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Research Article

FIELD-CULTIVATED PLANTS FROM *ACHILLEA MILLEFOLIUM* GROUP: TOTAL FLAVONOID CONTENT, ANTIRADICAL AND ANTIOXIDANT ACTIVITIES IN STEMS AND LEAVES, AND RATIO OF PLANT PARTS

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Summary: The medicinal plants of *Achillea millefolium* group are largely collected from their natural habitats this leading to both ecological risks and heterogeneity of the collected market samples. Our previous studies supplied evidence that field cultivation of wild species of the group (*A. collina* and *A. asplenifolia*) is a promising approach to obtain homogenous plant material without damage to the environment. In these studies inflorescences were the object of field experiments while in the present paper stems and leaves were investigated. The experimental design involved the above mentioned species (each with two populations) the seeds being collected from the natural habitats of plants. Cv. Proa bred for field cultivation was used as a standard; its seeds were purchased from the firm "Pharmasaat GmbH", Germany. The results confirm the findings of our previous work carried out with inflorescences, namely that stems and leaves contain flavonoids above the reference limit of European Pharmacopoeia (3 mg/g). This accounts for the high AO and AR activities as shown by the strong correlation between flavonoids and these items. Moreover, similarly to inflorescences, stems and leaves show low intraspecific variability and larger interspecific differences with regards to all characters studied. This suggests to the species studied being isolated and distant enough to serve as a homogenous source of raw yarrow material for the market. *A. asplenifolia* is distinguished by a higher potential of valuable components than *A. collina* while being closer by all parameters to cv. Proa. These data point to *A. asplenifolia* as a promising species for field cultivation. The weight ratio of plant parts shows that inflorescences dominate followed by stems and leaves.

Keywords: *A. collina*; *A. asplenifolia*; field cultivation; flavonoids; antioxidant and antiradical activities.

Abbreviations: AO – antioxidant; AR – antiradical; DPPH· – 2,2´-diphenyl-1-picrylhydrazyl radical; FRAP – ferric reducing antioxidant power.

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INTRODUCTION

The remarkable therapeutic properties of plants from *Achillea* genus (yarrow) have been recognized since ancient times. In their vast review Ali et al. (2017) summarize the curative effects of a group of *Achillea* plants (*A. millefolium*) such as anti-inflammatory, spasmolytic, choleretic, anxiolytic, antimicrobial, antinociceptive, anticancer etc. , and emphasize that these effects are underlain by the action of a large spectrum of secondary metabolites – phenolic acids, flavonoids, terpenoids including chamazulene in the essential oil, etc. Yarrow has been used as folk medicine by many cultures, reaching to modern times, and is applied in pharmaceutical industry as a drug and to design novel therapeutic products (Lakshmi et al., 2011; Saeidnia et al., 2011; Ali et al., 2017).

In previous publications (Vitkova et al., 2005; Edreva et al., 2017) we provided evidence that field cultivation of medicinal plants from *A. millefolium* group is a promising approach for obtaining high grade and homogenous plant material, rich in bioactive compounds, this being related to conservation of natural habitats of wild growing plants, and minimizing the ecological risk. However, we supplied data only on inflorescences while the results about stems and leaves which are also components of the herba were not presented. In the available literature data about separately studied stems and leaves are scarce (Kyslychenko, 2014), particularly about field-grown yarrow plants.

Thus, the aim of the present communication is to describe the data about the content of total flavonoids and antiradical and antioxidant activities in stems and leaves of two field-cultivated azulenogenic species of *A. millefolium* group – *A. collina* and *A. asplenifolia*, each presented by two populations, and to compare them with those of the cv. Proa bred for field cultivation. The weight ratio of plant parts – inflorescences, stems and leaves – was also evaluated.

MATERIALS AND METHODS

Plants

Seeds were collected from natural habitats of the two wild species near Sofia region – A. *collina* (2n 36) and *A. asplenifolia* (2n18). Each species was presented by two populations – *A. collina* 102 (Vitosha mountain) and *A. collina* 3802 (village Dolni Lozen), *A. asplenifolia* 9602 (village Kutina) and *A. asplenifolia* 10403 (village Bezden). Seeds of cv. Proa were purchased from the firm "Pharmasaat GmbH", Germany. Experiments were carried out in 2010. Seedlings were produced in a greenhouse in pots 12 cm in diameter and then transferred to the experimental field of the Institute of Plant Physiology and Genetics near Sofia at 570 m a.s.l. The type of soil was alluvial meadow with pH 5.6. Six-month seedlings were planted in the experimental field in 4 replicates, each of them containing 10 plants. The distance between plants and rows was 40 cm. Plants were harvested at the stage of full blossoming. After determination of the ratio of plant parts the dry material was milled for chemical analyses.

Total flavonoid content

The method of Zhishen et al. (1999) was used. One gram of material was

grinded in 10 ml 80% (v/v) aqueous ethanol. An aliquot of 1ml was mixed with 4 ml of water, 0.3 ml 5% $NaNO_2$, 0.3 ml 10% $AlCl_3$, 2ml 1M NaOH, and adjusted to 10 ml with water. The resulting red coloration was measured at 510 nm. Rutin (Sigma) was used as a standard.

Antiradical, or free radical scavenging activity

It was determined in 80% (v/v) aqueous ethanol extract (the same as for total flavonoids) by the 2,2′ –diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) test (Brand –Williams et al., 1995). A volume of 0.01 ml extract and 1.99 ml $DPPH^{\bullet}$ solution in methanol ($6x10^{-5}$ M) were mixed, and the decrease of absorbance was followed up at 515 nm. TROLOX (Sigma) was used as a standard.

Antioxidant activity

The method of Benzie and Szeto (1999) (the FRAP test) was applied using the same extract as for total flavonoid content. A volume of 0.05 ml extract was mixed with 1.50 ml FRAP reagent and 0.15 ml water. A blue coloration developed, the absorbance being read after 15 min at 593 nm. The FRAP reagent was a mixture of 0.3 M acetate buffer pH 3.6, 0.02 M $FeCl_3.6H_2O$, and 0.01 M TPTZ (2,4,6-tripyridyl-s-triazine) in 0.04M HCl in a ratio of 25:2.5:2.5. $FeSO_4$ was used as a standard.

Weight ratio of plant parts

The harvested plants were separated in inflorescences, stems, and leaves, dried at air temperature, and weighted to obtain the % ratio of the different parts.

Statistical analysis

Data are expressed as means ± standard error (±SE), n = 4. Different letters indicate significant differences assessed by the Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis. The statistical software package StatGraphics Plus version 5.1 for Windows was used.

Correlation analysis

The antioxidant and antiradical activities were subjected to correlation analysis with the content of total flavonoids by using ANOVA multifactor analysis.

RESULTS AND DISCUSSION

Total flavonoid content

The spasmolytic, choleretic and antispasmodial effects of yarrow are determined to a greater extent by phenylpropanoids such as flavonoids and cinnamic acid derivatives (Benedek et al., 2007)

In the stems of yarrow plants studied by us the total flavonoid content varies from 21.76 mg/g in *A.collina* 102 to 39.11 mg/g in cv. Proa. The populations of the species show similar values which is valid for populations of both *A. collina* and *A. asplenifolia*. The averages for the species are 22.00 mg/g and 27.56 mg/g for *A.collina* and *A. asplenifolia*, respectively. Cv. Proa is distinguished by the highest flavonoid level (39.11 mg/g), being approached by *A. asplenifolia* 9602 (Fig. 1).

In the leaves of the yarrow plants the total flavonoid content varies from 99.11 mg/g in *A.collina* 3802 to 144.89 mg/g in cv. Proa. The populations of the species show similar values observed in both

species. The averages for the species are 99.56 mg/g and 114.89 mg/g for *A.collina* and *A. asplenifolia*, respectively. Cv. Proa contains the maximum flavonoid compounds (144.89 mg/g) being approached by *A. asplenifolia* 9602 (Fig. 1).

Thus, for stems and leaves in both species the total flavonoid content is higher than the reference limit in European Pharmacopoeia (3 mg/g) (Benedek et al., 2008). Field cultivation appears hence as a promising approach to manipulate the flavonoid level in yarrow. Karlova (2006) observed good development of wild *A. collina* plants when cultivated in field conditions. Moreover, in flos and herba of these plants the author identified 7-O-glucosides of apigenin and luteolin as well as both aglycones. Our experiments confirming these findings are in progress. It has to be noted that the intraspecific variability of flavonoids is much lower than the interspecific variability in both stems and leaves.

If summarizing the flavonoid content in stems, leaves and inflorescences, the total flavonoid content of herba will be obtained. It amounts to 1034.68 mg/g dry mass. Thus inflorescences containing 321.34 mg/g flavonoids (Edreva et al., 2017), stems and leaves will account for 31.06%, 13.36%, and 55.58%, respectively, of total flavonoid content of herba. Leaves contain maximum flavonoids as compared to inflorescences and stems which can be accounted for by the chloroplasts being the site of flavonoid biosynthesis (Winkel, 2006).

Antioxidant and antiradical activities

The demand of products capable to counteract oxidative damage and diseases referred to as antioxidants and free radical scavengers is constantly increasing (Halliwell and Gutteridge, 1995). Medicinal plants including yarrow are rich sources of substances endowed with

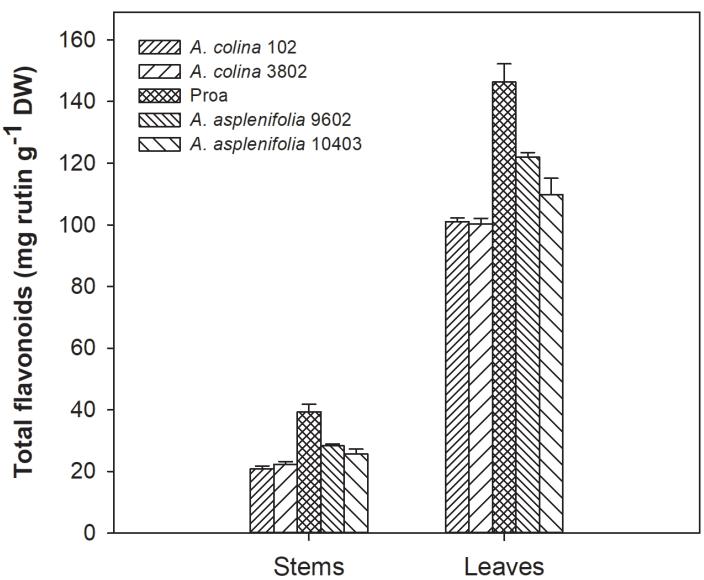

Figure 1. Flavonoid content (mg rutin g-1 DM) in stems and leaves of of *Achillea millefolium* group species (each with two populations) and cv. "Proa".

antioxidant and antiradical properties. Phenylpropanoids are regarded as important determinants of the antioxidant /antiradical potential of yarrow (Sokmen et al., 2004; Tuberoso et al., 2009; Vitalini et al., 2011).

In our experiments the antioxidant (AO) activity in stems varies from 114.29 mmoles FRAP in *A. collina* 102 to 200.00 mmoles FRAP in cv. Proa, whereas the antiradical (AR) activity is between 53.33 mmoles TROLOX in *A.collina* 3802 and 141.30 mmoles TROLOX in cv. Proa. The populations of the species show similar AO and AR activities. This is valid for populations of both *A. collina* and *A. asplenifolia*. The averages of AO activity are 120.00 mmoles FRAP and 147.14 mmoles FRAP for *A.collina* and *A. asplenifolia*, respectively. The averages of AR activity are 57.33 mmoles TROLOX and 105.54 mmoles TROLOX for *A. collina* and *A. asplenifolia*, respectively. Stems of cv. Proa have dominant position being closer to *A. asplenifolia* 9602 by both AO and AR activity (Fig. 2, 3).

The AO activity in leaves varies from 462.86 mmoles FRAP in *A.collina* 3802 to 814.29 mmoles FRAP in cv. Proa, whereas the variability of AR activity is between 378.67 mmoles TROLOX in *A.collina* 3802 to 666.67 mmoles TROLOX in cv. Proa. The populations of the species show similar AO and AR activities. This refers to both *A. collina* and *A. asplenifolia*. The averages of AO activity are 488.56 mmoles FRAP and 618.57 mmoles FRAP for *A. collina* and A. *asplenifolia*, respectively, and those of AR activity are 382.67 mmoles TROLOX and 530.67 mmoles TROLOX for *A.collina* and *A. asplenifolia*, respectively. Leaves of cv. Proa have the highest AO and AR activities, and are closer to *A. asplenifolia* 9602 by both AO and AR activity (Fig. 2, 3).

Noteworthy, the intraspecific variability of AO and AR activity is much

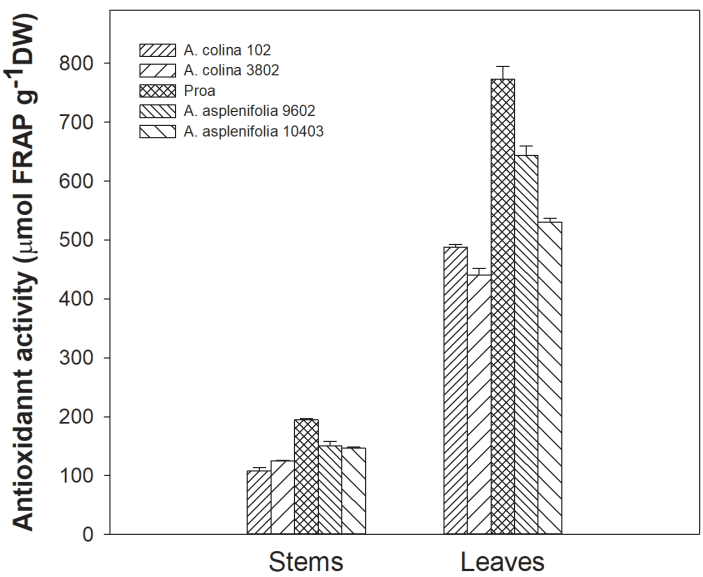

Figure 2. Antioxidant activity (µmoles FRAP g-1 DM) in stems and leaves of of *Achillea millefolium* group species (each with two populations) and cv. "Proa"

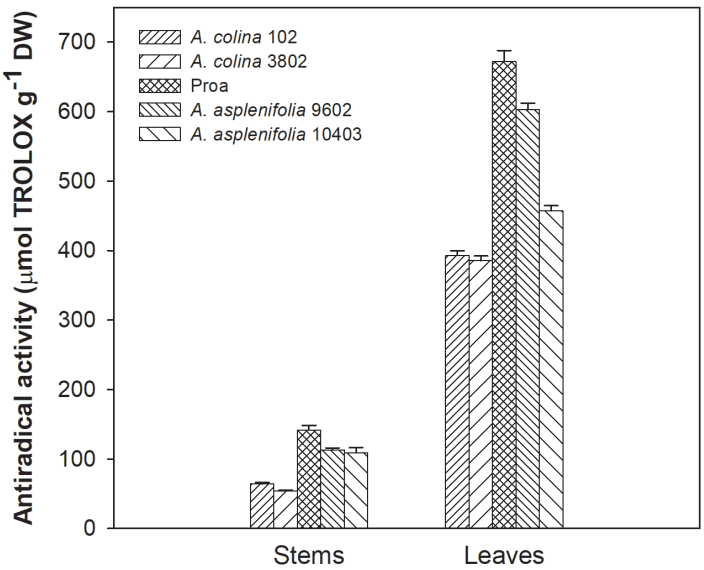

Figure 3. Antiradical activity (μmoles TROLOX g-1 DM) in stems and leaves of of *Achillea millefolium* group species (each with two populations) and cv. "Proa".

lower than the interspecific variability in both stems and leaves.

The DPPH• test is predominantly applied to evaluate the AR activity in different species of *Achillea*, such as *A. biebersteinii*, *A. alexandri-regis*, *A. ligustica*, *A. millefolium*, and *A. distans*. Positive relationships were established between the free radical scavenging activity and the antimicrobial capacity of yarrow as well as the power to protect cells from and prevent oxidative damage (Sokmen et al., 2004; Kundakovic et al., 2006; Tuberoso et al., 2009; Vitalini et al., 2011; Benedec et al., 2013). These properties explain and justify the large usage of yarrow in folk and modern medicine.

Weight ratio of different plant parts

The inflorescences are the predominant part of yarrow plants ranging from 45.37% in *A. asplenifolia* 10403 to 55.15% in *A. collina* 102. They are followed by stems (29.30% in *A.collina* 102 to 32.32% in *A. asplenifolia* 10403). The lowest position is taken by leaves (15.55% in *A.collina* 102 to 22.31% in *A. asplenifolia* 10403)

Table 1. Ratio of different plant parts (%) of *Achillea millefolium* group species (each with two populations) and cv. "Proa"

Species Plant parts	*A.collina* 102	*A. collina* 3802	cv. "Proa"	*A. asplenifolia* 9602	*A. asplenifolia* 10403
Inflorescences	55.15	53.39	51.61	52.41	45.37
Stems	29.30	29.51	31.30	32.00	32.32
Leaves	15.55	17.11	17.07	15.58	22.31

(Table 1). This ratio of herba is similar to the data reported by Kyslychenko (2014).

Correlation analysis

Our data show that strong correlations exists between the AO and AR activity (with correlation coefficients R^2=0.982), AO and the contents of total flavonoids (R^2=0.987), and AR and the contents of total flavonoids (R^2=0.962).

CONCLUSION

The results of the present paper (dealing with stems and leaves) confirm the findings of our previous work carried out with inflorescences, namely, that stems and leaves contain flavonoids above the reference limit of European Pharmacopoeia (3 mg/g) (Benedek et al., 2008). This accounts for the high AO and AR activities as shown by the strong correlation between these items, and suggests that field cultivation is a promising approach for obtaining yarrow plants with high bioactive potential. Similarly to inflorescences, stems and leaves show low intraspecific variability and larger interspecific differences with regard to all studied properties which points to the species studied being isolated and distant enough to serve as a homogenous source of raw yarrow material for the market. Leaves are the richest source of flavonoids and AO/AR activities but they account for the lowest part of herba. Irrespectively, neither stems nor leaves can be neglected as components of yarrow herba. *A. asplenifolia* is richer in valuable components than *A. collina*. This species is closer to the commercially supplied cv. Proa, and hence is a promising plant for field cultivation.

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