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Comparison of the chemical composition of four yarrow (*Achillea millefolium* L.) morphotypes

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Four morphotypes – white, pink, deep pink and red – of *Achillea millefolium* L. are cultivated in the collection of medicinal plants at the Kaunas Botanical Garden of Vytautas Magnus University. The colour of linguiform flowers may be affected by a different phenotype or genotype of plants, therefore phytochemical investigations of four yarrow morphotypes were started. The main goal in this work was to evaluate differences in the total content of flavonoids and essential oils and to identify and quantify principal components of essential oils in various *Achillea millefolium* L. morphotypes. Gas chromatography was used for quantifying essential oil components in flowers and herb. Flowers contained more essential oils than did herb in all morphotypes of *Achillea millefolium* L. The total content of flavonoids ranged within 0.05–0.07%. The highest content of flavonoids was determined in the deep pink morphotype, and the content of essential oil was highest in the white morphotype of *Achillea millefolium* L. The total content of flavonoids and the essential oil composition of the white morphotype of *Achillea millefolium* L. were determined at different vegetation periods.

Key words: *Achillea millefolium* L., gas chromatography, gas chromatography mass spectrometry, essential oil components, flavonoids

INTRODUCTION

The diversity of medicinal and aromatic plant species and varieties is important from both scientific and practical points of view. In the 21st century, attention is focused on the cultivation and preservation of medicinal and aromatic plants and on the evaluation of their quality [1]. It is important to increase the assortment of cultivated medicinal plants, to accumulate and study samples of introduced and acclimatized plants *ex situ* in the collections at present and in the future, thus preserving and enriching the genetic fund of useful plants of Lithuania [2]. Yarrow (*Achillea millefolium* L.) is a perennial herb of the *Asteraceae* family, *Dilleniidae* subclass, *Magnoliopsida* class, *Magnoliophyta* section, native in Asia and Europe including Lithuania [3].

Achillea millefolium L. (yarrow) is a well-known medicinal plant, widely used in folk medicine against gastrointestinal disorders, lack of appetite [4]. More than 120 chemical compoounds have been identified in *Achillea millefolium* L. [5]. The main active compounds in yarrow are flavonoids (apeginenin, rutin, lutelin, campherol) and essential oils (82 essential oil components have been identified). The main reported components of yarrow essential oil are azulene, camphor (18%), sabinene (12%), 1,8-cineol (10%) and α-pinene (9%) [6]. The content and composition of essential oils depends on many factors such as growing place, stage of development, etc. [5, 7]. The chemical composition of *Achillea millefolium* L. essential oils was investigated in many countries: Spain [8], Iran [9–11], Cuba [12], Yugoslavia [13], Russia [14], Estonia [15], Norway [7], India [16] and Lithuania [17–20]. In most cases, essential oil composition was compared in flowers and herb, however, only a few articles addressed the significance of flower colour in essential oil composition [18–20].

The aim of the study. In this study, we extend the phytochemical research of yarrow, focusing on the evaluation of essential oil composition and the total content of flavonoids in four morphotypes of *Achillea millefolium* L. (with white, pink, deep pink and red flowers) which had been bred in the same place in the collection of medicinal plants of the Kaunas Botanical Garden of Vytautas Magnus University since 1975.

MATERIALS AND METHODS

Object. Herb and flowers of four morphotypes of *Achillea millefolium* L. (with white, pink, deep pink and red flowers). The total content of flavonoids was determined and a quantitative and qualitative analysis of essential oils was performed for four morphotypes of *Achillea millefolium* L. Four wild morphotypes of *Achillea millefolium* L. were collected from natural habitats in Lithuania. The research was performed in 2005–2006 in Central Lithuania (Kaunas). The *Achillea millefolium* L. collection (area 22.8 m^2) was established within the collection of medicinal plants (area 1200 m^2) at the Research Division of Medicinal Plants of the Kaunas Botanical Garden of Vytautas Magnus University.

Materials. Sodium tetraborate, sodium hydroxide, methanol pure for analysis, acetic acid (Lachema, Czech Republic),

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heptane of chromatographic grade (Merck, Germany), liquid CO_2 (99.9%) (AGA, Lithuania), retention index standards mixture (aliphatic hydrocarbons ranging from C8 through C32) (Sigma, USA), aluminum chloride (Baker, New Jersey, USA), hexamethylenetetramine (South Korea, Fluka), rutin (Sigma, Germany).

Sample preparation and analysis method. Analysis of the yarrow extracts was performed by two methods. Gas chromatography with flame ionization detection and mass spectrometric detection was used for analysing of the composition of essential oils. The total content of flavonoids was determined using the spectrophotometric method described in Pharmacopea [21].

For determination of biologically active compounds, air-dried medicinal raw material was chopped with a food processor, Model 4258 (Braun AG, Germany) to 3-mm particles (stems were cut to 5 mm particles). Essential oil samples for gas chromatography were prepared using a supercritical fluid extraction apparatus HP7680T (Hewlett Packard, USA). Conditions for essential oil extraction were as follows: an extraction sample was 0.5 g of chopped air-dried raw material (14% of moisture in the mass), supercritical CO_2 density used for extraction 0.3 g/ml, extraction time 17 min, pressure 91 bars, trap column ODS (octadecylsilica), desorption solvent heptane. The volume of essential oil solution prepared in hexane was 0.7 ml.

The extraction of flavonoids from the chopped raw material (1 g) was carried out using 10 ml of aqueous methanol (70% v/v) shaking the mixture in an orbital mixer (Titertek, Flow Laboratories, Germany) for 15 hours. The extract was filtrated first through a paper filter and then through a membrane filter (0.45 μm). The total content of flavonoids was determined by the Pharmacopoeia method [21]. Sample solution for spectrophotometric measurement was prepared in an Eppendorf vial as follows: to 0.04 ml of methanolic yarrow extract were added solutions of 0.4 ml methanol, 0.02 ml of 33% acetic acid, 0.06 ml of 10% $AlCl_3$ and 0.08 ml of 5% hexamethylenetetramine. For a reference sample, 0.04 ml of extract was mixed with 0.4 ml of methanol and 0.02 ml of 33% acetic acid. Spectrophotometric analysis was performed at a 407 ± 2 nm wavelength after 30 min of incubation. For quantitative evaluation, the absorption differences between a sample solution and rutin solution (0.025 g / 50 ml) were compared. The total content of flavonoids in the yarrow extract, expressed in rutin concentration (%), was calculated according to the equation:

$$X = \frac{D \cdot m_0}{D_0},$$

where D is the absorption (optical density) of a sample, D_0 is the absorption of a standard rutin solution, m_0 is rutin weight (g) used to prepare a standard solution.

Gas chromatographic analysis of essential oils was performed using a gas chromatograph (HP5890A, Hewlett Packard, USA) equipped with a flame ionization detector and a DB-5 (I & W Scientific, USA), 25 m × 0.325 mm × 0.25 μm capillary column. Analysis conditions: injector temperature 250 °C, detector temperature 280 °C, injected volume 2 μl. Temperature gradient: from 50 °C (2 min) to 80 °C, 2 °C/min, from 80 °C to 160 °C, 5 °C/min, from 160 °C to 250 °C, 10 °C/min, and 3 min at a constant temperature of 250 °C. Gas chromatography / mass spectrometry was performed using a Clarus 600MS T gas chromatograph (Perkin Elmer, USA) with TurboMass spectrometer and RTX-5MS (30 m × 0.25 mm × 0.25 μm) capillary column. The temperature gradient was the same as for GC analysis with a FID detector. MS analysis conditions: IonSource temperature 250 °C, interface temperature 250 °C, scanning from 30.00 to 450.00 m/z, column flow 1.24 ml/min. Qualitative analysis was based on a comparison of retention indices and mass spectra with the data presented in databases (NIST Mass Spectral Database) and scientific literature [14–20].

RESULTS AND DISCUSSION

Total content of flavonoids. The optimal extraction time of flavonoids was determined. The highest content of flavonoids in the extract (for all four morphotypes of *Achillea millefolium* L.) was obtained after 15 hours of extraction (Fig. 1) and remained constant after 89 hours (data not shown).

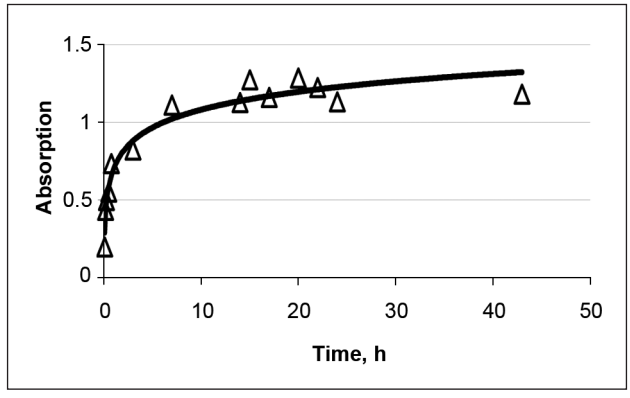

Fig. 1. UV absorption (λ = 407 nm) dependence on extraction time in deep pink yarrow extract

The highest level of flavonoids was determined in the herb of the deep pink morphotype (0.07%) and the lowest in the pink morphotype (0.05%) (Fig. 2). The difference between the lowest and the highest levels of flavonoids in four morphotypes of yarrow was 28.6%.

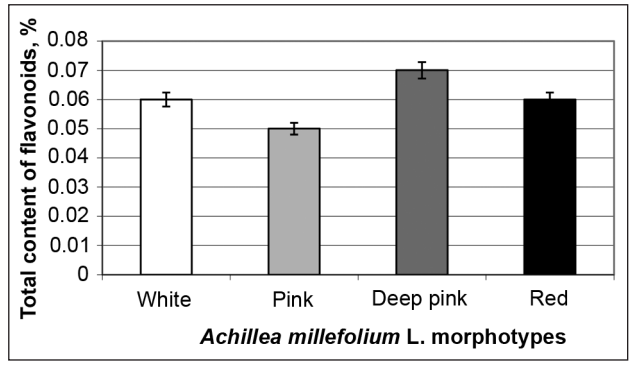

Fig. 2. Total content of flavonoids (%) in herb of four yarrow (*Achillea millefolium* L.) morphotypes (2005)

The relative standard deviation of sample preparation by aqueous methanol extraction (n = 3) for the spectrophotometric method did not exceed 4%.

The highest content of flavonoids accumulated in the white morphotype of yarrow during budding and at the end of blooming (0.037–0.038%) (Fig. 3). Quantitative and qualitative changes in the composition of phenolic compounds occur during plant development [22–24]. The content of flavonoids in leaves of *Ficaria verna* Huds. picked up before plant blossoming was also lowest [25] like in our case with *Achillea millefolium* L. According to the published results on the investigation of *Achillea collina* Rchb. Alba, the highest content of flavonoids was found at the beginning of flower differentiation [26]. In the study of Valkama et al., changes of flavonoid content in birch were revealed: in mature leaves a decrease in the total content of flavonoids was observed [27]. This fact can be explained by the reduction of synthesis and a simultaneous degradation of flavonoids or their transformation into insoluble, cell-wall-bound forms [27].

The total content of flavonoids in the white morphotype of yarrow (herb) in 2006 versus 2005 decreased by 36.7% (comparison of data in Figs. 2 and 3).

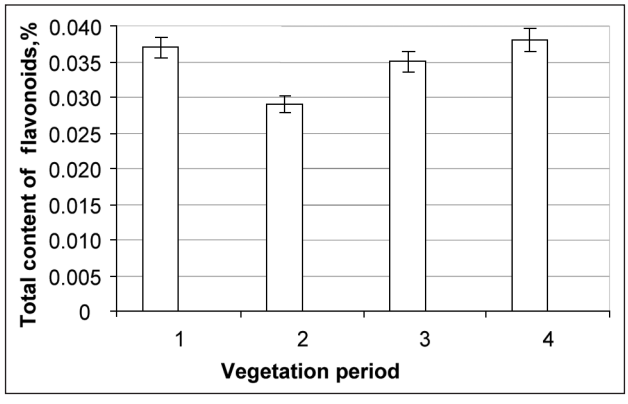

Fig. 3. Total content of flavonoids (expressed in rutin concentration, %) in herb of the white morphotype of *Achillea millefolium* L. at different vegetation periods: 1 – budding, 2 – beginning of blooming, 3 – intensive blooming, 4 – end of blooming (2006)

Essential oil analysis. The relative content of essential oil was determined and compared in four different morphotypes. The highest level of essential oil was found in the herb and flowers of the white morphotype of *Achillea millefolium* L. In all cases it was higher in flowers than in herb. The lowest content of essential oil was determined in pink yarrow (Fig. 4).

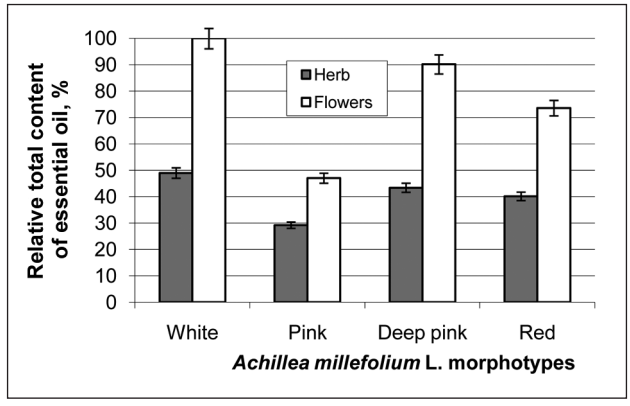

Fig. 4. Relative total content (%) of essential oil in four morphotypes of *Achillea millefolium* L., assuming the content of essential oil in the white yarrow morphotype to equal 100% (2005)

The content of essential oil in flowers of the white yarrow morphotype was more than twice as high as in the pink morphotype. Essential oil content in the herb of four yarrow morphotypes differed by up to 40%. The repeatability of sample preparation by supercritical fluid extraction (n = 5), expressed in the RSD of the total area of peaks in GC chromatogram, was less than 2%.

For the identification of essential oil components in flowers and herb of yarrow, mass spectrometry data and retention indices reported in the literature were used. Some of the components were identified only in herb or only in flowers (e. g., cadinene) (Table 1). The content and composition of essential oils was highly related to the colour of flowers and the anatomical part of a plant (flowers or herb).

Table 1. **Distribution of selected essential oil components in four morphotypes of *Achillea millefolium* L. (herb and flowers)**

Raw material	Morphotype	Essential oil compound									
		α-pinene	camphene	β-pinene	limonene	γ-terpinene	not identified	β-caryophyllene	α-humulene	Germacrene D	Cadinene
Herb	White	2.37	0.19	20.67	2.22	0.79	0	1.58	0.19	0.62	0.51
	Pink	0.6	0.46	3.9	4.55	0.17	1.09	1.32	0.19	0.51	0.32
	Deep pink	1.47	0.71	13.38	1.83	0.34	0.41	2.53	0.39	0.76	0.95
	Red	1.31	0.70	12.78	0.63	0.27	1.03	1.8	0.35	0.94	1.87
Flowers	White	5.25	0.4	42.16	6.4	3.26	0	2.1	0.35	1.33	0.99
	Pink	1.31	0.89	8.14	3.68	0.45	1.95	2.48	0.52	1.34	1.56
	Deep pink	3.53	1.88	28.43	3.29	1.47	3.92	6.3	1.23	2.55	0.07
	Red	2.29	0.79	21.53	7.52	1.17	0.56	4.22	0.65	2.75	5.87

Table 2. **Distribution of identified essential oil components during vegetation period in herb of white *Achillea millefolium* L. morphotype**

Essential oil compound	Vegetation period			
	Budding (June 01)	Beginning of blooming (July 05)	Intensive blooming (July 11)	End of blooming (July 27)
α-pinene	10.04	0.66	0.64	6.24
camphene	15.37	0.46	0.42	2.34
β-pinene	16.36	2.13	1.86	38.65
limonene	1.88	1.49	1.39	3.76
γ-terpinene	6.30	13.07	10.83	3.54
β-caryophyllene	4.35	7.64	8.07	13.79
α-humulene	0.42	1.12	0.94	2.34
Germacrene D	1.69	5.64	5.36	10.7
Cadinene	6.13	32.24	28.81	0.68

The content of identified essential oil compounds in all *Achillea millefolium* L. morphotypes comprised 50–65% of the total essential oil determined. In all essential oil samples, β-pinene prevailed, except the essential oil of flowers of the pink yarrow morphotype in which limonene was the dominating compound.

The content of essential oil increased during vegetation and at the end of blooming was three times higher than in the budding period (Fig. 5). The identified essential oil components comprised from 61% (budding period) to 82% (end of blooming) of the total essential oil content. The gas chromatography method repeatability (RSD) for the quantitative analysis of essential oils was 3.5% (n = 3).

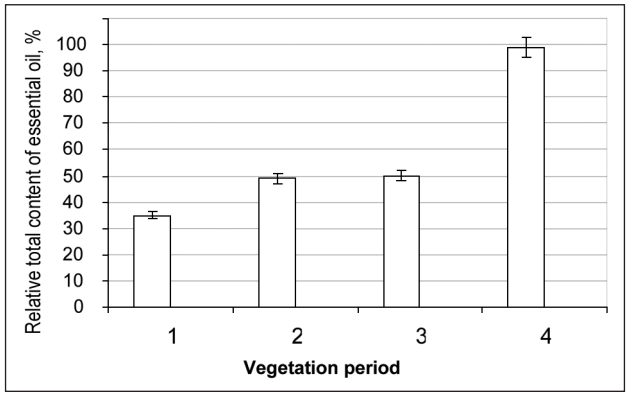

Fig. 5. Total content of essential oil (%) in herb of the white *Achillea millefolium* L. morphotype at different vegetation periods: 1 – budding, 2 – beginning of blooming, 3 – intensive blooming, 4 – end of blooming (2006)

In 2006, the white yarrow morphotype accumulated by 46.2% more of essential oil than in 2005. The content of α-pinene, camphene, β-pinene and limonene during the vegetation period changed parabolically, with the minimum levels in the middle of vegetation (Table 2).

The content of γ-terpinene and cadinene showed an inverse parabolic dependence on the vegetation time, with the maxima in the middle of vegetation. The content of β-caryophyllene, α-humulene and germacrene D continuously increased.

CONCLUSIONS

The total content of flavonoids was determined in four *Achillea millefolium* L. morphotypes: white, pink, deep pink and red. The highest content was determined in the deep pink (0.07%) yarrow morphotype and the lowest in the pink yarrow morphotype (0.05%). The total content of essential oil in flowers and herb was compared. Flowers contained more essential oil than did herb in all yarrow morphotypes. Essential oil components in flowers and herb were identified. The components and their content varied depending on the morphotype of yarrow and the part of a plant. The highest content of essential oil was determined in the white yarrow morphotype. During a vegetation periods (budding – starting of blooming – full blooming – end of blooming), the highest content of flavonoids was determined at the beginning and at the end of vegetation. The content of essential oil increased in the course of vegetation and reached its maximum by the end of blooming. The results of comparative investigations of *Achillea millefolium* L. morphotypes bred in the same place are important for differenting the phytochemical composition of the morphotypes. This study also provides data on the bioaccumulation dynamics of pharmacologically important compounds, which are of utmost importance in ensuring the high quality of raw material of this medicinal plant.

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KETURIŲ KRAUJAŽOLĖS (*ACHILLEA MILLEFOLIUM* L.) MORFOTIPŲ CHEMINĖS SUDĖTIES PALYGINIMAS

Santrauka

Keturi *Achillea millefolium* L. morfotipai yra auginami Vaistinių augalų kolekcijoje Vytauto Didžiojo universiteto Kauno botanikos sode: baltas, rožinis, tamsiai rožinis ir raudonas. Kadangi žiedų spalvą lemia skirtingas augalų fenotipas ar genotipas, buvo pradėti keturių kraujažolės morfotipų fitocheminiai tyrimai. Pagrindinis šio darbo tikslas – įvertinti bendro flavonoidų kiekio ir eterinių aliejų kiekio skirtumus bei nustatyti ir kiekybiškai įvertinti pagrindinius eterinių aliejų komponentus įvairiuose *Achillea millefolium* L. morfotipuose. Kiekybinis eterinių aliejų žieduose ir žolėje įvertinimas buvo atliktas dujų chromatografijos ir masių spektrometrijos metodu. Visų *Achillea millefolium* L. morfotipų žiedai kaupė daugiau eterinių aliejų nei žolė. Bendras flavonoidų kiekis sudarė 0,05–0,07%. Didžiausias flavonoidų kiekis buvo nustatytas tamsiai rožinėje kraujažolėje, o didžiausias eterinių aliejų kiekis buvo baltojoje *Achillea millefolium* L. Pastarojoje bendras flavonoidų kiekis ir eterinių aliejų sudėtis buvo nustatyti skirtingais vegetacijos tarpsniais.

Raktažodžiai: *Achillea millefolium* L., dujų chromatografija ir masių spektrometrija, eterinių aliejų sudėtis, flavonoidai