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INFLUENCE OF SPECIES COMPOSITION ON TOTAL PHENOLIC CONTENT AND ANTIOXIDANT PROPERTIES OF *HERBA ALCHEMILLAE*

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Abstract

VITKOVA, A., M. NIKOLOVA, M. DELCHEVA, Al. TASHEV, A. GAVRILOVA, I. ANEVA and D. DIMITROV, 2015. Influence of species composition on total phenolic content and antioxidant properties of *Herba Alchemillae. Bulg. J. Agric. Sci.,* 21: 990–997

Commercial product *Herba Alchemillae* is widely used in herbal medicine but it includes aerial parts of various species due to difficulties in their identification by herbalists. The present study determined the effect of *Alchemilla* species composition and the geographical distribution of species on total phenolic content and antioxidant activity of *Herba Alchemillae*. For that purpose a comparative analyzes of 22 samples of plant material collected from natural populations in 5 floristic regions of Bulgaria and 21 samples of the 8 common *Alchemilla* species was performed. The results showed that the content of total phenols and antioxidant activity depends mainly on the species composition and the quantitative participation of the species in plant materials. The study demonstrated greater percentage participation in *Herba Alchemillae* of species belonging to series *Vulgares*. All examined samples exhibited strong antioxidant activity, which is important from a practical point of view.

Key words: *Alchemilla* spp., medicinal plants, DPPH, radical scavenging activity, Bulgaria

Introduction

Genus *Alchemilla* L. (Lady's mantles) in Bulgarian flora is represented by 35 species (Asenov, 1973), 31 of them being considered medicinal plants (Medicinal Plants Act, 2000). The other 4 species are small plants or are distributed in remote areas, such as high mountains, with limited occurrence, thus possessing lower interest and not subjected of the study. Aerial parts and roots of the plants are used as remedy. The herbal drug contains mainly tannins, flavonoids, leucoanthocyanidins with expresses styptic and regenerating epithelium action (Nikolov et al., 2007). In recent years a number of phytochemical studies on endemic and widespread *Alchemilla* spp. were conducted (Ertűrk et al., 2011; Kaya et al., 2012; Duckstein et al., 2013). Several studies have been held in Bulgaria to establish the distribution of *Alchemilla* spp. (Gavrilova and Vitkova, 2010), biology of rare and endangered species and their conservation (Vitkova, 1997, 2009, 2012; Vitkova et al., 2011), possibility of cultivation (Vitkova et al., 2013a) and content of biologically active substances (Vitkova, 1996; Trendafilova et al., 2012; Nikolova et al., 2012).

Gavrilova (2014) reported eight types of habitats in Bulgaria of *Alchemilla* spp. Two of them are characterized by the greatest species diversity and high percentage of the projective cover (23-25%) of *Alchemilla* spp. - E5.33 Balkan mountain hay meadows and E5.41 Stripes and curtains of tall grasses near watercourse. These two habitats are reported as the potential source of economically significant resources of *Herba Alchemillae* in Bulgaria (Vitkova et al., 2013b; Gavrilova, 2014) and have therefore been selected as the subject of the study.

The present article is part of a comprehensive study of the distribution and resources of the *Alchemilla* spp. aimed to their sustainable use and protection in Bulgaria, Project – 7680/2009 funded by the Ministry of Environment and Water. In connection with the inventory of resources of *Alchemilla* spp. of great importance will be determination of the content of the main

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biologically active substances and antioxidant activity in plant material. It should be noted that *Herba Alchemillae* includes aerial parts of various species that are subsumed in *Alchemilla vulgaris* complex. Due to difficulties in the identification of the species, herbalists collect all species of lady's mantles occurring within the natural populations. More precise approach requires better characterization of *Herba Alchemillae*.

The purpose of present study was to determine the influence of *Alchemilla* species composition and the species' geographical distribution in Bulgaria on total phenolic content and antioxidant activity of *Herba Alchemillae*. To achieve this goal, we did comparative analyses of 22 samples of *Herba Alchemillae* collected from natural populations in 5 floristic regions of Bulgaria and 21 samples of the 8 widespread species.

Materials and Methods

Plant material

Forty-three samples of *Herba Alchemillae* were collected from 22 localities of *Alchemilla* spp. outside of protected areas in 5 floristic regions of Bulgaria – Stara Planina Mts. (West); Sredna Gora Mt. (Western); West Frontier Mountains (Osogovska Mt. and Vlahina Mt.); Pirin Mt. (Southern); Rhodopes Mts. (Western and Central) during the period 2011-2013 year (Table 1). The plants material was collected in the phase of mass flowering and was further dried under shade. Herbarium specimens of all identified species were deposited in the Herbarium of Institute of Biodiversity and Ecosystem Research, Sofia, Bulgaria (SOM). Identification the taxonomic status of *Alchemilla* spp. followed Asenov (1973). Additionally, the taxonomic scheme of genus *Alchemilla* proposed by Walters and Pawłowski (1968) was used (Table 2). The habitat types were determined by using the hierarchical classification scheme of EUNIS (European Nature Information System) of the European Centre for Nature Conservation and Biodiversity (European Topic Centre for Nature Protection and Biodiversity) (Davis et al., 2004).

Extraction of plant material

The extraction was performed from 1 g dry and ground plant material with 80% (3 x 30 mL) methanol by classical maceration for 24 h. After evaporation of the solvent the crude extract was subjected to subsequent analysis.

Determination of total phenolic content

Total phenolic content of methanol extracts was determined by employing the methods described in the literature involving Folin-Ciocalteu reagent and gallic acid as standard (Giorgi et al., 2009; Nićiforović et al., 2010). Plant extracts were diluted to a concentration of 1 mg mL^{-1}, and aliquots of 0.25 mL were mixed with 2.5 mL of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 ml of Na_2CO_3 (6%). After 1 h at room temperature, the absorbances of the samples were measured at 765 nm on spectrophotometer versus blank sample. Total phenols were determined as gallic acid equivalents (mg GA) per gram of extract.

DPPH radical scavenging activity

The effect of methanolic extracts on DPPH radical was estimated according to Stanojević et al., 2009. Different concentrations of plant extract (10, 20, 50, 100 and 200 μg mL^{-1}), in methanol were added at an equal volume (2.5 mL) to methanol solution of DPPH (0.3 mM, 1 ml). After 30 min at room temperature, the Ab values were measured at 517 nm on a spectrophotometer (Jenway 6320D) and converted into the percentage antioxidant activity using the following equation:

$$\text{DPPH antiradical scavenging capacity (\%)} = [1-(Ab_{sample}-Ab_{blank})/Ab_{control}] \times 100$$

Methanol (1.0 mL) plus plant extract solution (2.5 mL) was used as a blank, while DPPH solution plus methanol was used as a control. The IC_{50} values were calculated by Software Prizm 3.00.

Statistical analysis

Results are presented as mean ± standard deviations (SD) of tree independent analyzes (n = 3). Statistical analysis was carried out using Excel.

Total phenolic content and antioxidant activity of 23 samples were subjected to cluster analysis using Statistica software, version 7 (www.statsoft.com).

Results and Discussion

Species composition of Herba Alchemillae

Total thirteen *Alchemilla* species were identified in the samples of *Herba Alchemillae* as follows: section *Alchemilla* – 1 species of subsection *Calycantum*, series *Elatae* and 12 species of subsection *Heliodrosium*. Species of subsection *Heliodrosium* refered to two series – 4 species belonged to the series *Pubescentes* and 8 species to series *Vulgares* (Table 2). The species composition of the plant material colected from studied localities consisted of 1 to 6 *Alchemilla* species. Apparently these species form sympatric populations, a fact previously observed by several authors (Plocek, 1972; Glazunova, 1984).

The results showed that only species belonging to series *Vulgares* participate within samples of *Herba Alchemillae* collected from seven localities, and also prevailed species of the same series in the samples collected from other ten lo-

Table 1
Total phenolic content and antioxidant activity of studied *Herba Alchemillae* samples collected from different regions of Bulgaria

Number of samples according to Figure 1	Floristic region / studied localities	Habitat	GPS coordinates
1	Sredna Gora Mt. 1	E5.41 Stripes and curtains of tall grasses near watercourse	N 42.652477º E 24.392151º
15	Rhodopes Mts. 3	E5.33 Balkan mountain hay meadows	N 42.625914º E 24.326972º
22	Pirin Mt. 3	E5.33 Balkan mountain hay meadows	N 42.628704º E 24.324439º
5	Osogovska Mt. 1	E5.33 Balkan mountain hay meadows	N 42.601922º E 24.380561º
10	Stara planina Mts. 1	E5.41 Stripes and curtains of tall grasses near watercourse	N 42.188906º E 22.615777º
13	Rhodopes Mts. 1	E5.33 Balkan mountain hay meadows	N 42.166829º E 22.599241º
14	Rhodopes Mts. 2	E5.33 Balkan mountain hay meadows	N 42.208697º E 22.588356º
6	Osogovska Mt. 2	E5.33 Balkan mountain hay meadows	N42.184300º E 22.578640º
20	Pirin Mt. 1	E5.33 Balkan mountain hay meadows	N 41.777475° E 22.969338°
11	Stara planina Mts. 2	E5.33 Balkan mountain hay meadows	N43.108836º E 23.097295º
2	Sredna Gora Mt. 2	E5.41 Stripes and curtains of tall grasses near watercourse	N43.107775º E 23.096355º
9	Vlahina Mt.	E5.41 Stripes and curtains of tall grasses near watercourse	N 43.108150° E 23.143188°
4	Sredna Gora Mt. 4	E5.33 Balkan mountain hay meadows	N 41.838500° E 24.142280°
3	Sredna Gora Mt. 3	E5.33 Balkan mountain hay meadows	N 41.838910° E 24.142440°
12	Stara planina Mts. 3	E5.33 Balkan mountain hay meadows	N 41.83866° E 24.12769°
18	Rhodopes Mts. 6	E5.41 Stripes and curtains of tall grasses near watercourse	N 41.930034° E 24.681768°
17	Rhodopes Mts. 5	E5.41 Stripes and curtains of tall grasses near watercourse	N 41.445306° E 24.679172°
7	Osogovska Mt. 3	E5.41 Stripes and curtains of tall grasses near watercourse	N 41.400006° E 24.759306º
8	Osogovska Mt. 4	E5.33 Balkan mountain hay meadows	N 41.666793º E 24.578712º
16	Rhodopes Mts. 4	E5.33 Balkan mountain hay meadows	N 41.549339º E 23.645715º
21	Pirin Mt. 2	E5.41 Stripes and curtains of tall grasses near watercourse	N 41.521226º E 23.661258º
19	Rhodopes Mts. 7	E5.41 Stripes and curtains of tall grasses near watercourse	N 41.521802º E 23.660282º

	Altitude (m) /exposition	*Alchemilla* species composition	Total Phenols mg GAE g^{-1} extract*	DPPH scavenging activity IC50, µg mL-1
	1315/ Southwest	*A. bulgarica, A. erythropoda; A. crinita*	44.50±0.5075	34.03
	1352/ East	*A. gracilis, A. erythropoda, A. crinita, A. glabra, A. subcrenata, A. xanthochlora*	44.45±2.1370	33.53
	1368/ South	*A. monticola; A. xanthchlora*	45.48±3.8287	35.4
	1058/ West	*A. glaucescens, A.glabra, A. monticola, A. obtusa*	38.64±1.8360	38.07
	1620/ West	*A. glaucescens; A. glabra; A. monticola; A. obtusa, A. viridiflora*	37.84±0.7636	33.85
	1679/ East	*A. cinerea, A. crinita, A. monticola*	36.47±0.4257	35.41
	1937/ North	*A. cinerea, A. crinita, A. monticola*	36.55±3.4586	35.22
	1860/ Southeast	*A. glaucescens, A. subcrenata*	46.55±0.5692	47.74
	1720/ North	*A. bulgarica, A. erythropoda, A. monticola, A. xanthochlora*	47.53±1.7606	42.57
	1220/ South	*A. bulgarica, A. erythropoda, A. glabra, A. monticola*	33.75±1.9098	47.83
	1217/ East	*A. glaucescens, A. glabra, A. monticola*	61.13±1.2549	18.26
	1442/ West	*A. glabra*	60.96±0.9970	18.65
	1518/ South	*A. crinita, A. erythropoda A. monticola, A. subcrenata*	62.21±1.5703	17.46
	1521/ South	*A. glaucescens, A. erythropoda, A. crinita*	60.42±0.3111	23.93
	1513/ Southwest	*A. erythropoda, A. monticola, A. viridiflora*	64.85±2.6162	23.33
	1621/ East	*A. monticola*	64.66±1.4424	21.29
	1209/ Northeast	*A. acutiloba; A. obtusa; A. xanthochlora*	67.12±0.1344	17.85
	1345/ Northwest	*A. obtusa*	72.27±2.4924	13.08
	1139/ North	*A. glaucescens, A. erythropoda, A. subcrenata, A. xanthochlora*	71.88±2.3267	16.03
	1412/ North	*A. glaucescens, A. crinita, A. monticola, A. obtusa*	49.75±1.3145	20.09
	1647/ Southeast	*A. crinita; A. monticola; A. obtusa; A. xanthochlora*	53.37±4.5360	23.63
	1650/ Southeast	*A. glabra*	82.71±0.3606	15.57

Legend: *GAE - gallic asid equivalents

Table 2
Taxonomic position of the studied species of genus *Alchemilla* included in *Herba Alchemillae*

Section	Subsection	Series	Species
Alchemilla (Brevicaulon Rothm.)	*Calycanthum* Rothm.	*Elatae* Rothm.	*A. viridiflora* Rothm.
	Heliodrosum Rothm.	*Pubescentes* Buser.	*A. bulgarica* Rothm. *A. cinerea* Buser *A. erythropoda* Juz. *A. glaucescens* Wallr.
		Vulgares Buser.	*A. acutiloba* Opiz *A. crinita* Buser *A. glabra* Neygenf *A. gracilis* Opiz *A. monticola* Opiz *A. obtusa* Buser *A. subcrenata* Buser *A. xanthochlora* Rothm.

Table 3
Total phenolic content and antioxidant activity of the most common *Alchemilla* spp. in *Herba Alchemillae*

Sub-section	Series	Species	Localities	Total Phenols mg GAE g^{-1} extract* (Mean±SD)	Average phenolic content	DPPH scavenging activity IC_{50}, $\mu g.mL^{-1}$	Average antioxidant activity
Heliodrosum	Vulgares	*A. obtusa*	Osogovska Mt. 1	62.62±3.2217	62.38	21.99	26.35
			Pirin Mt. 2	71.94±0.9970		17.84	
			Osogovska Mt. 3	72.27±2.4924		13.08	
			Rhodopes Mts. 4	42.69±1.3145		52.35	
		A. monticola	Osogovska Mt. 1	43.85±4.8151	51.55	29.69	32.72
			Pirin Mt. 3	46.14±0.7636		47.19	
			Rhodopes Mts. 6	64.66±3.8960		21.29	
		A. glabra	Sredna Gora Mt. 2	74.70±4.8350	63.39	17.49	34.89
			Osogovska Mt. 1	57.03±7.0146		26.43	
			Rhodopes Mts. 3	41.56±1.8931		54.00	
			Vlahina Mt.	60. 96±4.5761		18.65	
			Rhodopes Mts. 7	82.71±0.3606		15.57	
		A. xanthochlora	Pirin Mt. 3	60.35±3.2217	51.18	27.68	41.78
			Rhodopes Mts. 3	42.00±3.3984		55.88	
		A. crinita	Pirin Mt. 2	46.71±0.7636	41.49	39.44	46.03
			Rhodopes Mts. 3	36.26±1.3081		52.62	
	Pubescentes	*A. erythropoda*	Rhodopes Mts. 3	54.96±0.5020	54.96	30.67	30.67
		A. glaucescens	Osogovska Mt. 1	59.95±4.8960	50.73	23.65	36.10
			Rhodopes Mts. 4	44.08±4.3539		52.00	
			Sredna Gora Mt. 2	48.18±4.7848		32.67	
		A. bulgarica	Sredna Gora Mt. 1	34.60±2.4852	34.60	75.63	75.63

calities. Only one species was found in *Herba Alchemillae* harvested in several localities: *A. glabra* (Rhodopes Mts. 7 and Vlahina Mt.), *A. obtusa* (Osogovska Mt. 3) and *A. monticola* (Rhodopes Mts. 6). These species belong to the series *Vulgares* too and are usually distributed along streams or marshy meadows. The plant samples collected from habitat E5.33 "Balkan mountain hay meadows" contained one or two species of series *Pubescentes* and the same number of series *Vulgares* (Sredna Gora Mt. 1, Sredna Gora Mt. 3, Stara planina Mts. 2) but no sample was composed only by species belonging to series *Pubescentes*. Result of the survey showed that in *Herba Alchemillae* the most predominant species belong to series *Vulgares* (up to five) compared to the series *Pubescentes* (one or two) (Table 1). It should be noted that the large number of species of series *Vulgares* always correlate with their quantitative participation in *Herba Alchemillae.*

The most common species in the plant material collected from the studied areas of the 5 floristic regions of Bulgaria were *Alchemilla monticola* (57%), *A. crinita* (39%), *A. glabra* (35%), *A. xantochlora* (27%) (series *Vulgares*) and *A. erythropoda* (39%), *A. glaucescens* (30%) (series *Pubescentes*) (Table 1). Literature data show that *A. monticola* is the most common species in northeastern Poland (Zukowski and Palus, 1982) and also in the Ukrainian Carpathians – *A. monticola* and *A. glabra* (Lovelius, 1987).

Phenolic content and antioxidant activity of Herba Alchemillae

Twenty-two samples of *Herba Alchemillae* (Table 1), as well as the twenty-one samples of eight most widespread *Alchemilla* species collected from the studied areas (Table 3) were examined for total phenolic content and antioxidant activity. It was found that total phenolic content ranged from 33.75 mg g^{-1} to 82.71 mg g^{-1} extract. Antioxidant activity presented as half maximal inhibitory concentration (IC_{50}) varied from 15.57 μg ml^{-1} to 96.91 μg ml^{-1}.

Cluster analysis based on the amount of total phenols and antioxidant activity (Figure 1) revealed that two groups of samples were formed. The first one roughly includes 10 samples. Eight of them belonged to the interval C1 to C15; however, without C2, C3, C4, C7, C8, C9 and C12, but included also C20 and C22. The second group included the remaining samples. In both groups, the number of species of series *Vulgares* (about 70%) was larger than that of series *Pubescentes*. There was also some differentiation based on the ecological conditions - in the first group 80% of the samples were collected in habitat E5.33 Balkan mountain hay meadows (Table 1). The habitat is of mesoxerothermic type and has greater number of species of series *Pubescentes* with higher abundance, resulting in the significant participation ratio in *Herba Alchemillae*. In regard to the phenols content in the samples of group I range from 33.75 mg g^{-1} to 47.53 mg g^{-1} extract. The antioxidant activity (IC_{50}) varies from 33.53 μg ml^{-1} to 47.83 μg ml^{-1}.

About 70% of the samples of second group were collected from habitat E5.41 Stripes and curtains of tall grasses near watercourse and dominated by species of the series *Vulgares*. The phenolic content in the samples from group II ranges respectively from 49.75 mg g^{-1} to 82.71 mg g^{-1}, and antioxidant activity from 13.08 to 23.93 μg mL^{-1}. The data showed higher phenolic content and antioxidant activity in the samples of group II than group I which can be explained by greater presence of the species of series *Vulgares* (Table 3).

Total phenolics and antioxidant activity were examined also in 21 samples of eight of the most common *Alchemilla* spp. in the surveyed areas (Table 3). These samples consisted of aerial parts of only one *Alchemilla* species (Table 3). The results showed that the species of series *Vulgares* have higher content of total phenols and antioxidant activity than those of the series *Pubescentes*. For clearer picture we studied average phenolic content and antioxidant activity obtained for total phenolics and antioxidant activity of all studied localities for each species. Data in Table 3 show that the average content of total phenols of the species of series *Vulgares* ranged from 41.49 mg g^{-1} to 63.39 mg g^{-1}, and series *Pubescentes* of 34.60 mg g^{-1} to 54.96 mg g^{-1} extract. The species *Alchemilla obtusa* and *A. glabra* (series *Vulgares*) and *A. erythropoda* (series *Pubescentes*) were with the highest total phenolics content and antioxidant activity.

The samples of *Herba Alchemillae* collected from various floristic regions and locations with the same or very similar species composition (Osogovska Mt. 1 – Stara planina Mts. 1; Rhodopes Mts. 5 – Pirin Mt. 2) showed also similar content of total phenolics and antioxidant activity. Samples of *Herba Alchemillae* collected from two floristic regions containing one species – *A. glabra* (Rhodopes Mts. 7 and Vlahina Mt.) showed similar content of total phenolics and antioxidant activity (Table 3). These results confirm that species composition has a greater influence on the quantity of phenolics and antioxidant activity. Despite this major trend we should note the single cases under which the phenolic content and antioxidant activity was significantly different in *Herba Alchemillae* containing the same species but collected from two different types of habitats (*A. obtusa* – Osogovska Mt. 3 and Rhodopes Mts. 4; *A. glabra* – Rhodopes Mts. 3 and Rhodopes Mts. 7). Samples of the above mentioned species collected in habitat E5.41 Stripes and curtains of tall grasses were richer in total phenols and higher antioxidant activity that these from habitat E5.33 Balkan mountain hay meadows. Though single, these examples confirmed the influence of local ecological conditions; a fact should not be ignored.

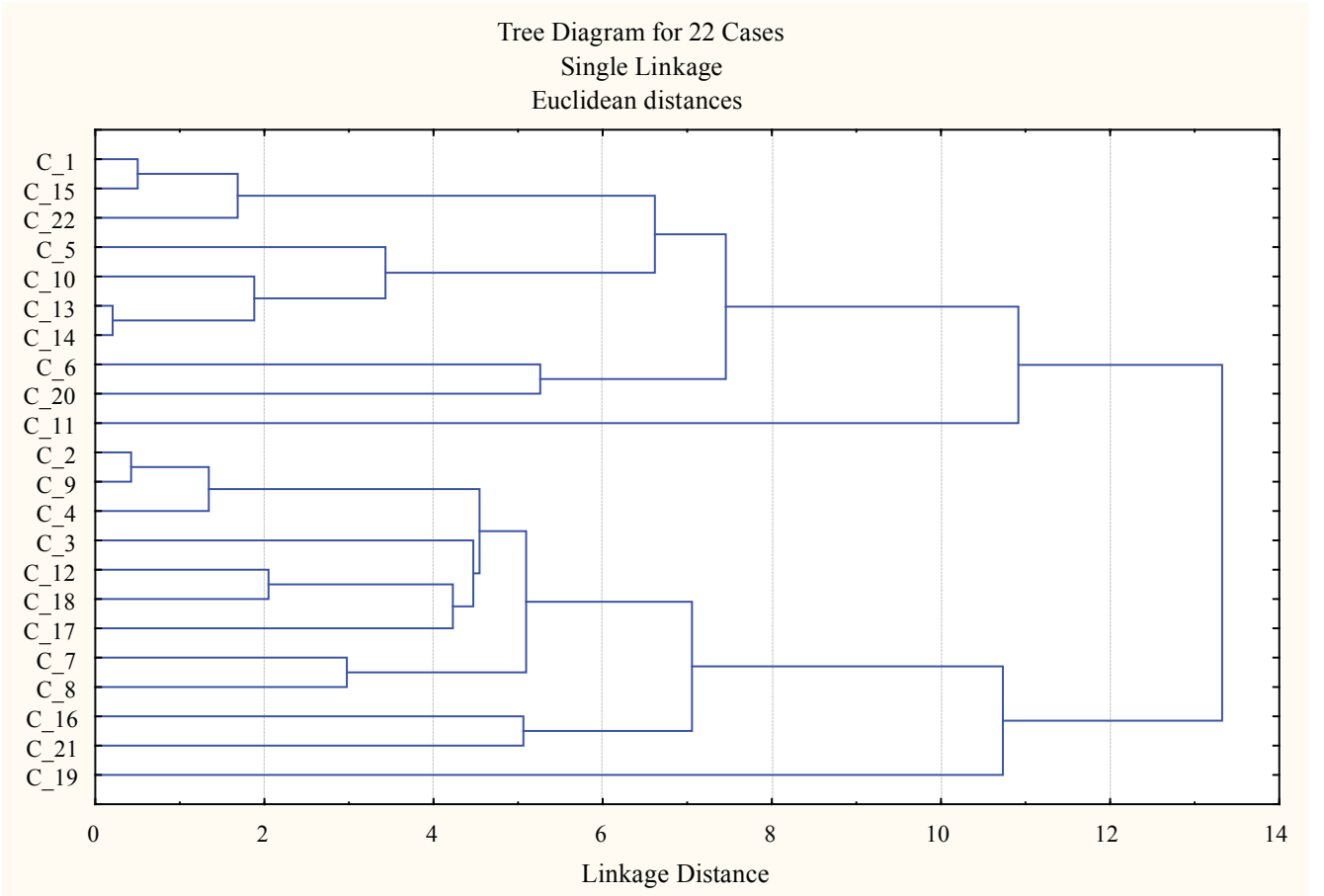

Fig. 1. Grouping of *Alchemilla* herba samples based on the amount of total phenols and antioxidant activity

The present study confirmed positive relationship between the content of total phenolics and antioxidant activity in plant extracts that was reported by other authors (Miliauskas et al., 2004; Shan et al., 2005).

Analyzes of all studied samples of *Herba Alchemillae* including different species as well as the samples of separated *Alchemilla* spp. demonstrated strong antioxidant activity according to classification scheme of Reynertson et al., 2005. All but one of the studied samples showed values of IC_{50} less than 50 μg ml^{-1}, which is an indicator for high antioxidant activity respectively for commercial product *Herba Alchemillae* in Bulgaria.

Conclusions

The studied samples of *Herba Alchemillae* collected from five floristic regions in Bulgaria included larger number of species of series *Vulgares*. Percentage contribution of these species was greater in samples collected in plant communities located along the streams. It was found that the studied species of series *Vulgares* contain a greater amount of total phenolics, and have a high antioxidant activity than the species of series *Pubescentes*. The quantity of total phenols and the level of antioxidant activity of *Herba Alchemillae* were influenced by the species composition of the sample and the proportion of the species of series *Vulgares* and *Pubescentes*. All studies samples had a high antioxidant activity which is a precondition for the good quality of commercial product *Herba Alchemillae* in Bulgaria.

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