue) containing 180 mM KCl, 10mM Tris/HCl, and 1 mM EGTA (pH 7.7) for 45 seconds. The homogenate was centrifuged at 750×g for 5 min and the supernatant obtained was centrifuged at 6800×g for 10 min. The mitochondrial pellet was resuspended in buffer containing 180 mM KCl, 20 mM Tris/HCl, and 3 mM EGTA (pH 7.35) to approximately 50 mg/mL protein, and stored on ice. The mitochondrial protein concentration was determined by the biuret method using bovine serum albumin as a standard [Gornal et al., 1949].

Measurements of mitochondrial respiratory rates. Oxygen uptake rates were recorded at 37°C by means of the Clark-type electrode system in a solution containing 150 mM KCl, 10 mM Tris/HCl, 5 mM KH_2PO_4, 1 mM $MgCl_2$, pH 7.2, with 5 mM pyruvate + 5 mM malate and 15 mM succinate (+2 mM amytal) as substrates. The solubility of oxygen was estimated to be 420 nmol O/mL. The final mitochondrial protein concentration in all experiments was 0.5 mg/ml. The basal (State 2) respiration rate (V_2) was measured with the respiratory substrates, then after the addition of 1 mM ADP State 3 respiration rate (V_3) was recorded. For investigation of mitochondrial function different amounts (2.5, 5, 10 and 15 μL) of yarrow extract (YE) (3.3 mg of dry extract of *A. millefolium* s.l. herb was dissolved in 1 mL 40 % ethanol) were added into 1.5 mL of incubation medium. After addition of 2.5 μL of this extract, final concentrations of phenolic compounds in incubation medium were: 100.9 ng/mL of chlorogenic acid,

9.3 ng/mL of vicenin-2, 7.9 ng/mL of luteolin-3',7-di-O-glucoside, 3.5 ng/mL of luteolin-7-O-glucoside, 16.9 ng/mL rutin, 10.4 ng/mL apigenin-7-O-glucoside, 53.2 ng/mL of luteolin and 74.2 ng/mL of apigenin. Amounts of solvent (ethanol) used in the experiments didn't have any effect on mitochondrial function. Mitochondrial respiration rates were expressed as nmol O/min/mg protein.

Statistical analysis of data. The results were statistically analyzed using the programmes SPSS version 16 for Windows (SPSS, USA) and SigmaStat version 3.00 for Windows (SPSS, USA). Graphical presentation of results was performed using the software SigmaPlot for Windows, version 10.0.

RESULTS AND DISCUSSION

Assessment of phytochemical composition and its quantitative and qualitative variation of *Achillea* species

Development of an RP-HPLC method for the analysis of phenolic compounds in *Achillea millefolium* L.

Extraction method optimization. In order to optimize the conditions for the whole extraction procedure two different solvent extraction methods (maceration and ultrasonic agitation) were assayed. The preliminary maceration studies were performed to determine the influence of extracting solvents on the recovery of phenolics by using aqueous ethanol as extractant. The concentration of ethanol in extractant was varied in the range from 40 to 96 % (v/v). In order to determine the influence of the extractant polarity on the recovery of phenolics optimized HPLC method was used. The results belonging to the assays which aim was to select the optimal concentration of ethanol in the aqueous ethanol as extractant are shown in Fig. 1. Although some individual compounds were better extracted with concentrations other than 70%, this concentration of aqueous ethanol was selected for further analyses as it afforded the highest total amount of identified phenolics.

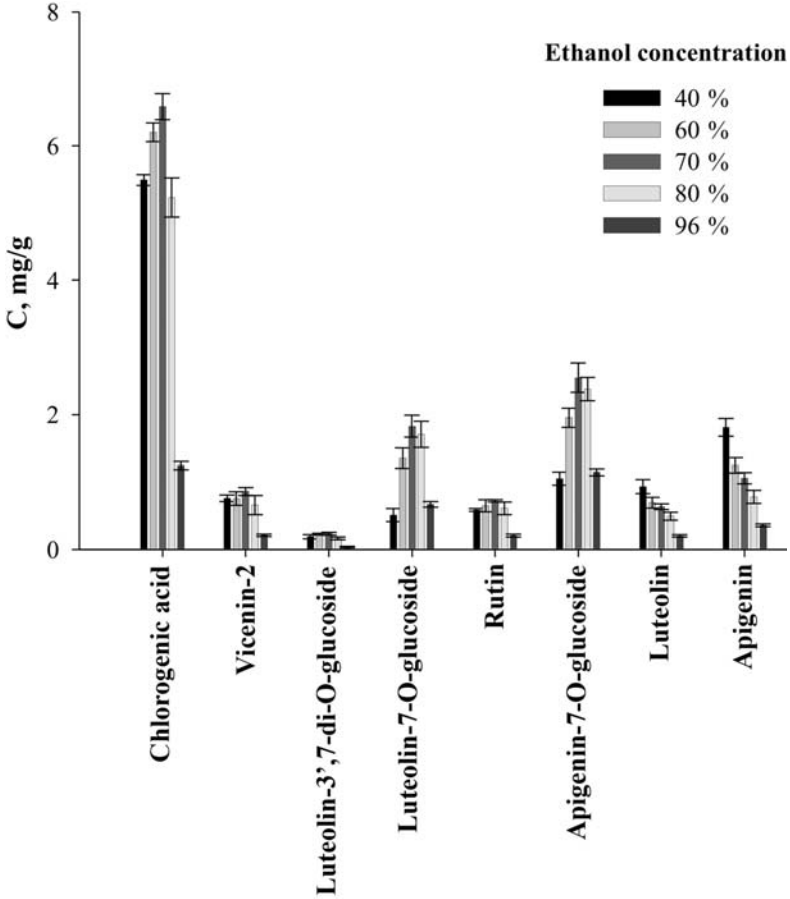

Fig. 1. *Mean values (mg/g) and error bars of the identified phenolics in aqueous ethanolic extracts of Achillea millefolium herb; (n=3).*

A comparison of two extraction techniques was conducted for several major constituents in yarrow. On the basis of the previous results the extractions of the same plant sample material were carried out by maceration and sonication at different time intervals (5, 10, 20, 30, 40, 50 and 60 min) using 70 % aqueous ethanol as an extractant. The comparison of the results from these extraction approaches permits the relative assessment of extraction efficiency. Preliminary studies indicated that even a 5 min period of sonication afforded higher total amount of identified phenolics (15.364 mg/g) than the extraction

yields with maceration (14.464 mg/g). Furthermore, extraction efficiency was observed to increase with time for sonication extractions (Fig. 2). The profile of the extracts obtained with different periods of sonication was the same in all cases. A 30 min period of sonication was selected for further analyses as it afforded the highest total amount of identified phenolics (19.726 mg/g). Whereas, longer sonication periods decreased the extraction yield.

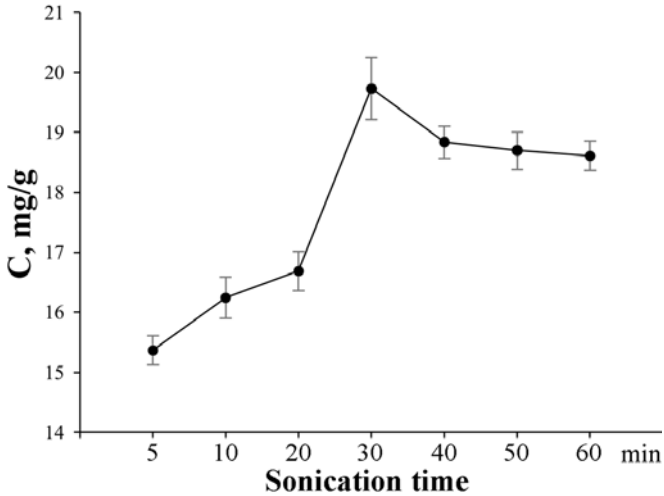

Fig. 2. *Mean values (mg/g) and error bars of total content of the identified phenolics in Achillea millefolium herb extracts obtained with different periods of sonication; (n=3).*

Chromatographic separation optimization. Broad characterization of plant matrix bioactive constituents was achieved using a gradient elution method performed with binary solvent system. Preliminary experiments were performed to obtain the best peak resolution and separation for a mixture of standards. Because of the complex botanical matrix containing analytes of various polarities stronger organic solvent acetonitrile was chosen. Moreover, in order to suppress the ionization of phenolic hydroxyl groups what results in higher chromatographic retention of analytes trifluoroacetic acid (TFA) was used. Two chromatographic columns, such as XTerra® RP_{18} (150×3.9 mm, 3.5 μm) Waters (Milford, USA) and Ascentis™ RP-Amide (150×4.6 mm, 5 μm)

SUPELCO (Bellefonte, USA), were screened with 0.1 % TFA solution in water (solvent A) and 0.1 % TFA solution in acetonitrile (solvent B) as mobile phase. The best selectivity and resolution was observed on the Ascentis RP-Amide column. The better selectivity of the latter column might be due to increased interaction of analytes with phenolic groups with polymeric amide-bonded phase [Gritti, Guiochon, 2006]. Therefore, this column was selected. Furthermore, in order to obtain sufficient resolution of real extract which is more difficult to separate comparing to model mixtures due to the effects of a complex matrix of the real samples the gradient elution method was additionally optimized. The chromatographic separation of 70 % (v/v) ethanolic extract of yarrow herb obtained using optimal HPLC conditions is illustrated in Fig.3. Optimization of gradient elution allowed an efficient separation of main phenolic compounds, such as phenolcarbonic acids (mono- and dicaffeoylquinic acids, DCQAs), flavonoid aglycones and their glycosides in extracts of yarrow in a single run.

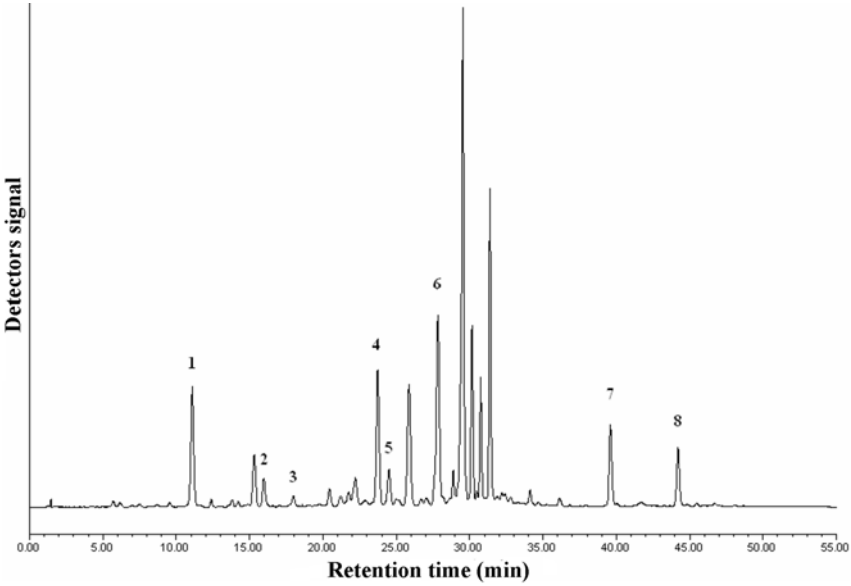

Fig. 3. *HPLC chromatographic separation of 70 % (v/v) ethanolic extract of Achillea millefolium herb. Peaks identified: 1-chlorogenic acid, 2-vicenin-2, 3-luteolin-3',7-di-O-glucoside, 4-luteolin-7-O-glucoside, 5-rutin, 6-apigenin-7-O-glucoside, 7-luteolin, 8-apigenin.*

Moreover, optimization of the chromatographic method enabled an efficient separation of luteolin-7-O-glucoside and rutin in real extracts with a resolution higher than 2.0. Linearity, limit of detection (LOD) and limit of quantification (LOQ) were evaluated for quantitative purposes (Table 1). Limits of detection and quantification were determined by calculation of the signal-to-noise ratio. LOD and LOQ values ranged from 0.04 to 0.46 µg/mL, and from 0.15 to 1.52 µg/mL, respectively, which suggested full capacity for the quantification of each bioactive compound investigated. R^2 values of the analytes were higher than 0.99, thus confirming the linearity of the method.

Table 1. *Regression curves, linearity, limit of detection (LOD) and limit of quantification (LOQ)*

Compound	Regression equation	Correlation coefficient (R^2)	LOD, (µg/mL)	LOQ, (µg/mL)
Chlorogenic acid	$Y=2.7\times10^3x-2.29\times10^3$	0.9940	0.46	1.52
Vicenin-2	$Y=5.29\times10^3x-2.63\times10^3$	0.9970	0.28	0.93
Luteolin-3',7-di-O-glucoside	$Y=1.06\times10^4x-7.8\times10$	0.9979	0.13	0.43
Luteolin-7-O-glucoside	$Y=1.2\times10^4x-2.92\times10^3$	0.9988	0.13	0.42
Rutin	$Y=9.4\times10^3x-9.56\times10^3$	0.9997	0.21	0.63
Apigenin-7-O-glucoside	$Y=1.13\times10^4x-2.68\times10^3$	0.9992	0.24	0.79
Luteolin	$Y=3\times10^4x-1.5\times10^3$	0.9993	0.04	0.15
Apigenin	$Y=1.52\times10^4x-2.26\times10^3$	0.9991	0.11	0.37

To verify the precision of the proposed HPLC method five replicate injections of a 70 % ethanolic yarrow extract were examined. It can be seen that reproducibility and repeatability for retention times (Table 2) are high and do not exceed 0.99%, while these parameters for peak area are acceptable, and do not exceed 4.97%.

Table 2. *Repeatability and reproducibility of HPLC method*[a]

Compound	RSD, %					
	Retention time			Integrated area		
	Run-to-run	Day-to-day	Extraction-to-extraction	Run-to-run	Day-to-day	Extraction-to-extraction
Chlorogenic acid	0.41	0.96	0.99	0.52	1.64	2.67
Vicenin-2	0.22	0.62	0.94	1.08	1.54	3.83
Luteolin-3',7-di-O-glucoside	0.18	0.56	0.87	1.13	1.61	3.75
Luteolin-7-O-glucoside	0.12	0.53	0.56	1.16	1.56	3.62
Rutin	0.09	0.48	0.52	1.18	1.47	4.15
Apigenin-7-O-glucoside	0.07	0.39	0.46	1.17	2.28	4.97
Luteolin	0.06	0.29	0.55	1.10	1.18	3.39
Apigenin	0.06	0.27	0.18	1.08	1.55	2.32

[a]n= 5 for run-to-run and day-to-day repeatability, and n=3 for extraction-to-extraction reproducibility.

The use of an endcapped polymeric RP-Amide column and optimized gradient profile enabled the flavonoid and phenolcarbonic acid complex to be separated, achieving better selectivity, sensitivity and precision.

Variation in the phenolic compounds composition of *Achillea millefolium* wild populations

The qualitative and quantitative composition of bioactive compounds is the essential parameter for the evaluation of the qualitative characteristics of herbal raw material. The phytochemical analysis of secondary metabolites present in herb of *A. millefolium* was conducted using the optimized HPLC method. The typical chromatogram of the ethanolic extract of yarrow herb is presented in Fig. 3. As it can be seen, the peaks of apigenin-7-O-glucoside, luteolin-7-O-glucoside and chlorogenic acid were dominant among the identified analytes in the chromatographic profile of ethanolic extract of yarrow herb.

The results from the phytochemical analysis indicate that wild populations of *A. millefolium* varied on the amount of phenolic compounds of the aerial parts of plant. It was determined that the total amount of the identified bioactive compounds in herb raw material ranged from 12.605 to 27.993 mg/g among wild populations of yarrow. The accumulation trends of chlorogenic acid and flavonoids highly varied and showed considerable quantitative heterogeneity among herb extracts of *A. millefolium*. The chlorogenic acid predominated (5.577–16.799 mg/g) in the composition of the complex of identified secondary metabolites. Regarding the composition of the flavonoid complex, the patterns of distribution in the analysed samples of yarrow herbs are characterized by the dominance of apigenin-7-O-glucoside (2.348–7.888 mg/g) and luteolin-7-O-glucoside (1.342–3.938 mg/g) (Table 3).

Table 3. *The composition of phenolic compounds and characteristics of its variability in herb raw material of Achillea millefolium different wild populations (n=80)*

Compound	Quantity, mg/g			Std. Error	RSD, %
	Min	Max	Mean		
Chlorogenic acid	5.577	16.799	9.303	0.266	25.55
Vicenin-2	0.587	1.750	1.034	0.030	26.26
Luteolin-3’,7-di-O-glucoside	0.069	0.588	0.191	0.011	29.98
Luteolin-7-O-glucoside	1.342	3.938	2.381	0.057	21.48
Rutin	0.282	1.515	0.674	0.026	34.85
Apigenin-7-O-glucoside	2.348	7.888	4.125	0.140	30.35
Luteolin	0.272	0.771	0.478	0.013	24.96
Apigenin	0.353	1.469	0.750	0.027	32.32

When the accurate nonparametric Kruskal-Wallis test for the independent samples was applied, the statistical comparison of the amount of phenolic compounds in the samples of *A. millefolium* herb gathered from different parts of Lithuania was done. Statistically significant differences regarding the amounts of chlorogenic acid, luteolin-3’,7-di-O-glucoside, rutin and apigenin in the raw materials from different

Lithuanian regions were determined. Dunn's multiple comparisons post-hoc test revealed that the amount of chlorogenic acid accumulated in herbal raw materials from western Lithuania was significantly larger ($p<0.05$) than the amount in starting materials collected from central part of the country. This test helped to determine that the level of luteolin-3',7-di-O-glucoside from central and northeastern parts was significantly higher ($p<0.01$) than in raw materials gathered from northwestern Lithuania. The levels of rutin accumulation were significantly higher in raw materials collected from northeastern ($p<0.01$) and western ($p<0.05$) parts than those from central part of the country. The content of apigenin in raw materials from western Lithuania was significantly higher ($p<0.01$) in comparison to its content in herbs from central part.

Hierarchical cluster analysis was applied to determine the relationship among wild *A. millefolium* populations on the basis of phenolic compounds accumulation. All populations ranged into two high level clusters (group I and II), according to the amounts of phenolic compounds in herbs collected from 80 different populations. The Kruskal-Wallis test allowed determining the difference among clusters to be significant, according to the contents of all phenolic compounds in herb of yarrows.

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. The plants of *Achillea* genus are characterized by high ecological plasticity; therefore they can grow in different locations. Soil chemical composition of investigated 80 habitats varied within a wide range. The analysed soils were poor in nitrogen (0.01–1.46 %) and amounts of potassium and phosphorus varied within ranges 20.90-427.80 and 6.30–4361.20 mg/kg, respectively. The quantities of humus varied from 0.61 to 25.44 %. The soil pH of analyzed habitats varied in the range from 4.61 to 8.18. More sensitive and comprehensive measures of the central tendency, such as median and 4 percentiles (10th, 25th, 75th and

90th), confirm that the soil in the majority of habitats was of neutral or alkaline reaction.

Concerning the composition of the flavonoid complex, the patterns of distribution in samples of *A. millefolium* herb are characterized by the predominant formation of flavone-O-glycosides, namely apigenin-7-O-glucoside and luteolin-7-O-glucoside. The phytochemical investigations on yarrow herbs from natural populations have revealed a considerable variation in accordance with phenolic compounds. The observed diversity could have a genetic basis, as well as it may be attributed to the environmental differences of growing habitats. *A. millefolium* habitats highly varied in soils' chemical composition that confirmed exceedingly broad ecological amplitude of the plant. Collecting the raw material it should be considered that an evaluation of the ecological conditions may be an important factor in predicting the patterns of secondary metabolites accumulation in the plant. Besides, the natural populations distinguishing by quantitative and qualitative composition of herbal raw material could be used for choosing of selective donors, this is important in order to obtain accessions of high quality.

Variation in the composition of flavonoids and phenolcarbonic acids among plant parts of *Achillea millefolium*

Defined by heterogeneous nature of yarrow raw material (*Millefolii herba*) botanical matrix, the phytochemical composition of the crude drug is complex and comprises of phytochemical profiles of flowers, leaves and stems.

Variability of the composition of phenolic compounds on flowers. Due to anatomical plant characteristics, the flowers constitute one of the most important and biggest parts of the raw material of yarrow. The chromatogram of the ethanolic extracts of *A. millefolium* flowers is presented in Fig. 4. The peaks of apigenin-7-O-glucoside, luteolin-7-O-glucoside, apigenin and luteolin dominate among the identified analytes in the chromatogram of ethanolic extract of yarrow flowers. In this regard the chromatogram of flowers differs from the

chromatogram of herb (Fig. 3), where three peaks are predominant – apigenin-7-O-glucoside, luteolin-7-O-glucoside and chlorogenic acid.

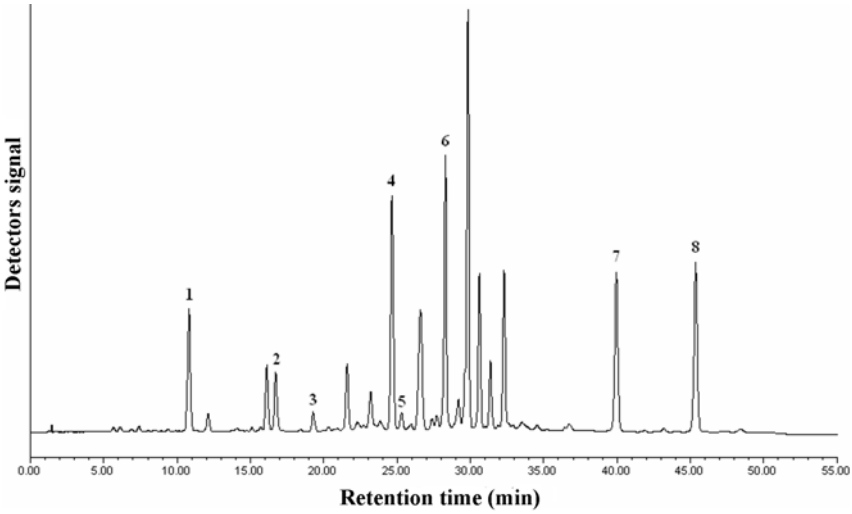

Fig. 4. *Characteristic HPLC chromatogram of a 70 % (v/v) ethanolic extract from Achillea millefolium flowers. Peaks identified: 1-chlorogenic acid, 2-vicenin-2, 3-luteolin-3',7-di-O-glucoside, 4-luteolin-7-O-glucoside, 5-rutin, 6-apigenin-7-O-glucoside, 7-luteolin, 8-apigenin.*

The results from quantitative evaluation of secondary metabolites on flowers from 34 wild yarrow populations indicated that the variation amplitude of total amount of the identified phenolics ranged from 13.290 to 27.947 mg/g. As it can be seen, chlorogenic acid (2.837–12.679 mg/g) predominated in the mixture of identified secondary metabolites (Table 4). Concerning the composition of flavonoid complex, the patterns of distribution with the predominant 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) are characteristic. Rather high amounts of apigenin and luteolin are of high importance in the pharmacological profile of yarrow,

particularly in spasmolytic and estrogenic effects. It was found that these aglycones exhibited the highest antispasmodic activities and also it was observed that in tea prepared from yarrow the concentrations of the flavonoids are high enough to exert a spasmolytic effect in the gut [Lemmens-Gruber et al., 2006]. Apigenin can stimulate estrogen receptors (ER)-dependent biological pathways by activating ERα and ERβ receptors. Luteolin showed a very slight effect on ERβ and does not seem to activate ERα at all [Innocenti et al., 2007]. The glycosidic forms of these aglycones displayed lower activity in spasmolytic assay and, on the contrary, higher activity in estrogenic activity assay.

Table 4. *The composition of phenolic compounds and characteristics of its variability in flowers of Achillea millefolium different wild populations (n=34)*

Compound	Quantity, mg/g			Std. Error	RSD, %
	Min	Max	Mean		
Chlorogenic acid	2.837	12.679	8.206	0.447	31.74
Vicenin-2	0.739	4.222	1.632	0.118	42.28
Luteolin-3',7-di-O-glucoside	0.056	0.515	0.203	0.015	43.10
Luteolin-7-O-glucoside	2.107	5.885	3.932	0.180	26.65
Rutin	0.180	0.582	0.303	0.017	32.34
Apigenin-7-O-glucoside	3.334	7.546	5.408	0.166	17.91
Luteolin	0.561	1.253	0.791	0.025	18.12
Apigenin	0.448	2.562	1.139	0.103	52.54

When the statistical analysis of *A. millefolium* flowers collected from different Lithuanian regions (central, western, southern and northern Lithuania) was conducted using the accurate Kruskal-Wallis test, it was determined that the regions were characterized by significant differences with regard to the amounts of rutin, apigenin and luteolin in raw materials. The Dunn's test revealed that according to the contents of aforementioned flavonoids, there were statistically significant differences between flowers collected from central and northern Lithuanian regions. The accumulated amounts of rutin were significantly higher in flowers gathered from central part ($p<0.05$) than from northern, while in the latter the levels of luteolin ($p<0.05$) and apigenin ($p<0.01$) were significantly higher if compared with their northern counterparts.

The results were submitted to a cluster analysis in order to reveal infraspecific variation of individual phenolics in flowers of *A. millefolium*. The application of hierarchical cluster analysis on the 34 populations' matrix by using amounts of phenolic compounds as clustering variables grouped flower extracts into two main clusters. The Kruskal-Wallis test revealed statistically significant differences among the groups of cluster analysis, according to the content of chlorogenic acid, luteolin-7-O-glucoside, rutin, apigenin-7-O-glucoside and apigenin in the samples of yarrow flowers.

Variability of the composition of phenolic compounds on leaves. The analysis of ethanolic extracts of *A. millefolium* leaves using HPLC method allowed determining the quantitative and qualitative composition of the phenolic compounds that is characteristic to the yarrow leaves collected from wild populations. Among the identified analytes in the chromatogram of ethanolic extract of yarrow leaves two peaks were dominant – chlorogenic acid and rutin (Fig. 5). In this regard it differs from the chromatographic profiles of herb and flowers extracts.

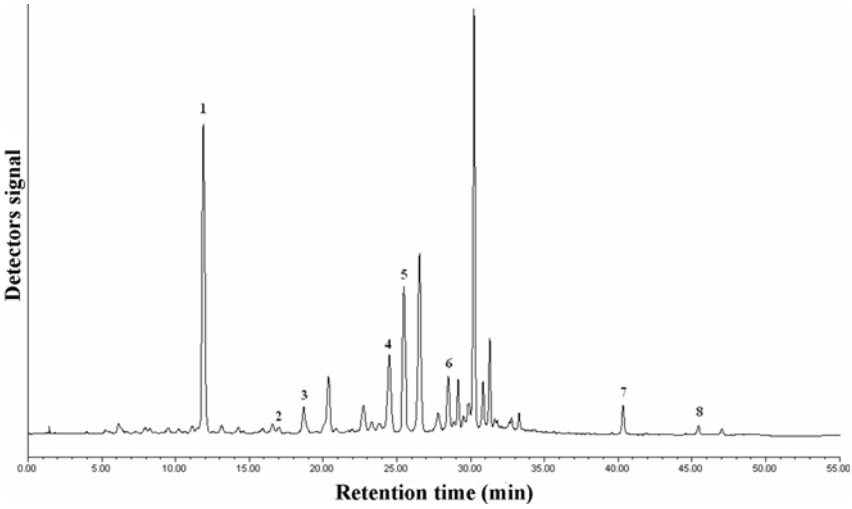

Fig. 5. *Characteristic HPLC chromatogram of a 70 % (v/v) ethanolic extract from Achillea millefolium leaves. Peaks identified: 1-chlorogenic acid, 2-vicenin-2, 3-luteolin-3',7-di-O-glucoside, 4-luteolin-7-O-glucoside, 5-rutin, 6-apigenin-7-O-glucoside, 7-luteolin, 8-apigenin.*

Comparison of the total amounts of identified secondary metabolites in yarrow leaves demonstrated that, they varied over a wide range (16.644–50.415 mg/g). This is confirmed by the results of descriptive statistics presented in Table 5. It was determined that the analyzed samples of yarrow leaves are characterized by clear predominance of one component – chlorogenic acid – in the composition of the identified secondary metabolites (12.676–42.464 mg/g). The chlorogenic acid is one of the most common phenylpropanoids among secondary plant metabolites and it was shown to exert antioxidant, anticancer, anti-inflammatory and analgesic activity. Therefore, chlorogenic acid exhibits similar to flavonoid fraction biological effects and might additionally contribute to the polyvalent pharmacological profile of *A. millefolium*. In the mixture of the identified flavonoids the flavonol glycoside rutin (1.363–6.652 mg/g) and luteolin-7-O-glucoside (0.496–3.744 mg/g) are predominant. Recent experiments have confirmed that hydroalcohol extracts from aerial parts (without flowers) of *A. millefolium* exert a moderate antinociceptive peripheral effect, which is believed to be mainly caused by rutin and esters of caffeic acid with quinic acid (chlorogenic and dicaffeoylquinic acids) [Pires et al., 2008].

Table 5. *The composition of phenolic compounds and characteristics of its variability on leaves of Achillea millefolium different wild populations (n=80)*

Compound	**Quantity, mg/g**			**Std. Error**	**RSD, %**
	Min	**Max**	**Mean**		
Chlorogenic acid	12.676	42.464	25.245	0.703	24.89
Vicenin-2	0.126	1.013	0.455	0.022	40.08
Luteolin-3’,7-di-O-glucoside	0.103	1.233	0.493	0.027	42.70
Luteolin-7-O-glucoside	0.496	3.744	1.405	0.072	45.75
Rutin	1.363	6.652	2.896	0.113	34.93
Apigenin-7-O-glucoside	0.272	1.694	0.860	0.041	43.12
Luteolin	0.031	0.222	0.084	0.004	44.91
Apigenin	0.035	0.195	0.078	0.004	45.07

The statistical analysis of the secondary metabolites of *A. millefolium* leaves collected from different Lithuanian regions (northeastern, western, northwestern and central Lithuania) was done using the accurate Kruskal-Wallis test; it was confirmed that the regions were characterized by statistically significant differences with regard to the amounts of vicenin-2, luteolin-7-O-glucoside, rutin, apigenin-7-O-glucoside and luteolin in raw materials. Posthoc Dunn's multiple comparison test revealed that the accumulated levels of vicenin-2 were significantly higher in leaves gathered from populations in northwestern part ($p<0.05$) than from ones in northeastern part of the country. The accumulated amounts of luteolin-7-O-glucoside were significantly higher in raw materials from populations of northwestern ($p<0.05$) and western ($p<0.01$) Lithuania if compared to populations located in central part. The content of rutin in leaves gathered from populations of western part were significantly higher than in leaves from central part ($p<0.05$), while the significantly higher concentrations of apigenin-7-O-glucoside were determined in leaves from central part ($p<0.05$) populations if compared to western part. The amount of luteolin accumulated in central region populations was significantly higher ($p<0.05$) if compared to ones from northeastern part.

Statistical evaluation was conducted by hierarchical cluster analysis using the contents of phenolics different populations as variables. Kruskal-Wallis test revealed that amounts of chlorogenic acid, luteolin-7-O-glucoside and rutin in raw materials differed significantly among the populations.

Variability of the composition of phenolic compounds on stems. The peaks of seven analytes in the chromatograms of yarrow stems were identified. Among them, peaks of chlorogenic acid and rutin are dominant, and in this regard they differ from the chromatographic profiles of herbs, flowers and leaves extracts.

The mean values of total contents of identified phenolics in the analyzed samples of *A. millefolium* stems collected from 80 wild popula-

tions ranged from 2.870 to 11.816 mg/g. The results of descriptive statistics are presented in Table 6. The level of chlorogenic acid accumulation is the highest in stems (1.835-9.813 mg/g), while rutin (0.299-2.132 mg/g) and apigenin-7-O-glucoside (0.062-0.802 mg/g) are predominant in the mixture of the identified flavonoids.

Table 6. *The composition of phenolic compounds and characteristics of its variability of Achillea millefolium stems from different wild populations (n=80)*

Compound	Quantity, mg/g			Std. Error	RSD, %
	Min	Max	Mean		
Chlorogenic acid	1.835	9.813	5.212	0.176	30.17
Vicenin-2	0.056	0.610	0.182	0.015	47.41
Luteolin-3’,7-di-O-glucoside	0.012	0.265	0.073	0.007	52.67
Luteolin-7-O-glucoside	0.037	0.559	0.182	0.013	66.06
Rutin	0.299	2.132	1.064	0.042	35.19
Apigenin-7-O-glucoside	0.062	0.802	0.273	0.019	61.79
Luteolin	0.006	0.039	0.015	0.001	48.21

To sum up, the presented results revealed that there is a variation in the accumulation patterns of phenolic compounds among different *A. millefolium* parts, indicating the organ-dependence of secondary metabolites accumulation in plants. Yarrow flowers are characterized by the dominance of apigenin-7-O-glucoside and luteolin-7-O-glucoside and their aglycones – apigenin and luteolin among the identified flavonoids. Rutin and luteolin-7-O-glucoside are predominant in mixture of identified flavonoids in *A. millefolium* leaves. The predominance of rutin and apigenin-7-O-glucoside is characteristic among the identified flavonoids in stems. The marked predominance of chlorogenic acid within the complex of identified secondary metabolites appeared to be characteristic to yarrow leaves and stems. The starting materials of yarrow with different chemical composition can be used for the development of phytopreparations of specific and predicted therapeutic effect.

Phenological variation of the composition of phenolic compounds

The present study was conducted to determine phenologic variation of chlorogenic acid and flavonoids content of *A. millefolium* growing in Lithuania. The plants were harvested at six phenological stages: rosette, vegetative, floral budding, full flowering, fresh fruiting and mature fruiting and dissected into stem, leaf and reproductive tissues and assayed for bioactive compounds by means of HPLC. Changes in chemical contents during the course of plant development varied with plant parts and phenolic compounds (Fig. 6). Having applied the accurate Kruskal-Wallis test for the independent samples, it was determined that differences in chlorogenic acid and flavonoids concentrations of plant tissues for each phenological stage were found to significant. Posthoc Dunn's multiple comparison test enabled us to specify which morphological parts of the plant differ significantly ($p<0.05$) in respect of amounts of the phenolic compounds.

Chlorogenic acid content of aerial plant parts decreased linearly with advancing of development stages and the highest levels were observed at the rosette stage in leaves (50.165 mg/g), at the vegetative stage in stems (18.717 mg/g), and at the floral budding stage in reproductive tissues (27.570 mg/g). The slight increase in the amount during the development of flowering buds is characteristic to the accumulation patterns of chlorogenic acid in *A. millefolium* leaves (Fig. 6 A). Whereas, contents of the majority of flavonoids in different plant parts increased during plant development, however, the highest total amounts of the identified flavonoids were observed at different stages of plant phenology for each tissue: at the floral budding stage in leaves (4.336 mg/g), at the fresh fruiting stage in stems (1.765 mg/g), and at the full flowering stage in reproductive parts (9.907 mg/g).

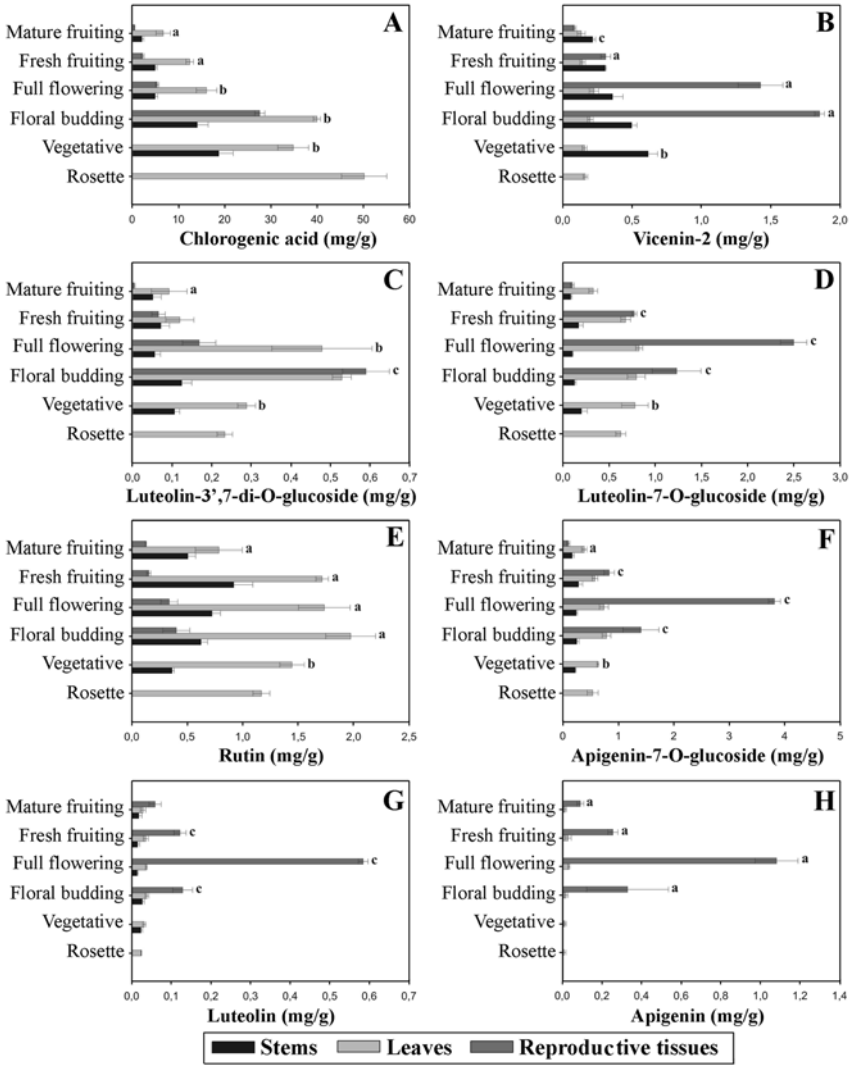

Fig. 6. *Phenologic changes of chlorogenic acid (A), vicenin-2 (B), luteolin-3',7-di-O-glucoside (C), luteolin-7-O-glucoside (D), rutin (E), apigenin-7-O-glucoside (F), luteolin (G) and apigenin (H) content in stem, leaf and reproductive tissues of Achillea millefolium. Small letters indicate differences among plant parts that are significant at p<0.05: a – leaves from reproductive tissues; b – leaves from stems; c – reproductive tissues from stems; (n=3).*

The concentrations of vicenin-2 varied from 0.084 to 1.852 mg/g depending on the plant part and development stage. The highest accumulation of this compound was detected in stems at the vegetative stage (0.616 mg/g) and in reproductive parts at the floral budding stage (1.852 mg/g), while its highest levels in the leaves were reached at full flowering (0.225 mg/g) (Fig. 6 B).

The amounts of luteolin-3',7-di-O-glucoside and rutin increased with advancing of developmental stages. The highest contents of these compounds in leaf and reproductive tissues were detected during the development of flowering buds (0.528 and 0.589 mg/g for luteolin-3',7-di-O-glucoside and 1.972 and 0.401 mg/g for rutin, respectively). The highest accumulation of luteolin-3',7-di-O-glucoside in stems was also observed at the floral budding stage (0.124 mg/g), while the highest levels of rutin were produced at the fresh fruiting stage (0.919 mg/g) (Fig. 6 C, E).

The amounts of luteolin-7-O-glucoside and apigenin-7-O-glucoside increased during the course of the phenological development of the plant. The highest contents of these compounds in the reproductive parts were observed at the full flowering stage (2.496 mg/g and 3.813 mg/g, respectively), while their highest levels in the vegetative organs were reached at different stages of plant phenology. The leaves accumulated the highest amounts of luteolin-7-O-glucoside and apigenin-7-O-glucoside at the full flowering (0.824 mg/g) and floral budding stages (0.786 mg/g), respectively, while the stems – at the vegetative (0.200 mg/g) and fresh fruiting stages (0.283 mg/g), respectively (Fig. 6 D, F).

The levels of luteolin and apigenin accumulation in yarrow increased during plant growth and development. The highest amount of luteolin was observed at the floral budding stage in leaves (0.039 mg/g) and stems (0.027 mg/g), and in the reproductive parts (0,584 mg/g) during flowering time. Whereas, the highest concentrations of apigenin (this compound was not detected in stems) were reached in leaves (0.033 mg/g) and flowers (1.082 mg/g) at the full flowering stage (Fig. 6 G, H).

The results from the present study indicate a close relationship between chlorogenic acid and flavonoids content of plant parts and de-

velopment stage during the phenological cycle of this species. The flavonoids' contents in all tissues increased with advancing plant phenology and generally higher accumulation levels were reached at flowering. Among different tissues, reproductive parts were found to be superior to stems and leaves with regard to vicenin-2, luteolin-3',7-di-O-glucoside, luteolin-7-O-glucoside, apigenin-7-O-glucoside, luteolin and apigenin accumulations, while leaves accumulated the highest levels of rutin and chlorogenic acid. Chlorogenic acid content of aerial plant parts decreased linearly with advancing of development stages.

Variation in the composition of phenolic compounds in other *Achillea* species

Investigations of the phenolics composition on spontaneous *A. cartilaginea* were carried out. The plant-part specific qualitative and quantitative variation in phenolic profiles in plants of this species was observed. Six phenolic compounds: chlorogenic acid and flavonoids, namely luteolin-7-O-glucoside, rutin, apigenin-7-O-glucoside, luteolin and apigenin, were identified in *A. cartilaginea* plant material.

It was determined that the total content of identified phenolic compounds in *A. cartilaginea* plant materials varied within a wide range: from 21.622 to 48.442 mg/g in herbs, from 9.562 to 15.027 mg/g in flowers, from 24.494 to 64.047 mg/g in leaves and from 4.673 to 13.102 mg/g in stems. Regarding the composition of the identified secondary metabolites in analysed samples of herb the patterns of phenolic distribution are characterized by the dominance of chlorogenic acid (18.328–42.248 mg/g), rutin (2.311–4.137 mg/g), apigenin-7-O-glucoside (0.647–1.308 mg/g) and luteolin-7-O-glucoside (0.241–0.572 mg/g). The highest amounts of luteolin-7-O-glucoside (1.726–2.970 mg/g), apigenin-7-O-glucoside (0.768–1.249 mg/g), luteolin (0.196-0.493 mg/g) and apigenin (0.116–0.237 mg/g) were accumulated in *A. cartilaginea* flowers. Leaves were found to be superior to other plant parts with regard to chlorogenic acid (21.015–55.600 mg/g) and rutin (2.797–7.021 mg/g) accumulations.

Luteolin-7-O-glucoside, rutin, apigenin-7-O-glucoside, luteolin and chlorogenic acid were identified in ethanolic extracts from herbs of *A. ptarmica*, which is indigenous but rare species in Lithuania. Chlorogenic acid amount decreased linearly with advancing of plant development and the highest level was detected at the rosette stage (82.150 mg/g). The highest total content (3.395 mg/g) of the identified flavonoids was observed at the floral budding stage. The highest level of luteolin-7-O-glucoside was reached at the rosette stage (0.533 mg/g), while the amounts of other flavonoids increased in herbs with advancing of developmental stages. The highest concentration of apigenin-7-O-glucoside was observed in herbs at the floral budding stage (0.956 mg/g), luteolin – at the full flowering stage (0.078 mg/g), and rutin – at the fresh fruiting (2.374 mg/g) stage.

The composition of the flavonoid and phenolcarbonic acid patterns was investigated in introduced species, namely *A. odorata*, *A. sibirica* and *A. filipendulina* and the trends of its variation during plant development were determined. Luteolin-7-O-glucoside, rutin, apigenin-7-O-glucoside, luteolin, apigenin and chlorogenic acid were identified in ethanolic extracts of *A. odorata* herb. Chlorogenic acid and rutin were the main compounds among the identified analytes of *A. sibirica* herb, accompanied with minor flavonoids as luteolin-7-O-glucoside and luteolin. Two phenolic compounds, as chlorogenic acid and luteolin were identified in extracts of *A. filipendulina* herb. The results from quantitative evaluation indicate significant variation in the concentrations of secondary metabolites of introduced yarrow species during their seasonal growth and development (Table 7). The highest levels of chlorogenic acid were observed at the vegetative stage of investigated species: *A. filipendulina* – 14.475 mg/g, *A. sibirica* – 33.450 mg/g and *A. odorata* – 39.803 mg/g. At the same stage aerial parts of *A. sibirica* and *A. odorata* accumulated the highest amounts of rutin 2.912 mg/g and 2.960 mg/g, respectively. The concentrations of luteolin-7-O-glucoside and luteolin in corresponding taxa increased with advancing of phenological development, and the highest contents of aforementioned flavonoids were reached during flowering time:

A. sibirica – 0.267 mg/g and 0.056 mg/g, respectively; *A. odorata* – 0.543 mg/g and 0.063 mg/g, respectively. *A. filipendulina* accumulated the largest amount of luteolin (0.040 mg/g) at the full flowering stage. Whereas the highest levels of apigenin-7-O-glucoside (0.973 mg/g) and apigenin (0.103 mg/g) were observed in herb of *A. odorata* at the full flowering stage.

Table 7. *Phenological variation of phenolic compounds (mg/g) in herb raw material of introduced species of Achillea*

Species	**Phenological stages**	**Chlorogenic acid**	**Luteolin-7-O-glucoside**	**Rutin**	**Apigenin-7-O-glucoside**	**Luteolin**	**Apigenin**
A. filipendulina	Rosette	13.012	–	–	–	–	–
	Vegetative	14.475	–	–	–	0.014	–
	Floral budding	7.174	–	–	–	0.029	–
	Full flowering	3.697	–	–	–	0.040	–
	Mature fruiting	3.390	–	–	–	0.016	–
A. sibirica	Vegetative	33.450	0.043	2.912	–	0.011	–
	Floral budding	25.492	0.062	2.239	–	0.008	–
	Full flowering	21.522	0.267	1.885	–	0.056	–
	Fresh fruiting	2.855	0.149	0.654	–	0.045	–
	Mature fruiting	1.760	0.103	0.206	–	0.034	–
A. odorata	Rosette	39.181	0.169	1.390	0.389	0.041	0.062
	Vegetative	39.803	0.233	2.960	0.288	0.043	0.040
	Floral budding	30.631	0.250	1.498	0.811	0.029	0.038
	Full flowering	7.314	0.543	0.472	0.973	0.063	0.103

The investigated taxa of *Achillea* genus exhibit differences not only in the content of flavonoids but also in their qualitative composition, thus indicating that the patterns of their distribution seem to be of chemotaxonomic significance. In addition, all investigated species of

Achillea are characterized by the presence of chlorogenic acid in high amounts (except *A. filipendulina*). With regard to the evaluation of the tendencies of morphological and phenological variability of chlorogenic acid and flavonoids in the analyzed taxa, one may conclude that they seem to be more or less similar to the patterns of distribution of phenolic compounds in *A. millefolium*.

Evaluation of the biological activity of extracts from plants of the genus *Achillea*

Assessment of free radical scavenging properties of extracts from plants of the genus *Achillea*

The plants of the genus *Achillea* have been found to possess antioxidative activity in various *in vitro* assays. The extracts from raw materials of *Achillea* species are a potential source of natural antioxidants for treatment and prevention of diseases in which oxidative damage takes place. Moreover, since some of the pharmacological properties of these plant extracts may be linked to their antioxidant potential, it is of high importance to investigate their antioxidant effectiveness. It is generally accepted that if a substance is weak antioxidant *in vitro*, it is unlikely to be any better *in vivo* [Hanasaki et al., 1994], consequently *in vitro* model systems represent a valuable tool for the evaluation of the antioxidant activity. The antioxidant activity of extracts from several *Achillea* species, in relation to free radical scavenging, was investigated by the on-line HPLC-DPPH assay. This coupled approach combines a separation by means of HPLC with fast post-column chemical detection and enables a rapid identification of active constituents of the studied plants. By calibration with reference antioxidant Trolox, we were able to use the on-line radical scavenging assay in a quantitative way.

Preliminary experiments were performed to optimize the reaction time by setting different flow rates (1, 1.5, 2 mL/min) of the working DPPH solution. The flow rate of 1.5 mL/min was chosen, because it gave relatively low base line disturbances and an acceptable peak height of active analytes. R^2 value of the Trolox was 0.998, thus confirming the linearity as well as suitability of the optimized HPLC-DPPH method for quantitative determinations.

The results of DPPH quenching showed the presence of radical - scavenging compounds in yarrow extracts, namely flavonoids and phenolcarbonic acids. HPLC-DAD-ESI/MS hyphenated technique was used to identify the main free radical scavenging components in the extracts of plants of the genus *Achillea*. This study revealed that the main components of the flavonoid complex that possessed radical scavenging properties were luteolin-7-O-glucoside, rutin, luteolin and luteolin-7-O-glucuronide. Regarding the phenolcarbonic acids, the mono- and dicaffeoylquinic acids (DCQAs) were shown to exhibit significant radical scavenging effects against DPPH. Four dicaffeoylquinic acids, namely 3,4-DCQA, 3,5-DCQA, 1,5-DCQA and 4,5-DCQA were identified in the extracts of *A. millefolium* herb on the basis of their elution patterns and ESI/MS spectra in both positive and negative ion detection modes.

The on-line HPLC-DPPH system was applied for screening and activity evaluation of ethanolic extracts from raw materials of native and introduced species of yarrow in Lithuania for radical – trapping components. The data obtained from DPPH on-line decolouration assay showed the presence of compounds within a complex of secondary metabolites in *A. millefolium*, which were capable of scavenging free radicals. Quantitative determination of contribution of individual compounds to radical scavenging properties of extracts from raw materials of yarrow was achieved by using Trolox calibration curve and results are presented in Table 8. According to the lower chromatographic profile (DPPH quenching chromatogram) it was observed that predominant radical scavengers in the extracts of yarrow were mono- and dicaffeoylquinic acids. The main contributors to antiradical activity of *A. millefolium* extracts among esters of caffeic acid with quinic acid were 3,5-DCQA, chlorogenic acid and 4,5-DCQA. While among

the identified flavonoids rutin, luteolin and its derivatives luteolin-7-O-glucoside and luteolin-7-O-glucuronide were shown to possess radical scavenging properties.

Table 8. *Radical scavenging activity of individual compounds in extracts of Achillea millefolium herb, flowers, leaves and stems*

No	Compound	Herb		Flowers		Leaves		Stems	
		E_T, µg	A_T, µg/g	E_T, µg	A_T, µg/g	E_T, µg	A_T, µg/g	E_T, µg	A_T, µg/g
1	Chlorogenic acid	1.055	5054.8	0.485	2302.1	1.198	5744.1	0.534	2592.7
2	Unidentified	0.163	780.8	0.129	612.6	–	–	–	–
3	Unidentified	0.091	436.5	0.067	318.5	0.107	512.8	–	–
4	Luteolin-7-O-glucoside	0.303	1453.6	0.259	1228.8	0.085	409.3	–	-
5	Rutin	0.093	446.9	–	–	0.129	617.7	0.073	351.6
6	Luteolin-7-O-glucuronide	0.171	817.4	0.125	593.4	0.266	1274.9	–	-
7	3,4-DCQA	0.286	1368.4	0.133	631.5	0.243	1165.6	0.113	547.0
8	3,5-DCQA	1.332	6382.6	1.445	6857.0	1.302	6240.8	0.251	1217.6
9	1,5-DCQA[a]	0756	3621.8	0.400	1899.8	0.325	1556.3	0.168	814.3
10	4,5-DCQA	1.313	6292.6	0.647	3070.8	0.933	4474.1	0.279	1358.1
11	Unidentified	0.093	443.3	0.054	257.1	0.069	329.2	–	-
12	Luteolin	0169	812.7	0.173	821.6	–	–	–	-
		5.826	27911.5	3.918	18593.2	4.656	22324.8	1.418	6881.2

E_T – Trolox content equivalent; A_T – antiradical activity equivalent. [a] An unidentified apigenin derivative was co-eluted with 1,5-DCQA in herb and flowers extracts.

Comparison of antiradical activity of extracts obtained from different parts of *A. millefolium* revealed that their radical scavenging capacities were variable and it was associated with the distribution of phenolic compounds, possessing radical scavenging activity, in the extracts (Table 8). The extracts of *A. millefolium* herb and leaves exhibited the highest antiradical activity (antiradical activity equivalent A_T 27911.5 µg/g and 22324.8 µg/g, respectively). The extracts from yarrow flowers showed lower scavenging properties (A_T 18593.2 µg/g), whereas extracts of stems possessed the weakest activity (A_T 6881.2 µg/g).

Screening of radical scavenging behaviour of extracts from the naturally growing but rare *A. cartilaginea* plants by on-line HPLC-DPPH

assay revealed that chlorogenic and dicaffeoylquinic acids were the main contributors to their antiradical activity. The extracts from *A. cartilaginea* leaves and herb possessed higher radical scavenging capacity (A_T 52598.8 and 35574.8 µg/g, respectively) than extracts from flowers and stems (A_T 20824.5 µg/g and A_T 11007.1 µg/g, respectively).

On-line DPPH quenching profile (Fig. 7) of extracts from the herb of rarely occurring in Lithuania *A. ptarmica* showed that many components within a complex of secondary metabolites possess radical scavenging properties. Esters of caffeic acid with quinic acid were found to be the principal radical scavengers in *A. ptarmica* extracts (Table 9).

Table 9. *Radical scavenging activity of individual compounds in extracts of Achillea ptarmica, A. odorata, A. sibirica and A. filipendulina herb raw materials*

No	Compound	*A. ptarmica*		*A. odorata*		*A. sibirica*		*A. filipendulina*	
		E_T, µg	A_T, µg/g	E_T, µg	A_T, µg/g	E_T, µg	A_T, µg/g	E_T, µg	A_T, µg/g
1	Chlorogenic acid	3.134	15410.1	1.253	6008.4	3.552	16932.4	0.564	2709.3
2	Unidentified	0.094	464.6	0.159	764.3	0.078	370.1	0.235	1129.7
3	Unidentified	0.231	1133.7	–	–	–	–	–	–
4	Luteolin-7-O-glucoside	–	–	0.127	606.6	0.079	375.5	–	–
5	Rutin	0.162	797.2	0.088	421.2	0.254	1208.6	–	–
6	Luteolin-7-O-glucuronide	–	–	0.108	518.2	–	–	–	–
7	3,4-DCQA	0.189	929.9	–	–	–	–	–	–
8	Unidentified	–	–	0.229	1099.8	–	–	–	–
9	3,5-DCQA	2.990	14702.4	1.698	8141.6	0.753	3591.4	1.783	8564.3
10	Unidentified	0.076	371.7	0.383	1835.2	0.062	295.5	0.140	672.9
11	4,5-DCQA	1.111	5462.8	0.647	3099.9	0.643	3063.6	0.821	3943.0
12	Unidentified	0.079	388.9	0.055	262.0	0.076	363.5	0.126	606.7
13	Luteolin	0.061	299.4	0.054	260.8	–	–	–	–
		8.128	39960.8	4.802	23018.0	5.496	26200.6	3.670	17625.9

E_T – Trolox content equivalent; A_T – antiradical activity equivalent.

The composition of antiradical phenolic constituents from raw materials of the introduced yarrow species (*A. odorata*, *A. sibirica* and *A. filipendulina*) varied (Fig. 7). However, according to corresponding negative peaks in the radical quenching chromatograms mono- and dicaffeoylquinic acids were the components contributing most to the radical scavenging activity of extracts. Quantitative evaluation revealed (Table 9) that *A. sibirica* extract possessed the most effective scavenging properties (A_T 26200.6 μg/g), *A. odorata* showed lower activity (A_T 23018.0 μg/g), while activity of *A. filipendulina* extract was the lowest (A_T 17625.9 μg/g).

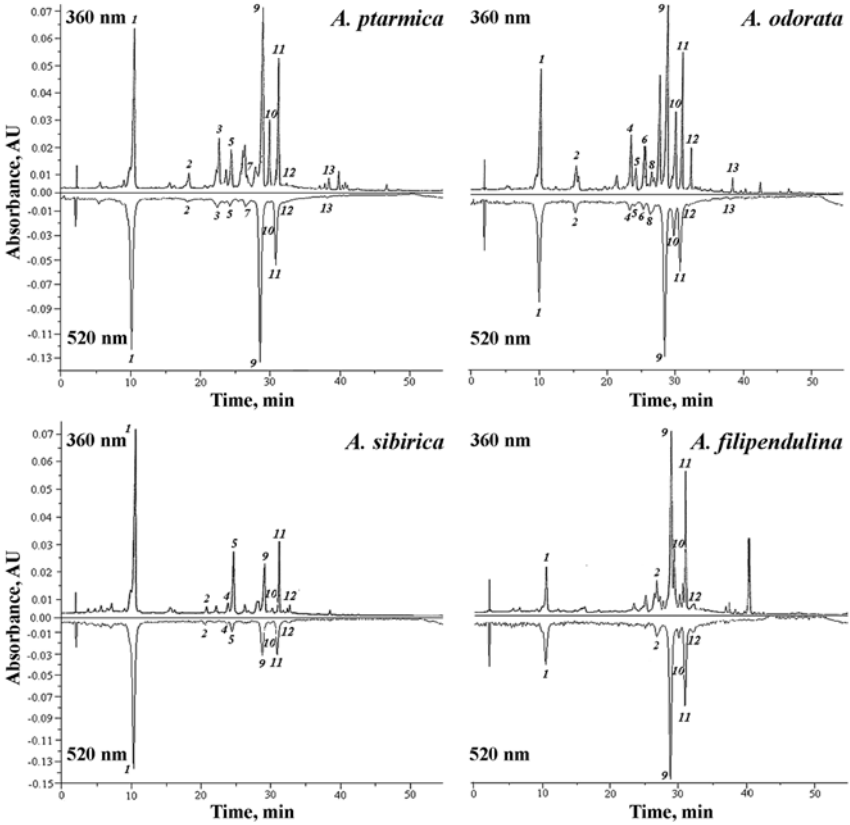

Fig. 7. *Chromatographic profiles of on-line HPLC-UV-DPPH quenching assay of extracts from Achillea ptarmica, A. odorata, A. sibirica and A. filipendulina herb raw materials (Refer to Table 9 for details of analytes).*

It can be concluded that developed on-line HPLC-DPPH method is sensitive and can be used for rapid, selective and reliable pinpointing of individual radical scavenging compounds, as well as for determination of their contribution to antiradical activity of complex mixtures. Extracts from the investigated taxa of the genus *Achillea* possessed antiradical activity which is due to the presence of radical scavenging components within a complex of secondary metabolites. The main radical scavengers in ethanolic extracts from plants of investigated yarrow species were confirmed to be esters of caffeic acid with quinic acid. Among the identified flavonoids o-dihydroxyl group-bearing rutin and luteolin derivatives, namely luteolin-7-O-glucoside and luteolin-7-O-glucuronide exhibited significant scavenging effects against DPPH radicals.

Effect of *Achillea millefolium* herb extract on the rat heart mitochondrial function

The influence of ethanolic extract from *A. millefolium* herb (YE) on the oxidative phosphorylation of rat heart mitochondria was investigated. The experiments showed that YE at the concentrations studied had no effect on the basal (State 2, not related to ATP synthesis) respiration rate (V_2) of mitochondria with complex I-dependent substrate- pyruvate + malate (Table 10). Addition of YE up to 2.5 μL/1.5 mL (total content of the identified phenolics 276.3 ng/mL) had no effect on State 3 respiration rate (V_3) as well (Fig. 8 and Table 10). However, the further increase in YE concentration caused the inhibition of State 3 respiration rate by 8 % and 12 % (5 and 10 μL/1.5 mL of YE, respectively). Higher amounts of YE (15 μL/1.5 mL) induced a significant decrease in State 3 respiration rate by 53 % (from 257.6±45.8 to 120.3±38.5 nmolO/min/mg; $p<0.05$). Accordingly, mitochondrial respiratory control index (RCI), i.e. the ratio between States 3 and 2 respiration rates, at lower used amounts of YE (2.5, 5 and 10 μL/1.5 mL) was unchanged, whereas the highest YE concentration induced a significant decrease in RCI from 6.06 (control) to 2.98 (15 μL/1.5 mL of YE).

Table 10. *Effects of Achillea millefolium herb extract (YE) on rat heart mitochondrial respiratory parameters when oxidizing pyruvate + malate*

	Substrate pyruvate + malate				
	Control	**YE (2.5 µL/ 1.5 mL)**	**YE (5 µL/ 1.5 mL)**	**YE (10 µL/ 1.5 mL)**	**YE (15 µL/ 1.5 mL)**
V_2	47.5±9.3	47.5±9.7	38.3±6.3	42.3±1.9	42.9±3.2
V_2 + YE	–	49.1±8.6	39.4±8.0	42.45±5.9	43.5±5.9
V_3	257.6±45.8	246.0±32.5	236.5±18.5	226.0±40.5	120.3±38.5*
V_{ATR}	39.8±12.7	38.0±7.1	25.8±5.5	30.5±13.5	33.2±5.2
$V_{ATR + Cyt\ c}$	86.7±25.2	95.6±19.9	80.6±37.5	84.0±39.1	93.3±18.8
RCI	6.06±1.7	5.87±1.7	6.27±0.6	5.31±0.7	2.98±1.2*

Measurements were performed in the presence of 5 mM pyruvate + 5 mM malate. V_2 – State 2 respiration rate in the presence of 0.5 mg/mL of mitochondria and substrates; V_3 – State 3 respiration rate in the presence of 1 mM ADP; V_{ATR} – State 4 respiration rate in the presence of 0.12 mM atractyloside; $V_{ATR+Cyt\ c}$ – State 4 respiration rate after the addition of 32 µM cytochrome *c*. Mitochondrial respiratory control index (RCI), i.e. the ratio between oxygen uptake rates in State 3 and State 2 (RCI = V_3/V_2). *p<0.05 vs. respective control; n=3.

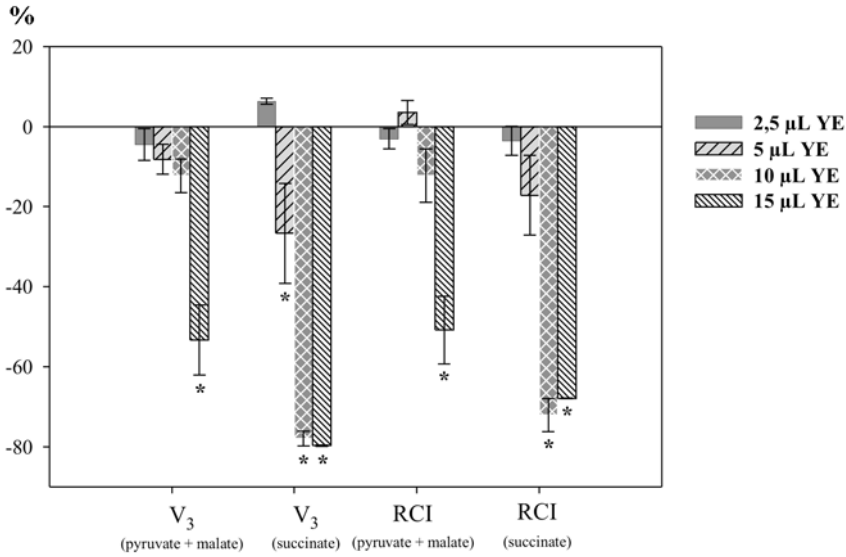

Fig. 8. *Effects of Achillea millefolium herb extract (YE) on mitochondrial State 3 respiration rate (V_3) and respiratory control index (RCI). Substrates were 5 mM pyruvate + 5 mM malate or 15 mM succinate (+ 2 mM amytal). For explanation see Table 10. *p<0.05 vs. respective control; n=3.*

Other experiments aimed to check the action of YE on complex II-dependent substrate- succinate oxidation. The obtained data showed that YE at the concentrations up to 2.5 µL/1.5 mL had no effect on State 3 respiration rate (Fig. 8 and Table 11). Higher amounts of YE induced significant inhibition of mitochondrial respiration, V_3 decreased by 27 %, 78 % and 80 % in the presence of 5, 10 and 15 µL/1.5 mL of YE, respectively. RCI decreased by 17 %, 72 % and 68%, respectively. The results described above demonstrate that succinate oxidation was more affected by YE, as compared to pyruvate + malate. In order to clarify the reason for YE induced diminishing of State 3 respiration rate, carbonyl cyanidine m-chlorophenylhydrazone (CCCP) was added, which acts as a classical uncoupler of oxidative phosphorylation and allows respiration to continue without the ATP synthesis. The results presented in Table 12 show that in the presence of the highest used amounts of YE V_{CCCP} decreased significantly by 25 % and 12 % (with pyruvate + malate and succinate, respectively; $p<0.05$).

Table 11. *Effects of Achillea millefolium herb extract (YE) on rat heart mitochondrial respiratory parameters when oxidizing succinate*

	Substrate succinate				
	Control	**YE (2.5 µL/ 1.5 mL)**	**YE (5 µL/ 1.5 mL)**	**YE (10 µL/ 1.5 mL)**	**YE (15 µL/ 1.5 mL)**
V_2	133.2±16.1	147.8±37.3	116.3±6.8	106.1±0.1	84.6±5.5
V_2 + YE	–	124.5±63.5	92.2±40.8	59.8±13.2*	61.2±4.6*
V_3	251.9±37.8	267.8±68.3	184.8±76.3*	55.6±9.4*	51.2±4.7*
V_{ATR}	140.7±23.5	131.1±61.9	87.3±33.7	52.3±8.6*	46.0±3.8*
$V_{ATR + Cyt\ c}$	218.0±18.6	176.5±21.5	224.5±23.5	95.4±19.6	74.6±13.4*
RCI	1.88±0.1	1.81±0.0	1.56±0.6	0.52±0.1*	0.60±0.0*

Substrate: 15 mM succinate (+ 2mM amytal). For explanation see Table 10. *$p<0.05$ vs. respective control; n=3.

Table 12. *Effects of Achillea millefolium herb extract (YE) on uncoupled from phosphorylation respiration rate of mitochondria energized with pyruvate + malate and succinate*

	Substrate pyruvate + malate		**Substrate succinate**	
	Control	**YE (15 µL/1.5 mL)**	**Control**	**YE (15 µL/1.5 mL)**
V_{CCCP}	447.0±12.6	336.0±28.9*	272.0±2.9	240.0±5.2*
$V_{CCCP+Cyt\ c}$	643.0±21.9	426.0±49.0*	453.5±19.5	369.5±8.5*

Measurements were performed in the presence of 5 mM pyruvate + 5 mM malate and 15 mM succinate (+ 2mM amytal) as substrates. When measuring uncoupled from phosphorylation respiration rate of mitochondria 0.19 µM of carbonyl cyanidine m-chlorophenylhydrazone (CCCP) was added into the incubation medium instead of ADP. V_{CCCP} – respiration rate in the presence of 0.19 µM CCCP; $V_{CCCP+Cyt\ c}$ – uncoupled from phosphorylation respiration rate of mitochondria in the presence of 32 µM cytochrome *c*. *p<0.05 vs. respective control; n=3.

Thus, one of the reasons for the inhibition of the State 3 respiration is the decreased activities of mitochondrial respiratory chain complexes I and II, rather than the lack of cytochrome *c*, since exogenous cytochrome *c* ($V_{CCCP+cyt\ c}/V_{CCCP}$) stimulates respiration of the control mitochondria by the same degree as in case of the presence of YE (1.44 and 1.27-fold with pyruvate + malate and 1.67 and 1.54-fold with succinate). This shows that YE did not affect the integrity of mitochondrial outer membrane.

Our studies on N,N,N‘,N‘,-tetramethyl-p-phenylenediamine (0.5 mM) and ascorbate (10 mM) oxidation showed that YE had no effect on the activity of complex IV, also called cytochrome oxidase (control, 600±50 nmolO/min/mg; in the presence of 15 µL/1.5 mL of YE, 690±40 nmolO/min/mg).

Because of a more pronounced decrease in V_3 than V_{CCCP} (-53 % and -25 % with pyruvate + malate; -80 % and -12 % with succinate, respectively), one may conclude that the inhibition of pyruvate + malate and succinate oxidation caused by YE may be not only due to diminished activities of respiratory chain complexes, but also due to the inhibition of F_1F_0ATP-ase and ADP/ATP translocator.

Further experiments were performed to study the effects of YE on the intactness of the inner mitochondrial membrane. It was found that the permeability of the inner mitochondrial membrane was unchanged when measured both in the presence and in the absence of YE, because the respiration rates in State 2 (V_2) and State 4 (V_{ATR}) were not increased in the presence of YE with substrate pyruvate + malate (Table 10). When oxidizing succinate, the permeability of the inner mitochondrial membrane was unchanged as well (Table 11), whereas higher YE concentrations (5, 10 and 15 μL/1.5 mL) induced a decrease in State 2 and State 4 respiration rates of mitochondria, which could be due to inhibition of mitochondrial respiratory chain. Moreover, YE had no effect on the mitochondrial State 4 respiration rate in the presence of exogenous cytochrome *c* ($V_{ATR+Cyt\ c}$ with both substrates: pyruvate + malate and succinate). This shows that YE had no effect on the integrity of the inner and outer mitochondrial membranes.

In order to evaluate whether the identified phenolic compounds from *A.millefolium* were responsible for the observed effects on mitochondrial function, the effects of mixture of pure phenolic compounds (MPC; such as 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 measured on mitochondrial respiration. Different amounts of MPC were added into the incubation medium during State 2 respiration of mitochondria: 1, 2, 5 and 10 μL/1.5 mL. It is interesting to note that MPC, in contrast to YE, had no effect on mitochondrial State 3 respiration rate (Table 13). Hence, YE induced decrease in State 3 respiration of mitochondria might be caused by additional, not further identified, plant compounds. Moreover, MPC was found to have stimulated the State 2 respiration rate (1.10-1.28-fold, $V_{2(+MPC)}/V_2$). This shows that MPC possesses a slight uncoupling effect on oxidative phosphorylation in mitochondria. Cytochrome *c* increased the State 4 respiration rate ($V_{ATR+Cyt\ c}/V_{ATR}$) in similar degree (1.8-1.6-fold) in both control mitochondria (without MPC) and mitochondria treated with MPC. Thus, MPC had no effect on the permeability of mitochondrial outer membrane, what is in accordance with our results obtained with YE.

Table 13. *Effects of mixture of phenolic compounds (MPC) on rat heart mitochondrial respiratory parameters when oxidizing pyruvate + malate*

	Substrate pyruvate + malate				
	Control	**MPC (1 µL)**	**MPC (2 µL)**	**MPC (5 µL)**	**MPC (10 µL)**
V_2	55.7±8.5	54.1±6.8	56.6±7.9	64.2±8.0	69.4±8.2
V_2 + MPC	–	62.0±6.2	62.1±6.7	72.9±7.5	88.5±8.3
$V_{2(+MPC)}/V_2$	–	1.15±0.1	1.10±0.0	1.14±0.1	1.28±0.2
V_3	357.7±40.9	376.0±37.9	358.0±33.6	395.0±38.5	349.0±31.2
V_{ATR}	61.0±9.9	60.2±9.1	57.4±8.2	60.3±8.9	74.4±9.6
$V_{ATR+Cyt\ c}$	112.0±5.3	106.0±4.8	101.0±4.1	102.0±4.3	117.0±5.0
RCI	6.42±0.9	6.95±0.7	6.33±0.6	6.15±0.6	5.03±0.5
$V_{ATR+Cyt\ c}/V_{ATR}$	1.8±0.2	1.8±0.2	1.8±0.2	1.7±0.1	1.6±0.0

Measurements were performed in the presence of 5 mM pyruvate + 5 mM malate. For explanation see Table 10; n=3.

The obtained data showed that the lowest used concentrations of YE had no effect on State 3 respiration rate of mitochondria with both used substrates: pyruvate + malate and succinate. Whereas, State 3 respiration rate was negatively affected by higher amounts of YE with all respiratory substrates. It has been reported that slight inhibition in State 3 respiration rate leading to increase in the generation of reactive oxygen species could be beneficial for the cell in mediating its protection, since ROS play an important role in many redox-sensitive signalling pathways [Halestrap et al., 2007; Kabir et al., 2005; 2006]. Moreover, inhibitory effects of YE on mitochondrial respiration adds new insights to the antimicrobial activity of *A.millefolium* since the mechanism of energy transduction is similar in mitochondria and in bacteria, one may assume that YE might inhibit bacteria respiration and energy production.

The obtained results showed that the inhibitory effects of YE on rat heart mitochondrial respiration were found to be depended on the concentration of YE. Hence, these findings in *in vitro* experiments give first insights into the molecular mechanism(s) of *A.millefolium* action on cellular energy metabolism. However, further studies are necessary for a more comprehensive evaluation of the complex effects of the pure phenolic compounds as well as *A.millefolium* extract as a multiple component mixture on mitochondrial function.

CONCLUSIONS

1. The RP-HPLC method for separation of the flavonoid and phenolcarbonic acid complex in ethanolic extract of *A. millefolium* was developed. The method proved to be suitable for determination of the qualitative and quantitative phenolic composition in the ethanolic extracts of yarrow. Furthermore, the main validation steps of HPLC method were evaluated. The method shows a good selectivity, linearity and precision, and it might be useful for the quality control analysis of yarrow raw materials and preparations.
2. The phytochemical investigations on *A. millefolium* herbs from wild populations have revealed a considerable variation in accordance with phenolic compounds. The total content of the identified phenolics in wild populations of yarrow varied from 12.605 to 27.993 mg/g. Chlorogenic acid (5.577–16.799 mg/g) predominated in the mixture of identified secondary metabolites. Regarding the composition of the flavonoid complex, the patterns of distribution in the samples of yarrow herb are characterized by the dominance of apigenin-7-O-glucoside (2.348–7.888 mg/g) and luteolin-7-O-glucoside (1.342–3.938 mg/g).
3. Significant qualitative and quantitative plant-part variations in composition of phenolic compounds on *A. millefolium* were detected, thus confirming organ-dependence of secondary metabolites accumulation. The patterns of flavonoid 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. Rutin and luteolin-7-O-glucoside were the main components among the identified flavonoids in yarrow leaves, while in stems the dominant flavonoids were rutin and apigenin-7-O-glucoside. Vegetative organs are characterized by significant dominance of chlorogenic acid within the complex of identified secondary metabolites.
4. The research data revealed that there is a close relationship between phenolic compounds composition in plant parts and the

development stage during phenological cycle of *A. millefolium*. Concentrations of flavonoids increased with advancing of plant seasonal development and the highest levels were reached at flowering time (on the average 15.475 mg/g). On the contrary, chlorogenic acid content of aerial plant parts decreased linearly with advancing of phenological stages and the highest levels were observed during the initial phases of plant development for respective plant parts: in leaves at rosette 50.165 mg/g; in stems at vegetative 18.717 mg/g; in reproductive parts at floral budding 27.570 mg/g.

5. The evaluated other species of *Achillea* genus, both spontaneous and introduced, displayed differences in the quantitative and qualitative composition of flavonoids. Hence, the pattern of flavonoid distribution seems to be of chemotaxonomic relevance.
6. Extracts from *Achillea* plants possessed antiradical activity, related to the presence of radical scavenging components within the complex of flavonoids and phenolcarbonic acids. Mono- and dicaffeoylquinic acids were confirmed to be the principal radical scavengers contributing most to the radical scavenging activity of extracts from *Achillea* plants. While among the identified flavonoids rutin, luteolin-7-O-glucoside, luteolin-7-O-glucuronide and luteolin were found to possess radical scavenging properties. Radical scavenging activities of extracts from spontaneous *Achillea* species were higher than that of extracts from introduced species.
7. Extract of *A. millefolium* herb in dose-dependent manner induces the decrease in State 3 respiration rate in rat heart mitochondria without any changes in the integrity of the outer and inner mitochondrial membranes. Oxidation of complex II dependent substrate succinate was more affected by yarrow extract, as compared to oxidation of complex I dependent substrate pyruvate + malate. The diminished capacity of mitochondria to synthesize ATP caused by yarrow extract could be due to decreased activities of mitochondrial respiratory chain complexes I and II, as well as due to the inhibition of F_1F_0ATP-ase and ADP/ATP translocator.

LIST OF PUBLICATIONS

Publications corresponding to the list of the Institute of Scientific Information (ISI):

1. **Benetis R, Radušienė J, Jakštas V, Janulis V, Malinauskas F.** Development of an RP-HPLC Method for the Analysis of Phenolic Compounds in *Achillea millefolium* L. *Journal of Liquid Chromatography & Related Technologies.* 2008; 31(4): 596-610.
2. **Benetis R, Radušienė J, Jakštas V, Janulis V, Puodžiūnienė G, Milašius A.** Quantitative HPLC determination of phenolic compounds in yarrow. *Pharmaceutical Chemistry Journal* 2008; 42(3): 153–6.
3. **Benetis R, Radušienė J, Janulis V.** Variability of phenolic compounds in flowers of *Achillea millefolium* wild populations in Lithuania. *Medicina* 2008; 44(10): 775-81.

Conference abstracts:

1. **Benetis R, Radušienė J, Janulis V.** Comparative study of sample preparation procedures and validation of an HPLC method for determination of phenolic compounds in *Achillea millefolium* L. 4th Nordic Separation Science Society (NoSSS) International Conference. Kaunas, August 26-29, 2007. Abstracts book and final program. p. 74.
2. **Benetis R.** Paprastosios kraujažolės (*Achillea millefolium* L.) augalų tyrimas ESC metodu. Jaunimas siekia pažangos: jaunųjų mokslininkų konferencijos straipsnių rinkinys. Akademija, 2007, Nr. 2. p. 118-21.

SANTRAUKA

Darbo aktualumas. *Achillea millefolium* L. yra vienas seniausiai vartojamų vaistinių augalų Lietuvoje, kuris plačiai vartojamas tiek liaudies, tiek šiuolaikinėje medicinoje [Radušienė, Gudaitytė, 2005]. Kraujažolės (*Achillea* L.) genties augalai yra labai plačios ekologinės amplitudės, plačiai paplitę tiek Lietuvoje, tiek pasaulyje. Lietuvoje natūraliai auga trys kraujažolių rūšys: *Achillea millefolium*, *Achillea cartilaginea* Ledeb. ex Rchb. ir *Achillea ptarmica* L. Savaiminės *A. cartilaginea* ir *A. ptarmica* aptinkamos retai ir dažniausiai tik tipinėse jų augavietėse, o *A. millefolium* plačiai paplitusi ir dažna visoje šalies teritorijoje [Galinis, 1980].

Europos šalyse kraujažolių vaistinė žaliava patenka tarp dažniausiai parduodamų vaistažolių [Hanlidou et al., 2004; Saukel, Kubelka, 1994]. Svarbiausias kriterijus vertinant vaistinės augalinės žaliavos kokybines charakteristikas yra biologiškai aktyvių junginių sudėties kokybiniai ir kiekiniai rodikliai. Kraujažolių žaliavoje nustatyta daug biologiškai aktyvių junginių grupių: terpenai, alkaloidai ir jų bazės, taninai, lignanai, kumarinai, sterolai, vitaminai, amino ir riebalų rūgštys. Mūsų nuomone, mokslinės literatūros šaltiniuose pateikti duomenys apie fenolinių junginių kiekinę ir kokybinę sudėtį, Lietuvoje ruošiamoje kraujažolių žaliavoje, nepakankami. Nėra duomenų apie šių junginių kaupimosi dėsningumus kraujažolių generatyviniuose ir vegetatyviniuose organuose. Tikslinga buvo nustatyti flavonoidų ir fenilpropanoidų kokybinę ir kiekinę sudėtį ir jos įvairavimo tendencijas. Biologiškai aktyvių junginių kaupimosi dėsningumų tyrimai atskirose augalo dalyse yra svarbūs vertinant galimybę juos vartoti gydymo tikslams ir siekiant ištirti cheminių junginių įvairavimo prigimtį bei jų svarbą metabolizmo procesuose.

Lietuvoje *A. millefolium* vaistinė žaliava paprastai renkama gamtinėse cenopopuliacijose, todėl reikalingos žinios apie žaliavų kokybės parametrus bei jų reikšmių variaciją skirtinguose krašto regionuose rinktoje vaistinėje augalinėje žaliavoje.

Flavonoidų ir fenilpropanoidų sudėties įvairavimo nustatymas skirtingose augavietėse yra svarbus siekiant racionaliau organizuoti žaliavų rinkimą gamtoje. Siekiant tinkamai vykdyti vaistinių augalinių

žaliavų paruošas, būtina įvertinti fenolinių junginių kokybinės ir kiekinės sudėties kitimo dinamiką augalų fenologinių tarpsnių kaitoje.

Farmakologinį augalinių preparatų poveikį sukelia ne viena, o kelios veikliųjų junginių grupės. Tokiu būdu pasireiškia sinergistinis poveikis, nulemiantis terapinį fitopreparatų aktyvumą [Capasso et al., 2000; Wagner, 1999]. Tai patvirtina ir didėjantis susidomėjimas *A. millefolium* fenoliniais junginiais (flavonoidais ir fenilpropanoidais). Jų sudėties kokybiniai ir kiekiniai rodmenys gali būti naudingi, kaip papildomi parametrai, kompleksiniam *A. millefolium* žaliavų ir iš jų gaminamų preparatų poveikio vertinimui.

Tikslinga ištirti *Achillea* genties rūšių augalinių žaliavų ekstraktų ir juose randamų fenolinių junginių antioksidantinį efektyvumą įvertinant jų gebėjimą sujungti laisvuosius radikalus bei poveikį mitochondrijų oksidacinio fosforilinimo sistemos funkcionavimui. Mitochondrijų oksidacinė pažaida yra susijusi su daugeliu ligų bei organizmo funkcijų pažeidimų, tokių kaip širdies ir kraujagyslių, neurodegeneracinės ligos, senėjimas, vėžys, cukrinis diabetas. Ekstraktų iš augalinių žaliavų, kaupiančių flavonoidus ir fenilpropanoidus, antioksidantinių savybių ir poveikio mitochondrijų funkcijoms mechanizmų ištyrimas suteiktų galimybę kryptingai veikti jose vykstančius procesus ir tokiu būdu apsaugoti ląsteles nuo oksidacinio streso sukeliamų pažaidų.

Vaistinių augalinių žaliavų kokybės ir biologiškai aktyvių junginių įvairavimo įvertinimui būtina taikyti šiuolaikinius analizės metodus bei įteisinti jų metodikas, kurios leistų patikimai, tiksliai bei atkartojamai tirti biologiškai aktyvių junginių pasiskirstymą augalų organuose bei fitopreparatuose. Kuriant analizės metodikas svarbu optimizuoti ekstrakcijos sąlygas, įvertinti ekstrahento poliškumo bei ekstrahavimo metodo įtaką bioaktyvių junginių sudėties kokybiniams ir kiekiniams rodmenims.

Flavonoidų ir fenilpropanoidų sudėties įvairavimo dėsningumų nustatymas augaluose bei šių junginių biologinių savybių pažinimo plėtojimas yra svarbūs veiksniai, padedantys kompleksiškai įvertinti augalinių žaliavų kokybines charakteristikas bei tikslines panaudojimo perspektyvas.

Darbo tikslas – ištirti Lietuvoje natūraliai augančių ir kultūroje auginamų kraujažolių (*Achillea* L.) augalinių žaliavų flavonoidų ir fe-

nilpropanoidų kokybinę ir kiekinę sudėtį, jos įvairavimą bei biologinį poveikį.

Uždaviniai:

1. Sukurti efektyviosios skysčių chromatografijos (ESC) metodiką flavonoidų ir fenilpropanoidų analizei kraujažolių žaliavose.
2. Ištirti natūraliose augavietėse surinktų *A. millefolium* vaistinės augalinės žaliavos ėminių fenolinių junginių sudėtį bei jos kokybinių ir kiekinių rodiklių įvairavimą skirtingose cenopopuliacijose.
3. Įvertinti flavonoidų ir fenilpropanoidų sudėtį bei jos rodiklių pokyčius *A. millefolium* stiebų, lapų ir žiedų augalinėse žaliavose fenologinių tarpsnių kaitoje.
4. Įvertinti kitų (*A. ptarmica*, *A. odorata*, *A. sibirica* ir *A. filipendulina*) rūšių fenolinių junginių sudėtį bei jos rodmenų kitimus fenologinių tarpsnių kaitoje.
5. Įvertinti Lietuvoje natūraliai augančių bei introdukuotų *Achillea* genties rūšių augalinių žaliavų ekstraktų antiradikalinį aktyvumą.
6. Ištirti *A. millefolium* žolės ekstrakto poveikį žiurkės širdies mitochondrijų oksidaciniam fosforilinimui.

Mokslinio darbo naujumas. Pirmą kartą nustatyta flavonoidų ir fenilpropanoidų kokybinė ir kiekinė sudėtis Lietuvoje augančių kraujažolių vaistinėje žaliavoje. Pirmąkart nustatytas fenolinių junginių sudėties įvairavimas skirtingų *A. millefolium* cenopopuliacijų žaliavose, įvertinta *A. millefolium* flavonoidų ir fenilpropanoidų sudėtis ir jos rodiklių kitimų dinamika atskiruose augalų organuose fenologinių tarpsnių kaitoje. Nustatytas flavonoidų ir fenilpropanoidų pasiskirstymo pobūdis bei įvairavimo dėsningumai kitų kraujažolių rūšių augalinėse žaliavose. Sukurta ESC metodika fenolinių junginių kiekiniam nustatymui *Achillea* genties rūšių augaluose, kurios dėka vykdomas efektyvus flavonoidų ir fenilpropanoidų išskirstymas panaudojant blokuotą amidinę atvirkščių fazių įkrovą. Kraujažolės genties rūšių augalinėse žaliavose identifikuoti pagrindiniai flavonoidų ir fenilpropanoidų komplekso komponentai sujungiantys laisvuosius radikalus bei įvertintas jų antiradikalinis aktyvumas.

Bendradarbiaujant su KMU BMTI Biochemijos laboratorijos moks-

lininkais nustatytas kraujažolių žolės ekstrakto poveikis žiurkės širdies mitochondrijų funkcijoms. Šie tyrimai yra nauji ir aktualūs, kadangi iki šiol nei Lietuvoje, nei pasaulyje tai nebuvo tyrinėta.

Praktinė ir teorinė reikšmė. Sukurta ESC metodika flavonoidų ir fenilpropanoidų kokybiniam ir kiekiniam nustatymui *Achillea* genties rūšių augalinėse žaliavose. Ji gali būti naudojama žaliavos kokybės įvertinimui, nustatant fenolinių junginių kokybinės ir kiekinės sudėties rodiklius.

Gauti rezultatai tiriant *A. millefolium* augalinių žaliavų ekstraktų ir juose randamų fenolinių junginių biologinį aktyvumą (poveikį ląstelės energetiniams procesams ir jų gebėjimą sujungti laisvuosius radikalus) yra neabejotinai naudingi kuriant naujus augalinius vaistinius preparatus, pasižyminčius priešuždegiminiu, antimikrobiniu, antioksidantiniu bei antidiabetiniu poveikiu.

Ištirta kitų *Achillea* genties rūšių augalinių žaliavų cheminė sudėtis, siekiant galimo šių augalų teikiamų žaliavų panaudojimo medicinos praktikoje ir plečiant medicinoje vartojamų kraujažolių rūšių asortimentą. Atlikti *A. millefolium* cenopopuliacijose surinktų augalinių žaliavų veikliųjų junginių kokybinės ir kiekinės sudėties tyrimai, kurie yra svarbūs detalesniam gamtinių augalų resursų įvertinimui, genetinės įvairovės išsaugojimui bei išsamesniam *Achillea* genties evoliucinių procesų dinamikos supratimui. Tyrimų rezultatai parodė, kad *A. millefolium* vaistinės augalinės žaliavos rinkimas natūraliose augavietėse, dėl jai būdingo cheminio heterogeniškumo, gali sąlygoti ženklų jos kokybę apibūdinančių rodmenų įvairavimą. Norint medicinos praktikoje vartoti homogenišką ir geros kokybės kraujažolių vaistinę žaliavą, tikslinga atrinktus augalus auginti kultūroje.

Gauti fenolinių junginių sudėties kitimų duomenys fenologinių tarpsnių kaitoje gali būti svarbūs kompleksiniam augalo vystymosi metu vykstančių antrinių metabolitų biosintezės ir jų transportavimo procesų dėsningumų supratimui. Įvertinus kitų *Achillea* rūšių augalinių žaliavų cheminės sudėties kokybinius ir kiekinius rodmenis nustatyta, kad jie ženkliai įvairuoja, o flavonoidų pasiskirstymo pobūdis gali būti svarbiu chemotaksonominiu požymiu.

Kritinių išvystytos ESC metodikos įteisinimo žingsnių įverčiai patvirtina jos tinkamumą patikimam kraujažolių vaistinių augalinių ža-

liavų bei iš jų gautų farmacinių preparatų kokybinių charakteristikų įvertinimui.

Atlikti *Achillea* genties rūšių augalinių žaliavų ekstraktų antiradikalinio aktyvumo tyrimai. Nustatyti stipriausiomis antiradikalinėmis savybėmis pasižymintys biologiškai aktyvūs junginiai. Kraujažolių ekstraktų gebėjimas sujungti laisvuosius radikalus priklauso nuo flavonoidų ir fenilpropanoidų komplekso kokybinės ir kiekinės sudėties. Tyrimų rezultatai bus naudojami kuriant preparatus, maisto papildus su išreikštu antiradikaliniu poveikiu ir juos įdiegiant į medicinos praktiką.

Darbo rezultatų aprobavimas. Tyrimų rezultatai pristatyti 4 konferencijose: Baltijos jūros regiono šalių genofondinių augalų panaudojimas selekcijai ir mainams (Kaunas, 2006 m. spalio 4–6 d.), Žmogaus ir gamtos sauga (Akademija, 2007 m. gegužės 16–19 d.), Jaunimas siekia pažangos (Akademija, 2007 m. balandžio 13–14 d.), 4th Nordic Separation Science Society (NoSSS) International Conference (Kaunas, 2007 m. rugpjūčio 26–29 d.). Tyrimų tematika paskelbti 3 moksliniai straipsniai.

Darbo apimtis ir struktūra. Disertaciją sudaro įvadas, literatūros apžvalga, tyrimo objektas ir metodai, tyrimų rezultatai ir jų aptarimas, išvados, literatūros sąrašas (335 literatūros šaltiniai), disertacijos tema paskelbtų darbų sąrašas. Darbe pateikta 22 lentelės, 34 paveikslai, 2 priedai. Disertacijos apimtis 130 puslapių.

IŠVADOS

1. Sukurta efektyviosios skysčių chromatografijos metodika, įgalinanti atrankų flavonoidų ir fenilpropanoidų komplekso komponentų nustatymą *A. millefolium* augalinėse žaliavose. Gauti kritinių metodikos įteisinimo žingsnių įverčiai patvirtina jos jautrumą, atrankumą, tiesiškumą ir tikslumą, todėl sukurta metodika gali būti taikoma kraujažolių vaistinių augalinių žaliavų bei iš jų gautų vaistinių preparatų kokybinių charakteristikų įvertinimui.
2. Gamtinių *Achillea millefolium* cenopopuliacijų vaistinės žaliavos flavonoidų ir fenilpropanoidų kokybinė ir kiekinė sudėtis parodė, kad identifikuotų antrinių metabolitų komplekso sudė-

tyje dominuoja chlorogeno rūgštis (5,577-16,799 mg/g), o tarp flavonoidų būdingas pasiskirstymas su vyraujančiais apigenin-7-O-gliukozidu (2,348–7,888 mg/g) ir liuteolin-7-O-gliukozidu (1,342–3,938 mg/g). Suminis identifikuotų fenolinių junginių kiekis skirtingų cenopopuliacijų vaistinėse žaliavose įvairavo nuo 12,605 iki 27,993 mg/g.

3. *A. millefolium* augalinėms žaliavoms būdingas fenolinių junginių kokybinės ir kiekinės sudėties kintamumas, tiesiogiai priklausantis nuo augalo morfologinės dalies. Žiedų žaliavoms charakteringas flavonoidų pasiskirstymas su vyraujančiais apigenin-7-O-gliukozidu ir liuteolin-7-O-gliukozidu bei aglikonais – apigeninu ir liuteolinu. Lapuose identifikuotų flavonoidų komplekse vyrauja rutinas ir liuteolin-7-O-gliukozidas, o stiebuose – rutinas bei apigenin-7-O-gliukozidas. Vegetatyviniuose organuose nustatytas žymus chlorogeno rūgšties dominavimas identifikuotų antrinių metabolitų komplekso sudėtyje.
4. *A. millefolium* fenolinių junginių sudėtis antžeminėse augalų dalyse tiesiogiai priklauso nuo sezoninio vystymosi fenologinių tarpsnių. Nustatyta, kad flavonoidų kiekiai didėjo augalui fenologiškai progresuojant, o didžiausios jų sankaupos pasiekiamos žydinčiuose augaluose (vidutiniškai 15,475 mg/g). Tuo tarpu, didžiausi chlorogeno rūgšties kiekiai rasti atitinkamos augalo dalies pradinėse vystymosi fazėse (lapuose skrotelės fazėje – 50,165 mg/g; stiebuose vegetatyvinėje fazėje – 18,717 mg/g; generatyviniuose organuose butonizacijos fazėje – 27,570 mg/g), o augalui vystantis jos kiekis, visose antžeminėse augalo dalyse, mažėjo.
5. Nustatyti kitų savaiminių Lietuvoje augančių ir introdukuotų *Achillea* rūšių augalinių žaliavų fenolinių junginių kiekinės ir kokybinės sudėties skirtumai. Flavonoidų pasiskirstymo pobūdis gali turėti chemotaksonominę reikšmę.
6. Nustatyta, kad *Achillea* rūšių augalinių žaliavų ekstraktų gebėjimas sujungti laisvuosius radikalus yra tiesiogiai susijęs su antiradikališkai aktyvių flavonoidų ir fenilpropanoidų pasiskirstymo dėsningumais augalinėse žaliavose. Mono- ir dikafeilchino rūgštys yra svarbiausios laisvųjų radikalų sujungėjos nulemiančios

antiradikalines tirtų žaliavų savybes. Identifikuotų flavonoidų komplekse gebėjimas sujungti laisvuosius radikalus būdingas rutinui, liuteolin-7-O-gliukozidui, liuteolin-7-O-gliukuronidui bei liuteolinui. Savaiminių *Achillea* rūšių augalinės žaliavos pasižymi stipresnėmis antiradikalinėmis savybėmis nei introdukuotų kraujažolių rūšių.

7. *A. millefolium* žolės ekstraktas *in vitro*, priklausomai nuo kiekio sukelia žiurkės širdies mitochondrijų kvėpavimo greičio trečioje metabolinėje būsenoje slopinimą, tačiau neveikia mitochondrijų vidinės bei išorinės membranos intaktiškumo. Kraujažolių ekstrakto poveikiui jautresnė yra mitochondrijų kvėpavimo grandinės II komplekso substrato sukcinato oksidacija, lyginant su I kvėpavimo grandinės komplekso substrato piruvato + malato oksidacija. Kraujažolių žolės ekstrakto sukeltas ATP sintezės slopinimas pasireiškia ne tik dėl mitochondrijų kvėpavimo grandinės I ir II kompleksų, bet ir dėl ATP sintazės bei ADP/ATP nešiklio slopinimo.

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